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Research Article

In Vitro Antimicrobial Effect of Phenolic Extracts and Resistant Starch on *Escherichia coli, Streptococcus* spp., *Bifidobacterium* and *Lactobacillus* spp.

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Abstract

The present study aimed to evaluate the antimicrobial activity of Grape Pomace Extract (GPE), Pistachio Peel Extract (PsPE), and Pomegranate Pomace Extract (PPE) with or without Resistant Starch (RS) as a prebiotic on gut microflora representative's *in vitro* conditions. For this purpose, the Resistant Starch (Fibersol2), grape pomace, pistachio peel, and pomegranate pomace were provided and the extracts of by-products were prepared. Folin-Ciocalteu method was used to determine the total phenolic content of extracts. The antimicrobial activity of extracts ± Resistant Starch against *Escherichia coli, Streptococcus* spp., *Lactobacillus* spp. and *Bifidobacterium* spp. were evaluated using Minimum Inhibitory Concentration (MIC) method. The total tannin and phenolic compounds of pomegranate pomace were more than the others. The results of MIC showed that 1600 and 3200 ppm of pistachio peel extract inhibited the *E. coli* growth. The growth inhibition of *Streptococcus* spp. by Resistant Starch was equal to 400 ppm dilution. *Streptococcus* did not grow in 50, 100, and 200 ppm of pistachio peel extract + Resistant Starch treatment. The dilution of 800, 1600, and 3200 ppm of grape pomace extract could prevent the growth of *Lactobacillus* spp., while *Bifidobacterium* increased in all treatments dilution except in 3200 ppm pistachio peel extract and 50 and 100 ppm pistachio peel extract + resistant starch.

Keywords: *Phenol, Resistant starch, Antimicrobial activity, Bacteria, MIC*

Fenolik Ekstrakt ve Dirençli Nişastanın *Escherichia coli, Streptococcus* spp., *Bifidobacterium* ve *Lactobacillus* spp. Üzerine İn-vitro Antimikrobiyal Etkisi

Öz

Bu çalışma Üzüm Posası Ekstraktının, Fıstık Kabuğu Ekstraktının ve Nar Posası Ekstraktının Dirençli Nişasta ile birlikte veya ayrı olarak *in vitro* şartlarda mide mikroflorası bileşenlerine bir prebiyotik olarak antimikrobiyal etkilerini araştırmak amacıyla yapılmıştır. Bu amaçla, dirençli nişasta (Fibersol 2), üzüm posası, fıstık kabuğu ve nar posası elde edilerek ekstraktları hazırlandı. Ekstraktlardaki fenolik miktarını belirlemek amacıyla Folin-Ciocalteu metodu kullanıldı. Minimum inhibe edici konsantrasyon metodu kullanılarak *Escherichia coli, Streptococcus* spp., *Lactobacillus* spp. ve *Bifidobacterium* spp. etkenlerine karşı ekstraktlar ± dirençli nişastanın antimikrobiyal etkisi araştırıldı. Nar posasının toplam tanin ve fenolik bileşikleri diğerlerinden daha fazlaydı. Minimum inhibe edici konsantrasyon sonuçları, 1600 ve 3200 ppm düzeyindeki fıstık kabuğu ekstraktının *E. coli* üremesini inhibe ettiğini gösterdi. Dirençli nişastayla birlikte Streptococcus'u büyüme inhibisyonu 400 ppm dilusyonda gerçekleşti. *Streptococcus* dirençli nişastayla birlikte 50, 100 ve 200 ppm fıstık kabuğu ekstraktı uygulamasında üremedi. Üzüm posası ekstraktının 800, 1600 ve 3200 ppm dozları *Lactobacillus* spp. üremesini önlerken *Bifidobacterium* spp. üremesi 3200 ppm fıstık kabuğu ekstraktı ile 50 ve 100 ppm fıstık kabuğu ekstraktı ile birlikte dirençli nişasta uygulamaları haricinde tüm uygulamalarda arttı.

Anahtar sözcükler: *Fenol, Dirençli nişasta, Antimikrobiyal aktivite, Bakteri, Minimum İnhibe edici konsantrasyon*

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INTRODUCTION

Polyphenols are known as natural compounds that could be found in foods like fruits, vegetables, cereals, etc.[1]. Phenolic compounds are not involved in metabolic pathways of plants, and they are a kind of secondary plant substances [2]. These compounds can act as anti-inflammatory, antimicrobial, and antioxidant factors [3]. In this regard, using natural antibacterial compounds such as residual plant rich extracts in phenolic compounds, as food preservatives, has increased due to concerns about food safety [4]. The industrial by-products were used in livestock feed too. Since animal's intestinal microorganisms can have an effect on the energy harvested of diet, adjustment of gut microbiota opens up an opportunity for promoting digestive health [5]. Recently, researchers have paid a lot of attention to the industrial pomaces especially those containing phenolic compounds [6]. Colonic microbiota has an effect on the absorption of dietary polyphenols in small intestine [7]. Some bacterial species (e.g. *Escherichia coli*, *Bifidobacterium* spp., *Lactobacillus* spp. etc.) are catalyzing the metabolism of phenolic [8] and some of phenolic extracts like Grape Pomace Extract (GPE) [9], Pistachio Peel Extract (PsPE) [10] and Pomegranate Pomace Extract (PPE) [11] have bioactive properties including antimicrobial activity. Not only can the colonic microbiota have an effect on using phenolic compounds, but also it can change by prebiotic substrates. One of the reasons for the effectiveness of prebiotic is that prebiotics are fermented using the intestinal flora, and the commensal microorganisms increase. Therefore, diseases decrease by moderating the intestinal microflora and controlling the pathogenic microorganisms [12].

The term of Resistant Starch (RS) was defined by Asp [13] as "the starch or starch degradation products that escapes digestion in the small intestine and may be completely or partially fermented in the large intestine". Hence, RS is one of the substrates that increases the concentrations of beneficial bacteria through diet. The prebiotic properties of RS can be due to its non-digestibility of carbohydrate fractions for cecal and colonic microbiota that influence the host gut health in animal studies [14]. RS is used by *Lactobacilli Bifidobacteria* and promotes the *Lactobacilli Bifidobacteria* colonization, and it can also reduce the intestinal pathogen levels [15].

However, there are several reports about antimicrobial activity of phenolic extracts of different food sources against common animal colonic bacteria. But there are no available reports about synchronic effect of phenolic compounds and prebiotics on these bacteria. Therefore, this investigation was carried out to evaluate the antibacterial effect of GPE, PsPE and PPE phenolic compound extracts on *E. coli*, *Streptococcus* spp., *Bifidobacterium* and *Lactobacillus* spp. as common animal's gut microflora with or without RS.

MATERIAL and METHODS

Raw Material

Pomegranate pomace and grape pomace were purchased from Nariran Co., Saveh and SunSunShahd Co., Urmia, Iran, respectively. Pistachio peel was purchased from Nut and Pistachio Peel Commerce Co., Mashhad, Iran. The peels and pomaces were air-dried under ambient conditions. Then, they were milled (0.5 mm) and stored in 4°C for the following tests and extractions. The RS (Fibersol2) was purchased from Karen Nutrilife Co., Yazd, Iran.

Preparing Extracts and Determining Total Phenolic Content

To prepare the extracts, 50 g of air-dried and powdered pomegranate pomace, grape pomace and pistachio peel were extracted separately with 300 mL of methanol (99.5%), and kept 30-32 h at room temperature by shaking every 30 min. Then, the extracts were filtered through Whatman 42 mm and kept at water bath under sterile air condition. Afterwards, the extracts were collected and weighed after combination and evaporation of all methanolic fractions. Finally, the extracts were kept at -20°C for the next experiments. It should be noted that Folin-Ciocalteu method was used to determine the total phenolic content [16].

Determining Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the extracts in combination or without RS was determined through National Committee for Clinical Laboratory standards suggestion (NCCLS, 2000) by using Micro Broth Dilution method (96-well plates) in duplicates. Briefly, in order to prepare the stock solution, 0.02 g of each extract and 0.02 g of RS was added separately to 2 mL sterile Brain Heart Infusion (BHI) Broth medium, and it was vortexed well to reach a final concentration of 10^4 ppm. Two-fold dilutions were prepared to obtain concentrations of 50, 100, 200, 400, 800, 1600, and 3200 ppm to each extract in 2 mL BHI broth + DMSO (dimethyl sulfoxide). The standard bacterial strains including *E. coli* ATCC 35218, *Streptococcus* spp. (*S. sobrinus* ATCC 33478), *Bifidobacterium* spp. ATCC 29521, and *Lactobacillus* spp. *(L. acidophilus* ATCC 43121) were cultivated on LB broth for the activation of bacteria (Luria Bertani Broth, Sigma-Aldrich). Then, the bacterial suspensions were prepared in turbidity equal to 0.5 McFarland standard tubes (5×10^5 cfu/mL). Then, 200 µl of each dilution (GPE, PsPE, PPE, RS, GPE + RS, PsPE + RS, PPE + RS) with 6 µL of bacterial suspensions of each bacterium was added to each well. Finally, the plates were incubated at 37°C for 24 h in aerobic atmosphere (except for *Bifidobacterium* strain that was incubated in anaerobic condition). After incubation period, ELISA Microplate Reader was used to measure the absorbance of each well at 630 nm (BIOTEK ELX 800). MIC came to be the lowest concentration of extracts (with or without RS) which prevented visible growth of bacteria [17].

Statistical Analysis

The data was recorded at 0 h (at the time of innoculation) and 24 h (after incubation), and analyzed through t-tests (P≤.05) using SAS (9.1) to determine the difference between the growth of bacteria in two-hour intervals.

RESULTS

Since phenolic compounds of grape pomace, pomegranate pomace, and pistachio peel play an important role in their antibacterial activity, the tannin and total phenolic compounds of their extracts were measured. As shown in *Table 1*, total tannin and phenolic compounds of pomegranate pomace were more than the others, while phenolic compounds of grape pomace and tannin content of pistachio peel were the lowest.

The results MIC on GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS for *E. coli* are shown in *Table 2*. According to this table, *E.coli* could grow in culture media containing all dilution of GPE, PPE, RS, GPE + RS, PPE + RS, PsPE + RS. While the dilution of 1600 and 3200 ppm of PsPE could restrain its growth. In *Table 3*, the MIC results of GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS are shown for *Streptococcus* spp., according to which, 200, 1600 and 3200 ppm dilution of GPE could act as an inhibiting factor for growing *Streptococcus* spp., while *Streptococcus* spp. could not grow in 50 and 800 ppm dilution of PsPE; RS prevented the *Streptococcus* spp. growth in 400 ppm dilution. Therefore, the MIC of RS for *Streptococcus* spp. was 400 ppm dilution (80 µL of medium + extract or RS in 2 mL BHI Broth + DMSO). 1600 ppm of GPE + RS also could prevent *Streptococcus* spp. growth. On the other hand, *Streptococcus* spp. bacteria did not grow in 50,100 and 200 ppm of PsPE + RS, while it could grow in 400, 800, 1600, and 3200 ppm.

The results of MIC treatments on *Lactobacillus* spp. are shown in *Table 4*, according to which, 200, 800, 1600 and 3200 ppm of GPE could prevent *Lactobacillus* spp. growth. This bacterium did not grow in 800 and 600 ppm of PsPE treatment. On the other hand, 100, 200, 400, and 3200 ppm of RS could prevent *Lactobacillus* growth. 1600 ppm and 3200 ppm of GPE + RS and PsPE + RS treatment also prevented the growth of *Lactobacillus*, respectively. In *Table 5*, the MIC results of GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS for *Bifidobacterium* spp. are shown. Accordingly, *Bifidobacterium* increased in all treatment dilutions except 3200 ppm PsPE and 50 and 100 ppm PsPE + RS *(Table 6)*.

Table 1. *The phenolic compounds of grape pomace, pomegranate pomace, and pistachio peel (% of DM)*

Extracts	Tannin	Total Phenol
Grape pomace	2.167	2.700
Pomegranate pomace	3.643	14.939
Pistachio peel	1.906	11.739

DISCUSSION

The by-products of food industry and some peels are tannin-rich sources which are used in animal feed in many developed countries. The antimicrobial activity of peels has been confirmed against pathogenic bacteria [18]. It appears that polyphenols show prebiotic like effects on modulation of the gut microbiota [19]. Several *in vitro* studies have shown that some polyphenols can change the composition of gut microbiota, while some bacteria may be inhibited; others can be developed [20]. Shoko et al.[21] noticed the antimicrobial activity of methanol extract from grape seeds. Similarly, Tzounis et al.[22] reported that phenolic compounds significantly increased the growth of *E. coli*, while the growth of *Bifidobacterium* and *Lactobacillus* were unaffected. Yamakoshi et al.[23] also discussed that a proanthocyanidin-rich (a type of phenolic compound) extract from grape seeds significantly increased the number of *Bifidobacteria* of gastrointestinal tract. *Bifidobacteria* is one of the potentially beneficial bacteria due to its beneficial effects on the immune system and metabolism [24] which is a non-pathogenic bacterium [25]. Secondary metabolites of plants and by-products such as phenolic compound, carotenoids, flavonoids etc. with biological activity may have some resistance mechanisms including enzymatic inactivation, target site modifications, and decrease in intracellular drug accumulation [26]. In this regard, Tabasco et al.[27] argued that using different phenolic extracts and sensitivity of *Bifidobacteria* is different, and *B. lactis* showed the highest sensitivity towards the phenolic extract. Mir Ahmadi and Davari [28] reported that the antimicrobial effects of tea leaf extract against *E.coli* were 750 ppm. In their study about the effects of phenolic compounds on probiotic and pathogenic bacteria, Pacheco-Ordaz et al.[29] concluded that phenolic compounds, without affecting the viability of probiotics *(L. rhamnosus, L. acidophilus)*, can selectively restrain the growth of pathogenic bacteria *(E. coli, S. typhimurium)*. Similarly, Vega-Vega et al.[30] evaluated the effects of rich extracts of the phenolic compounds on the growth of some pathogenic bacteria and reported the antimicrobial benefits of the mixture of phenolic extracts.

The factors which may have a crucial impact on the bacterial growth include the structure of polyphenols, the microorganism strain, and the estimated dosage [31]. The differences of bacterial resistant to polyphenolic compounds are possibly due to bacteria wall composition differences. For example, Puupponen-Pimia et al.[32] reported that the Gram-positive bacteria are more sensitive to polyphenols than Gram-negative bacteria. Hence, in the present study, *E. coli* as Gram-negative bacteria can easily grow in the culture medium containing the phenolic extract. Kemperman et al.[33] stated that the mode of action of polyphenols on bacteria may be due to binding

Table 2. *The MIC results of GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS for E. coli*

Dilutions	Maen-0h	Mean-24h	F-Value	V. Equal Test	T-Value	Significant
GPE 50	0.076	0.643	0.1745	Equal	0.0007	*
GPE 100	0.059	0.572	0.2103	Equal	<.0001	*
GPE 200	0.097	0.593	0.0935	Equal	0.0047	*
GPE 400	0.152	0.599	<.0001	Unequal	0.0263	*
GPE 800	0.320	0.659	0.3642	Equal	0.0007	*
GPE 1600	0.374	0.784	0.4027	Equal	0.0050	*
GPE 3200	0.698	0.940	0.2717	Equal	0.0156	*
PPE 50	0.101	0.669	0.3119	Equal	<.0001	*
PPE 100	0.120	0.712	0.2615	Equal	0.0004	*
PPE 200	0.139	0.776	<.0001	Unequal	0.0010	*
PPE 400	0.134	0.811	0.3119	Equal	0.0005	*
PPE 800	0.315	0.924	0.3390	Equal	0.0004	*
PPE 1600	0.361	1.066	0.3140	Equal	0.0108	*
PPE 3200	0.877	1.516	0.8731	Equal	0.0013	*
PsPE 50	0.108	0.649	0.1491	Equal	0.0002	*
PsPE 100	0.152	0.585	0.1463	Equal	0.0009	*
PsPE 200	0.229	0.592	0.6500	Equal	0.0015	*
PsPE 400	0.377	0.626	0.9234	Equal	0.0137	*
PsPE 800	0.659	0.776	0.8997	Equal	0.0492	*
PsPE 1600	1.115	1.028	0.3469	Equal	0.1314	NS
PsPE 3200	1.327	1.226	0.8810	Equal	0.1564	NS
RS 50	0.070	0.923	0.0953	Equal	0.0006	*
RS 100	0.071	0.988	<.0001	Unequal	0.0578	*
RS 200	0.066	0.862	0.0493	Equal	0.0093	*
RS 400	0.071	0.876	0.0352	Equal	0.0124	*
RS 800	0.073	0.857	0.0369	Equal	0.0077	*
RS 1600	0.068	0.821	0.0235	Equal	0.0201	*
RS 3200	0.071	0.812	0.0368	Equal	0.0134	*
GPE+RS 50	0.073	0.5345	0.2290	Equal	0.0001	*
GPE+RS 100	0.0745	0.6115	0.0977	Equal	0.0001	*
GPE+RS 200	0.0795	0.5005	0.1474	Equal	0.0026	*
GPE+RS 400	0.0985	0.4705	0.1807	Equal	0.0023	*
GPE+RS 800	0.124	0.476	0.2784	Equal	0.0027	*
GPE+RS 1600	0.1724	0.6	0.681	Equal	0.0043	*
GPE+RS 3200	0.2755	0.7355	1.000	Equal	<.0001	*
PPE+RS 50	0.073	0.786	0.0707	Equal	0.0006	*
PPE+RS 100	0.0855	0.615	0.2103	Equal	<.0001	*
PPE+RS 200	0.1045	0.6505	0.3276	Equal	0.0003	*
PPE+RS 400	0.1235	0.745	0.0163	Equal	0.0039	*
PPE+RS 800	0.157	0.852	0.8193	Equal	<.0001	*
PPE+RS 1600	0.2315	0.9315	0.7312	Equal	0.0002	*
PPE+RS 3200	0.3375	1.0557	0.4778	Equal	0.0006	*
PsPE+RS 50	0.093	0.494	0.3119	Equal	<.0001	*
PsPE+RS 100	0.1125	0.8325	0.0137	Equal	0.0041	*
PsPE+RS 200	0.1615	0.7215	0.0606	Equal	0.0004	*
PsPE+RS 400	0.245	0.924	<.0001	Unequal	0.0009	*
PsPE+RS 800	0.4255	1.3055	0.2627	Equal	0.0006	*
PsPE+RS 1600	0.6505	1.632	0.3417	Equal	0.0004	*
PsPE+RS 3200	1.2535	2.007	0.3060	Equal	0.0105	*

** Significant difference in bacterial growth between 0 h and 24 h (P≤0.05); NS: Not significant difference in bacterial growth between 0 h and 24 h (P>0.05)*

Table 3. *The MIC results of GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS for Streptococcus spp.*

Dilutions	Maen-0h	Mean-24h	F-Value	V. Equal Test	T-Value	Significant
GPE 50	0.0845	0.3105	0.1409	Equal	0.0004	*
GPE 100	0.0965	0.3425	0.2331	Equal	0.0031	*
GPE 200	0.1335	0.137	0.3119	Equal	0.6285	NS
GPE 400	0.1555	0.2615	0.4973	Equal	0.0074	*
GPE 800	0.2725	0.6405	0.9556	Equal	0.0263	*
GPE 1600	0.49	0.512	0.8959	Equal	0.6617	NS
GPE 3200	0.7485	0.7575	0.2313	Equal	0.7525	NS
PPE 50	0.1065	0.2685	0.6289	Equal	0.0021	*
PPE 100	0.1195	0.2765	0.6881	Equal	0.0003	*
PPE 200	0.1585	0.3925	0.0386	Unequal	0.0446	*
PPE 400	0.2275	0.454	0.2845	Equal	0.0025	*
PPE 800	0.304	0.619	0.7487	Equal	0.0012	*
PPE 1600	0.557	0.8675	0.1371	Equal	0.0036	*
PPE 3200	0.796	1.161	1.000	Equal	0.0005	*
PsPE 50	0.146	0.144	1.000	Equal	0.2929	NS
PsPE 100	0.237	0.251	<.0001	Unequal	0.0454	*
PsPE 200	0.331	0.3725	0.9152	Equal	0.0160	*
PsPE 400	0.627	0.7855	0.7487	Equal	0.0032	*
PsPE 800	1.005	1.128	0.7897	Equal	0.0612	NS
PsPE 1600	1.526	1.29	0.1521	Equal	0.0112	*
PsPE 3200	1.91	1.67	0.9810	Equal	0.0090	*
RS 50	0.08	0.3585	<.0001	Unequal	0.0034	*
RS 100	0.082	0.371	<.0001	Unequal	0.0044	*
RS 200	0.0785	0.2955	0.0948	Equal	0.0231	*
RS 400	0.0825	0.0905	0.1409	Equal	0.2193	NS
RS 800	0.0815	0.0835	1.0000	Equal	0.1056	NS
RS 1600	0.0865	0.09	0.7112	Equal	0.5354	NS
RS 3200	0.0865	0.0895	1.000	Equal	0.0513	NS
GPE+RS 50	0.705	0.295	0.1896	Equal	0.0020	*
GPE+RS 100	0.291	0.3035	0.0509	Equal	0.0030	*
GPE+RS 200	0.0855	0.3135	0.0411	Unequal	0.0430	*
GPE+RS 400	0.1095	0.3165	0.1889	Equal	0.0423	*
GPE+RS 800	0.162	0.32	0.6358	Equal	0.0062	*
GPE+RS 1600	0.263	0.288	0.9470	Equal	0.5384	NS
GPE+RS 3200	0.364	0.407	0.5903	Equal	0.0106	*
PPE+RS 50	0.089	0.276	<.0001	Unequal	0.0086	*
PPE+RS 100	0.102	0.2135	0.1016	Equal	0.0124	*
PPE+RS 200	0.1135	0.28	0.1583	Equal	0.0143	*
PPE+RS 400	0.1335	0.3035	1.000	Equal	<.0001	*
PPE+RS 800	0.1755	0.3515	0.3476	Equal	0.0054	*
PPE+RS 1600	0.3565	0.623	0.7776	Equal	0.0005	*
PPE+RS 3200	0.4715	0.8195	0.6573	Equal	0.0037	*
PsPE+RS 50	0.098	0.101	0.8193	Equal	0.6094	NS
PsPE+RS 100	0.112	0.1135	0.5903	Equal	0.3118	NS
PsPE+RS 200	0.195	0.21	0.9152	Equal	0.2937	NS
PsPE+RS 400	0.316	0.37	1.000	Equal	0.0061	*
PsPE+RS 800	0.486	0.561	1.000	Equal	0.0404	*
PsPE+RS 1600	0.966	1.0955	0.4243	Equal	0.0111	*
PsPE+RS 3200	1.450	1.287	0.2268	Equal	0.1651	*

** Significant difference in bacterial growth between 0 h and 24 h (P≤0.05); NS: Not significant difference in bacterial growth between 0 h and 24 h (P>0.05)*

Table 4. *The MIC results of GPE, PPE, PsPE, RS, GPE+RS, PPE+RS, PsPE+RS for Lactobacillus spp.*

Dilutions	Maen-0h	Mean-24h	F-Value	V. Equal Test	T-Value	Significant
GPE 50	0.0795	0.181	0.1059	Equal	0.0035	*
GPE 100	0.1005	0.1855	0.0977	Equal	0.0491	*
GPE 200	0.118	0.3165	0.0777	Equal	0.2257	NS
GPE 400	0.171	0.249	0.5325	Equal	0.0156	*
GPE 800	0.248	0.3135	0.3261	Equal	0.0945	NS
GPE 1600	0.4615	0.6765	0.0700	Equal	0.2992	NS
GPE 3200	0.839	0.9455	0.6711	Equal	0.2704	NS
PPE 50	0.104	0.2125	0.0727	Equal	0.0251	*
PPE 100	0.13	0.183	<.0001	Unequal	0.1644	NS
PPE 200	0.1535	0.252	0.6402	Equal	0.0132	*
PPE 400	0.217	0.331	0.2397	Equal	0.0334	*
PPE 800	0.322	0.466	0.4475	Equal	0.0120	*
PPE 1600	0.497	0.746	0.2290	Equal	0.0020	*
PPE 3200	0.789	1.166	0.9636	Equal	0.0095	*
PsPE 50	0.112	0.114	0.9674	Equal	0.8729	NS
PsPE 100	0.157	0.163	0.8193	Equal	0.3530	NS
PsPE 200	0.2715	0.3155	0.1154	Equal	0.0154	*
PsPE 400	0.4715	0.5575	0.9092	Equal	0.0131	*
PsPE 800	0.7485	0.8735	0.4097	Equal	0.1123	NS
PsPE 1600	1.284	1.252	0.5074	Equal	0.0876	NS
PsPE 3200	1.795	1.636	0.3119	Equal	0.0007	*
RS 50	0.081	0.307	0.5903	Equal	<.0001	*
RS 100	0.08	0.288	<.0001	Unequal	0.0972	NS
RS 200	0.077	0.2735	0.0359	Unequal	0.1136	NS
RS 400	0.08	0.319	<.0001	Unequal	0.0690	NS
RS 800	0.081	0.301	0.1104	Equal	0.0108	*
RS 1600	0.0815	0.2815	1.000	Equal	<.0001	*
RS 3200	0.083	0.3	<.0001	Unequal	0.0701	NS
GPE+RS 50	0.079	0.152	1.000	Equal	0.0015	*
GPE+RS 100	0.0845	0.144	0.8591	Equal	0.0029	*
GPE+RS 200	0.0865	0.16	0.8846	Equal	0.0028	*
GPE+RS 400	0.1085	0.1415	0.6457	Equal	0.0235	*
GPE+RS 800	0.1975	0.146	<.0001	Unequal	0.0309	*
GPE+RS 1600	0.2765	0.315	0.8877	Equal	0.5374	NS
GPE+RS 3200	0.396	0.4665	0.6123	Equal	0.0322	*
PPE+RS 50	0.0875	0.183	0.5903	Equal	0.0001	*
PPE+RS 100	0.1045	0.1455	0.3390	Equal	0.0188	*
PPE+RS 200	0.12	0.1725	0.5325	Equal	0.0087	*
PPE+RS 400	0.1535	0.219	0.2615	Equal	0.0333	*
PPE+RS 800	0.2025	0.2955	0.0848	Equal	0.0065	*
PPE+RS 1600	0.381	0.49	1.000	Equal	0.0027	*
PPE+RS 3200	0.4975	0.693	0.7776	Equal	0.0087	*
PsPE+RS 50	0.0895	0.0905	1.000	Equal	0.8586	NS
PsPE+RS 100	0.1375	0.1425	1.000	Equal	0.0194	*
PsPE+RS 200	0.2275	0.233	0.4845	Equal	0.1778	NS
PsPE+RS 400	0.2845	0.3355	0.2726	Equal	0.0493	*
PsPE+RS 800	0.487	0.608	<.0001	Unequal	0.0368	*
PsPE+RS 1600	0.8705	0.977	0.4441	Equal	0.0030	*
PsPE+RS 3200	1.385	1.195	0.0238	Unequal	0.1742	NS

** Significant difference in bacterial growth between 0 h and 24 h (P≤0.05); NS: Not significant difference in bacterial growth between 0h and 24 h (P>0.05)*

Table 5. *The MIC results of GPE, PPE, PsPE, RS, GPE + RS, PPE + RS, PsPE + RS for Bifidobacterium spp.*

Dilutions	Maen-0h	Mean-24h	F-Value	V. Equal Test	T-Value	Significant
GPE 50	0.085	0.5585	<.0001	Unequal	0.0181	*
GPE 100	0.097	0.527	0.0993	Equal	0.0055	*
GPE 200	0.151	0.552	0.3895	Equal	0.0097	*
GPE 400	0.194	0.591	0.6881	Equal	0.0002	*
GPE 800	0.323	0.752	0.0771	Equal	0.0059	*
GPE 1600	0.551	0.938	0.7172	Equal	0.0290	*
GPE 3200	0.857	1.217	0.2513	Equal	0.0005	*
PPE 50	0.1135	0.567	0.0848	Equal	0.0003	*
PPE 100	0.146	0.6645	0.0652	Equal	0.0014	*
PPE 200	0.179	0.7725	0.1037	Equal	0.0017	*
PPE 400	0.2365	0.8895	0.2513	Equal	0.0001	*
PPE 800	0.3715	1.0685	0.2661	Equal	0.0006	*
PPE 1600	0.6425	1.267	0.7014	Equal	0.0040	*
PPE 3200	0.955	1.483	0.5432	Equal	0.0001	*
PsPE 50	0.1375	0.1535	1.000	Equal	0.0171	*
PsPE 100	0.2065	0.2525	1.000	Equal	0.0002	*
PsPE 200	0.3475	0.5465	0.8417	Equal	0.0008	*
PsPE 400	0.600	0.888	0.3922	Equal	0.0016	*
PsPE 800	0.8485	0.515	0.1583	Equal	0.0013	*
PsPE 1600	1.429	1.671	0.4568	Equal	0.0003	*
PsPE 3200	1.983	2.047	0.1556	Equal	0.3811	NS
RS 50	0.0725	0.6275	0.1807	Equal	<.0001	*
RS 100	0.0775	0.6305	0.0143	Equal	0.0064	*
RS 200	0.0795	0.6115	0.0153	Equal	0.0060	*
RS 400	0.0795	0.6095	0.0161	Equal	0.0055	*
RS 800	0.079	0.6225	0.0877	Equal	0.0064	*
RS 1600	0.079	0.6305	0.0331	Equal	0.0048	*
RS 3200	0.0805	0.633	0.0277	Equal	0.0017	*
GPE+RS 50	0.074	0.537	0.2513	Equal	0.0001	*
GPE+RS 100	0.079	0.5175	0.1104	Equal	0.0062	*
GPE+RS 200	0.0865	0.506	0.1095	Equal	0.0048	*
GPE+RS 400	0.11	0.499	0.1409	Equal	0.0022	*
GPE+RS 800	0.1395	0.528	0.1059	Equal	0.0022	*
GPE+RS 1600	0.22	0.7265	0.2374	Equal	0.0028	*
GPE+RS 3200	0.336	0.8185	0.3625	Equal	0.0020	*
PPE+RS 50	0.0875	0.558	0.2103	Equal	0.0004	*
PPE+RS 100	0.11	0.525	<.0001	Unequal	0.0383	*
PPE+RS 200	0.128	0.6335	0.0499	Unequal	0.0318	*
PPE+RS 400	0.164	0.7355	<.0001	Unequal	0.0228	*
PPE+RS 800	0.2365	0.9295	0.2673	Equal	0.0020	*
PPE+RS 1600	0.3585	0.4855	0.5115	Equal	0.0009	*
PPE+RS 3200	0.5	1.174	0.3613	Equal	0.0014	*
PsPE+RS 50	0.088	0.085	0.8193	Equal	0.6094	NS
PsPE+RS 100	0.1205	0.123	0.8193	Equal	0.4226	NS
PsPE+RS 200	0.166	0.21	0.2513	Equal	0.0132	*
PsPE+RS 400	0.2705	0.396	<.0001	Unequal	0.0004	*
PsPE+RS 800	0.4915	0.8705	0.3390	Equal	0.0056	*
PsPE+RS 1600	0.7935	1.28	0.7487	Equal	0.0007	*
PsPE+RS 3200	1.345	1.752	0.4501	Equal	0.0119	*

**:Significant difference in bacterial growth between 0 h and 24 h (P≤0.05); NS: Not significant difference in bacterial growth between 0 h and 24 h (P>0.05)*

Table 6. *Observed bacterial growth in different dilutions of extracts±RS (brief)*

Bacterial Strains		Dilutions						
		50	100	200	400	800	1600	3200
E. coli	GPE	+	+	+	+	+	+	+
	PPE	+	+	+	+	+	+	+
	PsPE	+	+	+	+	+	-	-
	RS	+	+	+	+	+	+	+
	GPE+RS	+	+	+	+	+	+	+
	PPE+RS	+	+	+	+	+	+	+
	PsPE+RS	+	+	+	+	+	+	+
Streptococcus spp.	GPE	+	+	-	+	+	-	-
	PPE	+	+	+	+	+	+	+
	PsPE	-	+	+	+	-	+	+
	RS	+	+	+	-	-	-	-
	GPE+RS	+	+	+	+	+	-	+
	PPE+RS	+	+	+	+	+	+	+
	PsPE+RS	-	-	-	+	+	+	-
Lactobacillus spp.	GPE	+	+	-	+	-	-	-
	PPE	+	-	+	+	+	+	+
	PsPE	-	-	+	+	-	-	+
	RS	+	-	-	-	+	+	-
	GPE+RS	+	+	+	+	+	-	+
	PPE+RS	+	+	+	+	+	+	+
	PsPE+RS	-	+	-	+	+	+	-
Bifidobacterium spp.	GPE	+	+	+	+	+	+	+
	PPE	+	+	+	+	+	+	+
	PsPE	+	+	+	+	+	+	-
	RS	+	+	+	+	+	+	+
	GPE+RS	+	+	+	+	+	+	+
	PPE+RS	+	+	+	+	+	+	+
	PsPE+RS	-	-	+	+	+	+	+

+ The bacteria grew; - The bacteria did not grow

polyphenols to cell membranes of bacteria. Therefore, it can disturb the function of membrane, and prevent cell growth. Hattori et al.[34] also reported that polyphenols can produce hydrogen peroxide and change the permeability of microbial membrane. On the other hand, some of phenolic compounds can interact with lipids and proteins and change the permeability of the membrane [33].

A study done by Roozegar et al.[35] showed that the leaf extract of *P. atlantica* was phenol compound-rich which was implied to associate with antibacterial properties. This extract had an antimicrobial effect on *Streptococcus* spp. In his evaluation of the antimicrobial activity of pomegranate pomaces, Al-Zoreky [36] found that 80% of methanolic extract of peels were a strong inhibitor for *E. coli*. In another study, Hosseini et al.[37] stated that *P. atlantica* extracts has an antibacterial activity against *S. mutans*. The pomegranate fruit skin extracts were shown by Sadeghian et al.[38] as a strong antimicrobial activity against the microorganisms (e.g. *S. aureus*; *P. aeruginosa*; *C. albicans)*. Rodriguez et al.[39] noticed that *L. plantarum* has several enzymatic activities such as tannase, phenolic acid decarboxylase, and benzyl alcohol dehydrogenase that make it able to have an effect on degradation of some phenolic compounds. Importantly, RS is a type of prebiotic that functions by binding the bacteria to the granule surface [40]. This could improve the viability of beneficial bacteria such as *Bifidobacteria* at the end of the digestive area [41]. Wronkowska et al.[42] stated that RS has beneficial effects on the growth of *Bifidobacteria* in the intestine. Our findings are similar to the findings of Li [43] who mentioned RS as a prebiotic based on its ability to enrich *Bifidobacterium* and *Lactobacillus* spp. Therefore, RS is completely fermented by gut microflora and selectively

used by *Lactobacilli Bifidobacteria* followed by decrease in intestinal pathogen levels [15]. Roberfroid et al.[44] showed that RS can stimulate the growth of *Bifidobacterium* and *Lactobacillus* ssp. as beneficial bacteria. However, our study indicates that RS had an effect on *Bifidobacterium,* but it could not stimulate the growth of *Lactobacillus* ssp.

We concluded that, on the one hand, PsPE could inhibit *E. coli* growth, while GPE + RS, PPE + RS and PsPE + RS were inactive against *E.coli*. On the other hand, GPE and RS were active against *Streptococcus* spp. growth. The MIC of GPE and RS was 1600 and 400 ppm, respectively. GPE inhibited *Lactobacillus* spp. growth. *Bifidobacterium* (as beneficial bacteria) could increase in all mixtures of extracts and RS. Since the industrial by-products of this study are used in animal feed, their individual phenolic compounds can be identified and quantified. It will be beneficial for optimizing extraction to be used with RS as a prebiotic in livestock industry.

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Research Article

Atretic Ovarian Follicles Morphology and Immunolocalization of Active Caspase-3 in Algerian Bedouin Goat *(Capra hircus)* Ovaries

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Abstract

This study aims to highlight the cellular and molecular events of atresia in the Bedouin goat ovarian follicles. Ovaries collected from slaughterhouses during breeding and non-breeding seasons were submitted to morphological studies and immunolocalization of active caspase-3. The results showed that ovaries were heavier ($P<0.05$) during the breeding season. The atresia occurred at all stages of follicular development in breeding and non-breeding seasons. At the preantral stage, degeneration affected primarily oocyte and active caspase-3 immunoreactivity was detected in the oocyte with a few staining in follicular cells. At the antral stage, atresia affected in first the granulosa layer cells which were characterized by an abnormal indentation of nuclei and positive active caspase-3 immunolabeling. At the early stage of atresia, the granulosa cells exhibited a few pycnotic nuclei and lost their cellular integrity, by getting scattered; these cells were positive to active caspase-3. At the late stage, the scattered cells invaded the antral cavity and the dead cells were eliminated by phagocytic cells. The oocyte seemed to undertake a long process of atresia via an autophagic pathway and it was the last to die. Finally, the follicle was replaced by a collagen fiber forming a scar of atresia. In conclusion, important morphological changes occurred in atretic follicles mainly in antral stage by apoptosis via caspase-3 signaling pathway, in breeding and non-breeding seasons.

Keywords: *Atresia, Apoptosis, Active caspase-3, Breeding season, Non-breeding season, Bedouin goat*

Cezayir Bedevi Keçisi *(Capra hircus)*'nde Atretik Ovaryum Folikül Morfolojisi ve Aktif Kaspaz-3 İmmunolokalizasyonu

Öz

Bu çalışmada Bedevi keçisi ovaryum foliküllerinde gelişen atrezide meydana gelen hücresel ve moleküler olaylar tanımlanmıştır. Çiftleşme sezonunda veya dışında kesimhanelerden toplanan ovaryumlar morfolojik çalışmalar ve aktif kaspaz-3 immunolokalizasyonu için kullanıldı. Elde edilen sonuçlar ovaryumların çiftleşme sezonunda daha ağır olduğunu gösterdi ($P<0.05$). Çiftleşme sezonunda veya dışında tüm foliküler gelişme evrelerinde atrezinin oluştuğu belirlendi. Prenatal evrede, dejenerasyon başlıca oositi etkilemekteydi, ve aktif kaspaz-3 immunoreaktivitesi az sayıda foliküler hücrelerde boyanma şeklinde oositlerde tespit edildi. Antral evrede, atrezi çekirdeğin anormal girinti oluşturması ve pozitif kaspaz-3 immunoboyanması ile karakterize olup ilk sıra granuloza tabakası hücrelerini etkilemekteydi. Atrezinin erken evresinde, granuloza hücreleri az sayıda piknotik çekirdek göstermekte olup hücresel bütünlükleri kaybolmuştu. Bu hücreler dağılmış halde olup aktif kaspaz-3 pozitiftiler. Geç evrede, dağılmış hücreler antral boşluğa yerleşmiş olup ölü hücreler fagosit hücreler tarafından elimine edilmişlerdi. Oosit otofajik yol ile uzun bir atrezi süreci geçirmiş olup son ölendi. Son olarak, folikül kollajen fiber ile doldurularak atrezi yarası oluşturmuştu. Sonuç olarak, hem çiftleşme sezonunda hem de dışında atrezik foliküllerde ve özellikle de antral evrede kaspaz-3 uyarı yoluyla gelişen apoptozis ile karakterize önemli morfolojik değişiklikler oluşmaktadır.

Anahtar sözcükler: *Atrezi, Apoptozis, Aktif kaspaz-3, Çiftleşme sezonu, Çiftleşme dışı sezon, Bedevi keçisi*

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INTRODUCTION

The Bedouin goat *(Capra hircus)* represents a great value for most of people in the western south of Algeria, due to its meat, milk and fibers production. This breed acquires its importance from its perfect adaptation to the harsh environmental conditions of its biotope [1]. This has been confirmed by various ecophysiological and metabolic studies [2-4]. Considering the paramount importance for this community, goat's production level must be maintained properly by increasing fertility and conception rate [5]. This is essential to gain a comprehensive knowledge on the reproductive physiology of this breed and on the mechanisms by which female regulates its reproduction and its follicular pool.

In most mammals, the phenomenal loss of germ cells during folliculogenesis is governed by a physiological process called atresia or follicular involution, a degenerative process that allows the elimination of follicles from the follicular pool. Many studies on follicular atresia have been carried out where the classification criteria for atresia are mainly based on morphological studies [6]. The mechanism of follicular cell death has not been fully elucidated. However, several studies have shown that in mammalian ovaries follicular atresia is under the exclusive control of apoptosis [7], genetically programmed [8] and morphologically distinct form of cell death which is initiated and executed by proteases like the family of cysteine-dependent aspartate-directed proteases [9] and characterized by oligonucleosomal fragmentation of DNA [10]. Active caspase-3, is the main effector caspase of the apoptotic cascade within cells [11]. The detection of this epitope should be a unique and sensitive indicator of apoptosis [12]. Other studies suggested that non apoptotic forms of programmed cell death like autophagy occurred; this process promote cell death by excessive self-digestion and degradation of damaged cellular proteins and organelles [13], characterized by a double-membrane vesicle that encloses organelles and portions of the cytosol [14]. Furthermore, recent studies have demonstrated that autophagy could be triggered by various stimuli inducing apoptosis suggesting that this phenomenon is closely related to apoptosis induction and is gonadotropin dependent [8,15].

In the present study we described the morphological changes in the atretic follicles and the immunolocalization of the active caspase-3 in different cell components of atretic follicles to highlight the cellular and molecular events of atresia in the Bedouin goat ovarian follicles during breeding and non-breeding seasons.

MATERIAL and METHODS

Twenty five paired ovaries from adult cyclic Bedouin goats were collected from Bechar slaughterhouses (South West of Algeria, 31°.62' N., 2°.22' W.). This experimentation was conducted on 7 females from non-breeding and 18 females from breeding seasons, aged from 2 to 5 years. Ovaries were removed, degreased, weighed then transported to the laboratory in warm (37°C) physiological saline solution within 2 h. After that, they were rinsed with PBS (Phosphate Buffer Saline), transferred to Petri dishes for macroscopic observations then fixed in 4% (w/v) paraformaldehyde at room temperature for 24 h; samples were sectioned along the axis of the helium and then dehydrated in a graded series of ethanol, clarified in xylene, embedded in paraffin and sectioned at 4 µm. Sections were mounted in order and put on slides for histological studies, hydrated and stained with Hematoxylin-Eosin (H&E) and Heidenhain azan. Ovarian sections were analysed by light microscopy (Optika B-350) using a computer program Ts View connected to a digital camera (Hirocam MA88-500).

Follicles Classification

The histological classification of ovarian follicles stages was evaluated according to the methods described by Rodgers and Irving-Rodgers [16]. The differentiation between healthy and atretic follicles was determined according to the classification criteria of follicular atresia proposed by the Nomenclature Committee on Cell Death (NCCD) [6]. The follicle was considered as atretic when one of the following morphologic criteria was observed: 1- Follicular cells loose the integrity of their plasma membrane and some apoptotic bodies appeared; 2- The connections in granulosa layers became loose; 3- Granulosa cells were massively eliminated; 4- Residual follicular cells and oocyte were degenerated.

Immunolocalization of Active Caspase-3

The presence of active caspase-3 in the ovaries was investigated by immunohistochemistry using Avidin-Biotin Complex method (ABC) (vectastain Elite ABC kit, Vector Laboratories, Burlingame). After a deparaffinization and hydration, sections were permeabilized at room temperature in saponin (Fischer Scientific UK) and proteinase K (Eurobio) (0.2 mg/mL) mixture; this was followed by endogenous peroxidase blocking using H_2O_2 (3%) in PBS (0.1 M; pH=7.2) at room temperature. Tissue sections were washed in PBS. The antigen was blocked at room temperature with normal horse serum in a wet chamber. All sections were incubated for 1 h at room temperature with the primary antibody: a rabbit monoclonal anti- active caspase-3 (ab32042, Abcam, Cambridge, UK), which was diluted at 1:100 in PBS. Tissue sections were washed and incubated with a biotinylated mouse anti-rabbit IgG antibody (Ca 94010, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame) at 1:200 dilution for 1 h at room temperature. The samples were washed and incubated for 1 h at room temperature with horse-radish peroxidase-conjugated-streptavidin. After washing, labeling was visualized with 3, 3'-diaminobenzidine-tetra-hydrochloride chronic substrate (SK-4100, DAB substrate kit for peroxidase; Vector Laboratories). Sections were counterstained with hematoxylin (Hematoxylin QS, H-3404;

Vector lab, Burlingame, A, USA) and analyzed by light microscopy. Sections incubated with normal horse serum rather than primary antibody were used as negative controls. Immunohistochemical staining was evaluated using semi -quantitative methods and estimated at four levels: -/ no labeling, +/- variable, +/ moderate, ++/ intense.

Transmission Electron Microscopy Analysis

Antral follicles (≥ 5mm) dissected out under a binocular loupe, rinsed in PBS and cut into small pieces (1 mm^3) were prefixed in Karnovsky fixative mixture (2.5% Glutaraldehyde, 4% Paraformaldehyde) diluted in PBS for 2 h at 4°C; then post-fixed in 1% osmium tetroxide diluted in PBS, dehydrated in graded ethanol series (50°, 70°, 90°, 100°), cleared with acetone and finally embedded in araldite (Agar Scientific Ltd.). Ultrathin sections (0.8 nm; ultramicrotome LKB V) mounted on copper grids (200 mesh) were double contrasted with uranyless (R&D-Delta Microscopies-France) and lead citrate (Wako). The sections were examined and photographed using a transmission electron microscope (Hitachi H7700, Hitachi, Tokyo, Japan) equipped with high-resolution camera.

Statistical Analysis

Results were expressed as mean±SEM. Statistical significance of differences between different parameters was examined by Mann Whitney test using R statistical software (R 3.4.2); $P<0.05$ was considered to be statistically significant.

RESULTS

Ovaries Weight

The weight of the paired ovaries was 1.6±0.27 g in non breeding season and 2.5±0.17 g in breeding season *(Fig. 1)* and was significantly different between the two seasons ($P<0.01$).

Morphological Analysis of Ovaries

The ovaries were found almond shaped with pale colored structures localized in the edge of the mesovarium near the lateral margin of the pelvic inlet *(Fig. 2)*. The ovaries located within and on the floor of the pelvic cavity were ovoid shaped and flattened. Their surface was irregular by the presence of follicles and the corpora lutea in breeding season and was smoothy, devoid of any follicles in non-breeding season.

The ovaries were enclosed in a fibrous capsule the *tunica albuginea (Fig. 3a, b)*. The cortex contains many follicles at different stages of folliculogenesis. The medulla consisted of connective tissue with many blood vessels *(Fig. 3c)*. The ovarian follicles were classified into preantral and antral follicles according to the presence or not of the antral cavity containing follicular fluid.

The healthy preantral stage regrouped primordial, primary and secondary follicles *(Fig. 4a-c)*. At the primordial follicle *(Fig. 4a)*, the small oocyte was surrounded by flattened not replicating granulosa cells. The primary follicle *(Fig. 4b)* has a large oocyte surrounded by zona pellucida and then by one layer of cuboidal granulosa cells resting on basal lamina called membrane of Slavjansky *(Fig. 4b)*. The secondary follicle *(Fig. 4c)*, was centered by oocyte, which enclosed nucleus and germinal vesicle and was surrounded with zona pellucida *(Fig. 4c)*; this zone separated granulosa layers from oocyte; at this stage, we observed a differentiation between theca interna and theca externa cells *(Fig. 4c)*. In the antral follicles, the antrum filled with the follicular fluid *(Fig. 5a)* was delimited by granulosa cells which were replicated massively into apical and basal cells *(Fig. 5b)*. At this stage, we observed the oocyte centered by the

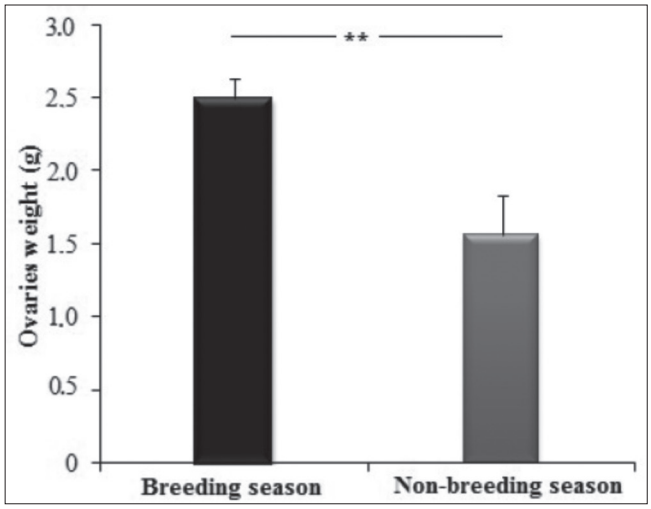

Fig 1. Ovaries weight during breeding and non-breeding seasons in Bedouin goat. Data were expressed as mean ± ESM. ** $P<0.01$, statistically significant

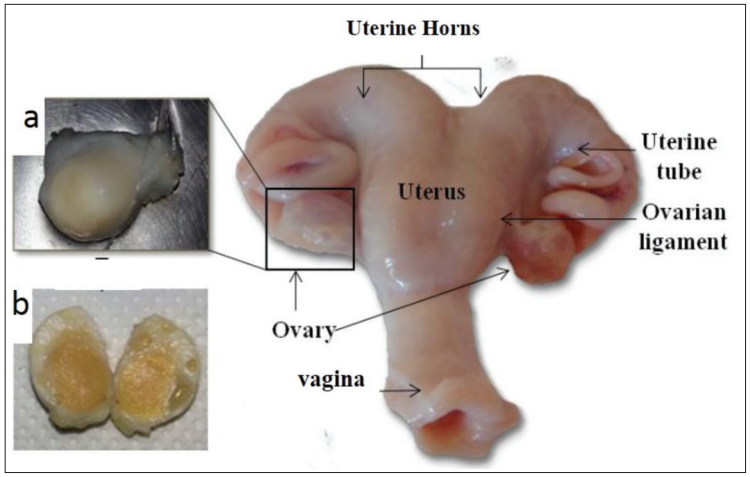

Fig 2. Morphology of female reproductive tractus in Bedouin goat showing the ovaries localization; (a): Ovary; (b): Longitudinal ovary section

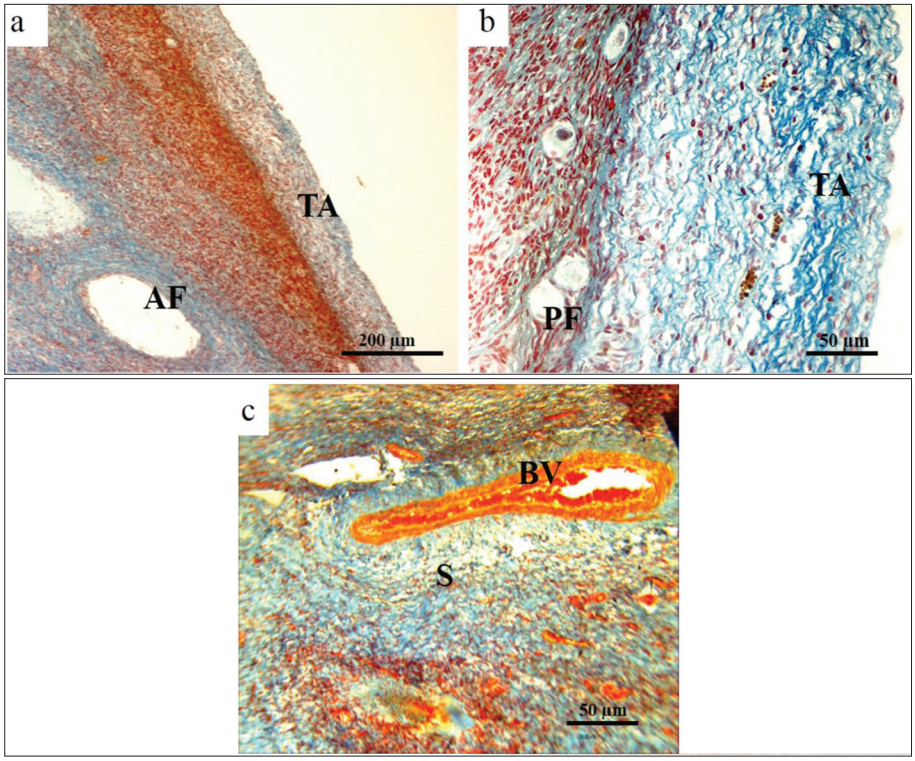

Fig 3. Histology of Bedouin goat ovary. (a) Ovarian cortex; (b) Peripheral cortex with primordial follicles; (c) Ovarian medulla reaches in blood vessels *(arrowhead);* AF: Antral Follicle; PF: Primordial Follicle; S: Stroma; TA: Tunica Albuginea; BV: Blood Vessels

nucleus, surrounded by corona radiata cells and regular zona pellucida *(Fig. 5c)*.

The vast majority of those all ovarian follicles observed were touched by atresia, in breeding and non-breeding seasons. We noticed that atresia in the preantral stage was different from that occurred in the antral stage *(Fig.4, Fig. 5)*.

At the preantral stages, atresia occurred principally in the oocyte, characterized by vacuolization of oocyte cytoplasm, eccentric nucleus *(Fig. 4d)* and irregular outline of oocyte followed by irregularities of zona pellucida *(Fig. 4e,f)*. The follicular cells remained unchanged.

At the antral stage, a few follicles were found to be healthy. The early stage of atresia was characterized mainly by several changes in the follicular wall including the basal lamina, which became thick with irregular contour *(Fig. 5d)*; transformed to a vitreous membrane or "membrane of atresia", devoid of any cell type, which ended up dispersing in the ovarian stroma *(Fig. 5d)*. In the granulosa layer, we observed few cells with pycnotic nuclei *(Fig. 5e)*, followed by the destruction of the layers of the membrana granulosa closest to the antrum and a loss in connections between granulosa cells *(Fig. 5h)*. This cells detached and scattered in the antrum were eliminated massively by phagocytic cells *(Fig. 5h)*. At the late stage of atresia, the antral cavity was invaded completely by the scattered cells *(Fig. 5d)*. The detached cells from the follicle wall induced morphological changes of the basal layer of the granulosa cells *(Fig. 5e)*. The oocyte degenerated very late, characterized by the irregular contour with irregular zona pellucida and marginal nucleus *(Fig. 5f)*; the oocyte cytoplasm was completely vacuolised and its nucleus disappeared *(Fig. 5i)*. At the end of atresia process, the follicles disappeared totally leaving behind a scare of atresia *(Fig. 5g)*.

The ultrastructure of the atretic follicle wall showed in the granulosa layer *(Fig. 6)*, cells with abnormally indented nuclei with condensed chromatin forming small clumps *(Fig. 6a)* and neighboring cells with cytoplasmic prolongations surrounding dead cells *(Fig. 6b)*. The healthy granulosa cells adjacent to the antrum presented the nuclei centered by the nucleoli with the fibrillar center and the dense fibrillar component *(Fig. 6c)*; in its cytoplasm a lot of lipid droplets and cytoplasmic organelles were observed *(Fig. 6d)*. The theca interna contained many homogenous cells, blood vessels and numerous collagen fiber and leukocytes *(Fig. 6e)*. The theca externa was constituted with elongated homogenous cells with elongated nuclei *(Fig. 6f)*.

The percentage of healthy and atretic follicles at different stages and seasons (breeding and non-breeding) was reported in *Fig. 7*. The number of atretic primordial follicles was lower than the healthy ones. The follicular stage affected by atresia was principally the tertiary stage and showed a significant increase in number of atretic follicles compared to healthy follicles in all seasons *(Fig. 7)*. At this stage, the number of atretic follicles was significantly higher ($P<0.001$) in breeding and non-breeding seasons *(Fig. 7)*.

Immunohistolocalization of Active Caspase-3

The active caspase-3 observed in preantral and antral stages, compared to negative control, was observed during the breeding and non-breeding seasons *(Fig. 8; Fig. 9)*.

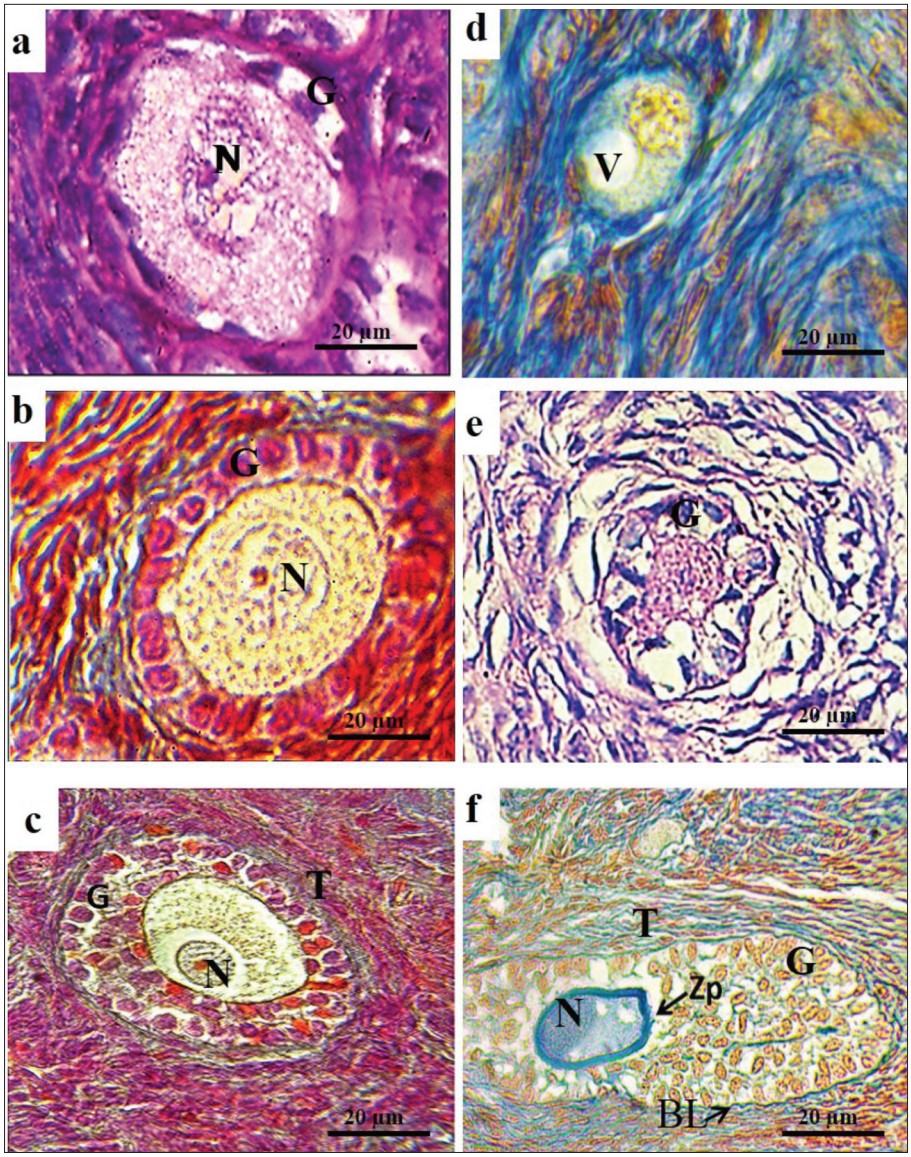

Fig 4. Histology of atretic preantral follicles compared to the healthy follicles. (a) Healthy primordial follicle; (b) Healthy primary follicle; (c) Healthy secondary follicle; (d) Atretic primordial follicle; (e) Atretic primary follicle; (f) Atretic secondary follicle; BL: Basal Lamina; G: Granulosa; N: nucleus; T: Theca; V: Vacuole; Zp: Zona pellucida; (a) and (e): stained with H&E; (b, c, d and f): stained with azan

At the preantral stage, active caspase-3 immunoreactivity was observed in the cytoplasm of the oocyte *(Fig. 8)* and few staining in nuclear follicular cells *(Fig. 8a,d)*. The intensity of oocyte labeling was variable from primordial to primary follicles *(Fig. 8a,c; Table 1)*; no labeling was observed at the oocyte of secondary follicle *(Fig. 8e, Table 1)*. At this stage, positive cells appeared in granulosa and theca cells *(Fig. 8e,f; Table 1)*.

At the antral stage, no staining was detected in the oocyte. A cytoplasmic immunoreactivity of granulosa, theca, and cumulus oophorus cells was observed with a little nuclear immunoreactivity *(Fig. 9, Table 1)*.

DISCUSSION

The weight of Bedouin goat ovaries during non-breeding season was similar to those reported in Sahel and Sokoto Red goat breeds [17] and higher than that reported in Bengal and local Andhra Pradesh goat breeds [5,18,19]. In breeding season, the weight of ovaries was higher than that reported in several goat breeds [5,17-19]. This difference in weight was explained by a cyclic formation of corpus luteum characterized by a hypertrophy of luteinized granulosa cells, a fibroblast hyperplasia of the connective tissues and a vascularity which contribute to an increase in the size of the corpus luteum in breeding season [20].

Morphological aspect of ovaries was similar to that reported in Black Bengal goat [5,17]. During breeding and non breeding seasons, the folliculogenesis was accompanied by a physiological regulation process called atresia described in detail for the first time by Logothetopoulos et al.[21], this process induced many structural changes in the different components of the follicle according to the follicular stage. In our goat breed, the detection of signs of the atretic process in all follicular stages confirmed that atresia can occur at all stage of the development. Similar observations have

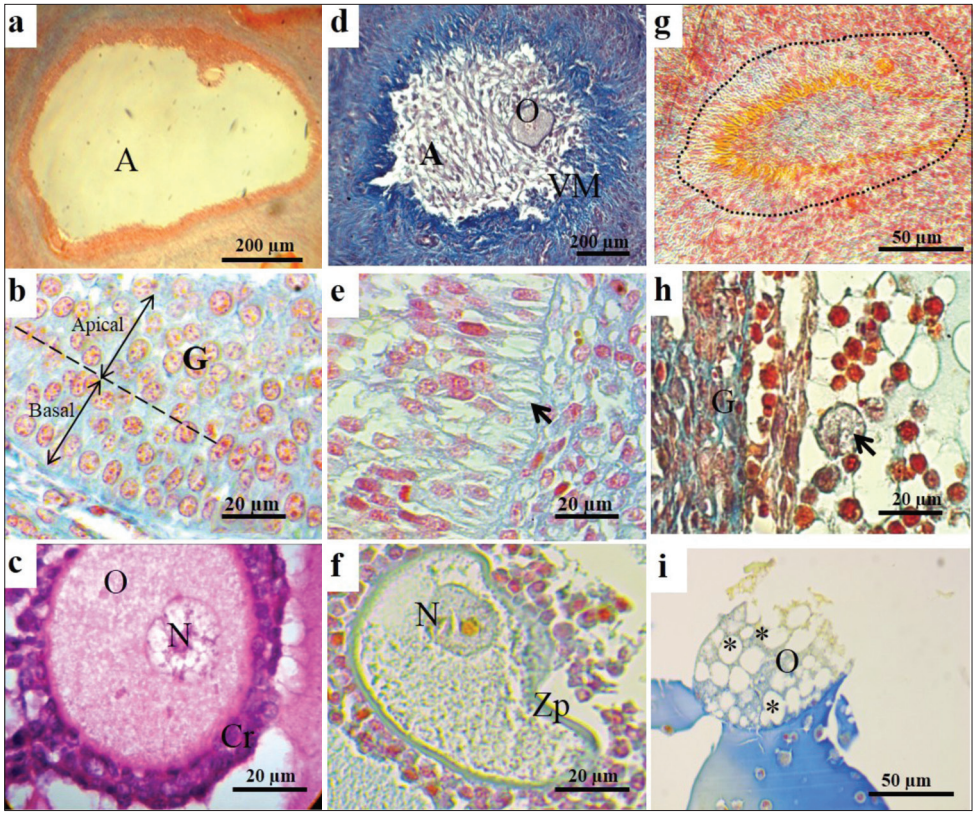

Fig 5. Histology of atretic antral follicles showing early and late stages of antral atretic follicles compared to healthy follicles. (a) Healthy large antral follicle; (b) Healthy granulosa layer with cuboidal cells resting on basal lamina; (c) Oocyte from healthy follicle. (d) Atretic follicle: granulosa invaded the antral cavity with intact oocyte, basal lamina transformed into vitreous membrane; (e) Granulosa layer from early atretic follicle: disorganization of follicular cells and extension of the cells adjacent to the basal lamina; (f) Oocyte from early atretic follicle: eccentric nuclei and irregularity in outline of oocyte accompanied with irregularity of zona pellucida; (g) The hatched line shows a scare of follicle atresia; (h) Granulosa layer from late atretic follicle: scattered cells with pycnotic nuclei and their migration in antral cavity, observation of phagocytic cells *(arrow-head);* (i) Appearance of some autophagic vacuoles in oocyte cytoplasm *(asterisk);* A: Antrum; Cr: Corona radiata; O: Oocyte; VM: Vitreous Membrane; N:nucleus; G: granulosa; Zp: Zona pellucida. (a, b, d, e, f g, h and i) stained with Azan, (c) stained with H&E

also been reported in other vertebrate species such as the rat [22], sow [23], human [24] mice [25], pig [6], cow [16], ewe [26] and goat [27].

During the preantral stage, the first event of atresia observed in the oocyte of follicle induced a degeneration of the oocyte nucleus and a vacuolization of oocyte cytoplasm as reported in Indian goat [28] and in sow [6]. These vacuoles may contain mitochondria and inflated endoplasmic reticulum [29]. In the Brazilian local goat, it was reported that massive swelling of the mitochondria with disappearing ridges and increased size of the endoplasmic reticulum were the first signs of degeneration of the preantral follicles [30]. According to Kerr et al.[31], and Sharma and Bhardwaj [28], the second event of atresia in preantral follicle concerned the granulosa cells. The compaction and condensation of the chromatin giving the dense appearance to the nuclei is attached to the wall of the nuclear envelope giving it a very irregular appearance. This was depicted in the granulosa of the secondary follicles which seems more sensitive to degeneration than the primordial and the primary follicles, as reported in Brazilian goat [32].

At antral stages, the degeneration of follicles was more complex. In contrast to the preantral stage, in first, our results showed some morphological changes in the wall of the atretic follicles while the oocyte remains unchanged. At early stage of atresia, Escobar et al.[13] reported that the oocyte was particularly resistant to the factors inducing apoptosis. It only degenerated very late with appearance of multiple vacuoles like autophagic vesicles or autophagosomes. This would suggest that oocyte undergo another form of cell death probably by autophagic process. In granulosa layer of atretic antral follicles, we observed in Bedouin goat, cells with abnormally indented outline of nuclei; as reported in hamster [33]. At the late stage of atresia, the phagocytic cells removed the degenerating cells in the antral cavity by macrophages and in follicular wall by the neighboring intact cells as observed in pig and mice [23,34]. In Bedouin goat we noticed the presence of leukocyte in the theca interna layer cells probably removed by apoptosis as observed in sheep [35]. Finally, our results showed that oocyte from late antral atretic follicle was the last to undergo morphological changes, so it has been shown that changes never occur in oocyte during early stage of atresia [36]. The elimination of oocyte observed in our results was probably due to a physiological process involving an autophagic pathway [13]. This elimination required a micro RNAs regulators implicated in the crosstalk between autophagy and apoptosis according to Xu et al.[15].

The initiation of atresia process was associated with a crosstalk of cell apoptosis, autophagy and ferroptosis rather than change of typical apoptosis markers such as FAS (Fas cell surface death receptor), BAX (BCL2-associated X protein) or caspases [8]. In our study, the localization of active caspase-3 was observed in oocyte cytoplasm and some nuclei of granulosa cells in atretic preantral follicles. Inversely, active caspase-3 was never observed in granulosa cells of preantral follicles. These results were

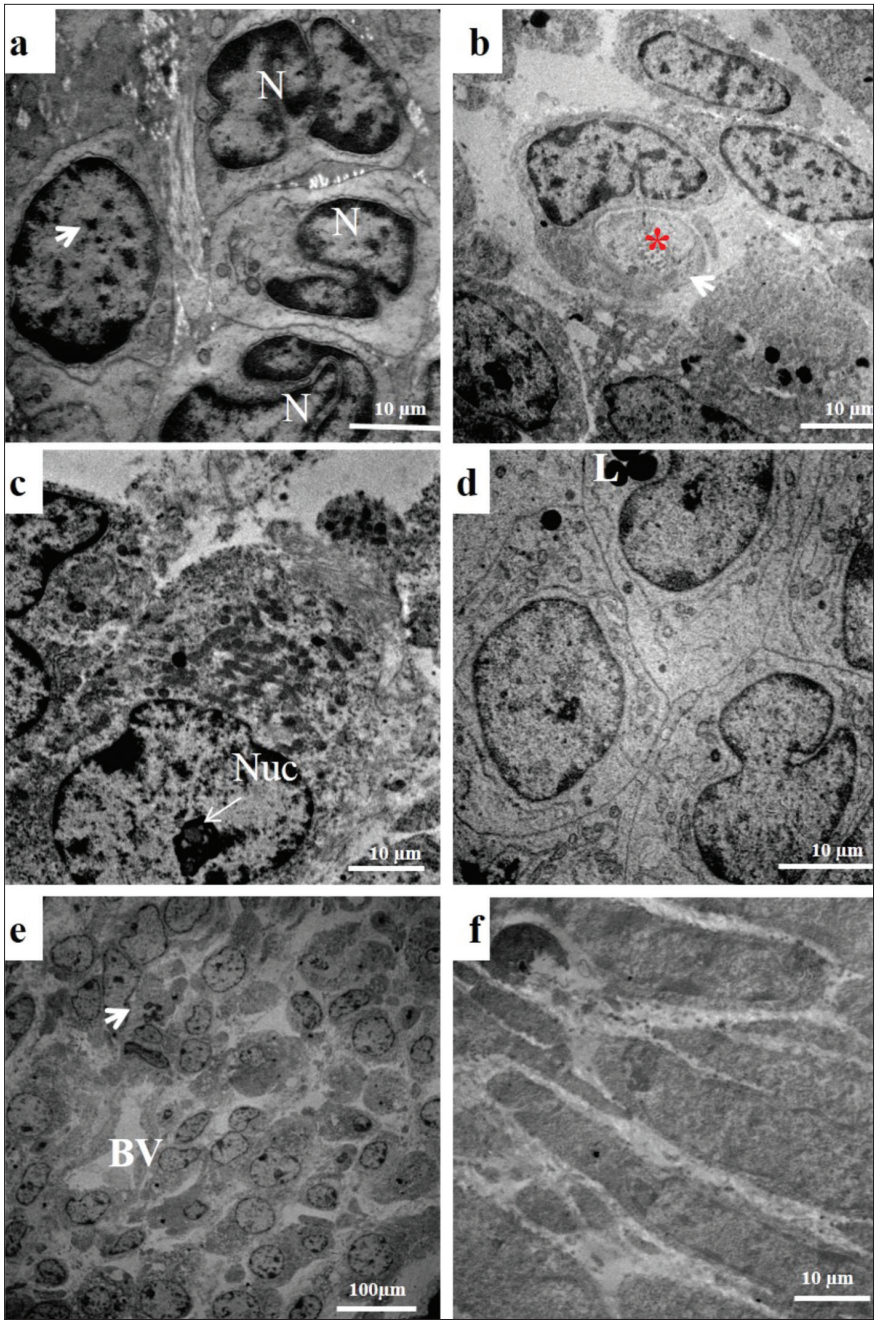

Fig 6. Electron micrographs of the granulosa and theca layers showing different characterization of atresia. (a) Granulosa cells from the atretic follicle have irregular and abnormally indented outline of nuclei with condensed chromatin forming small clumps *(arrow-head);* (b) Phagocytic cell in granulosa cell from advanced atretic follicle eliminates the cell debris *(asterisk)* containing cytoplasmic projections *(arrow-heads);* (c) Healthy granulosa cell with a nucleolus presents the fibrillar center and the dense fibrillar component; (d) Lipid droplets and cytoplasmic organelles were observed similar to the granulosa cells of normal developing follicle; (e) Theca interna layer showing the leukocyte invasion; (f) Theca externa; L: Lipid droplet; N: Nucleus; Nuc: Nucleolus; BV: Blood Vessels

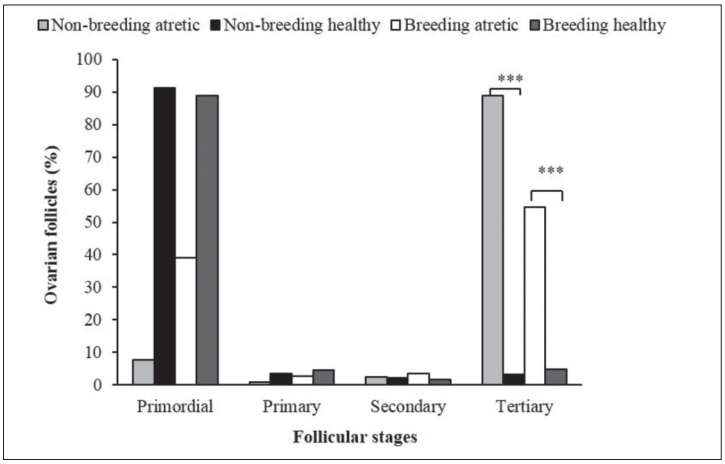

Fig 7. Percentage of follicles at different stages of development during breeding and non-breeding seasons. The difference between atretic and healthy follicle at tertiary stage was significant (*** P<0.001) determined by Mann Whitney test

Table 1. *Immunolocalization of active caspase-3 in ovarian Bedouine goat follicles*				
Cells	**Preantral Follicles**			**Antral Follicles**
	Primordial	**Primary**	**Secondary**	
Oocyte	++	+/-	-	-
Cumulus oophorus	-	-	-	++
Granulosa	-	+/-	+	++
Theca	-	-	+/-	+
Symbols are as follows: - negative, +/- variable, + moderate, ++ strong				

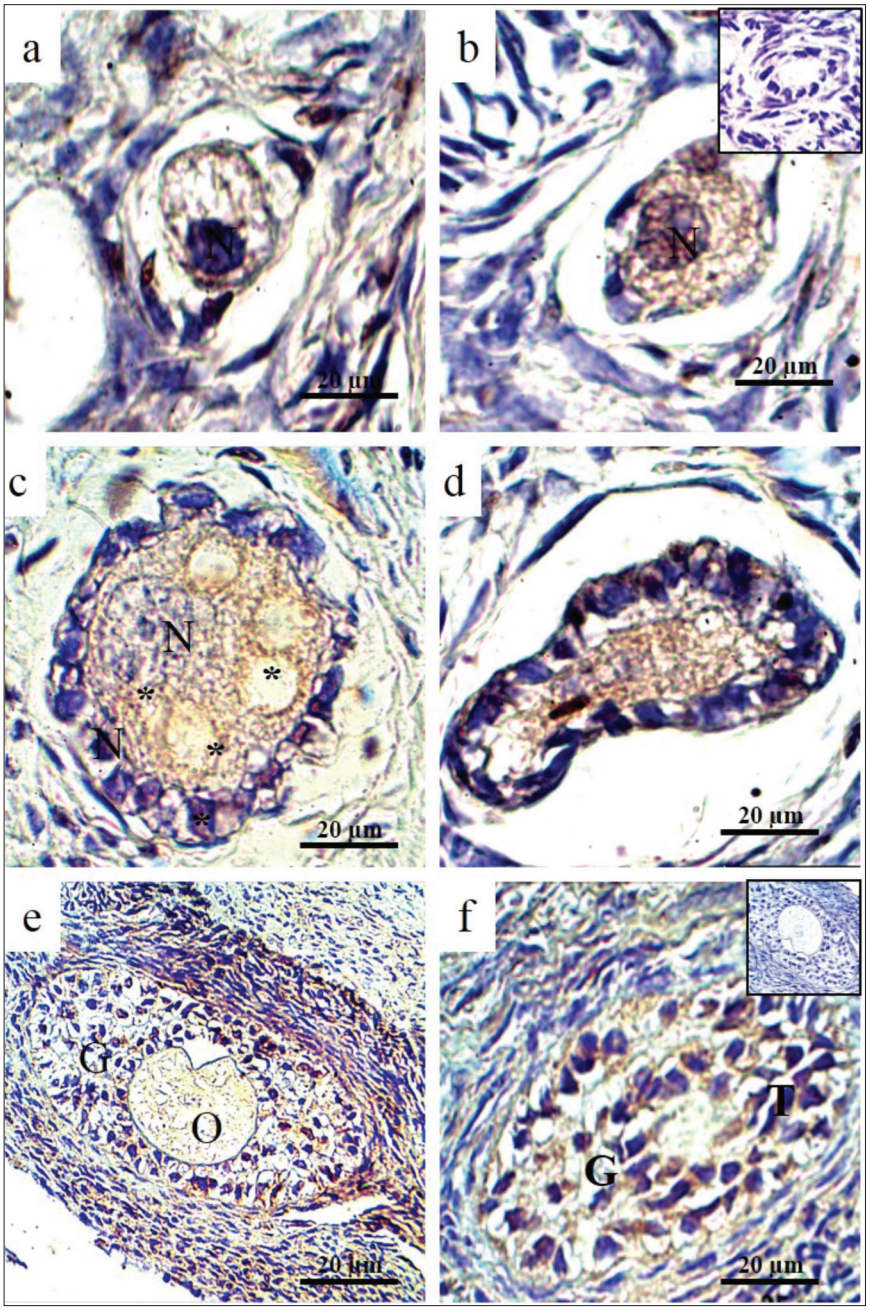

Fig 8. Immunolocalization of active caspase-3 in atretic preantral follicles in Bedouin goat ovaries. (a, b) primordial follicles surrounded by a few flattened follicular cells; (c, d) primary follicles, surrounded by one layer of cuboidal follicular cells; (e, f) secondary follicles. No immunostaining was observed in negative controls (inset); G: Granulosa; N: Nucleus; O: Oocyte; T: Theca

consistent with those found in small follicles of human ovary [24]. In the antral follicles, no labeling was detected in the oocytes despite the morphological abnormalities observed. However, a more frequent positive signal was detected in the granulosa cell, cumulus oophorus and theca cells; similar results were reported in rat [37]. Some

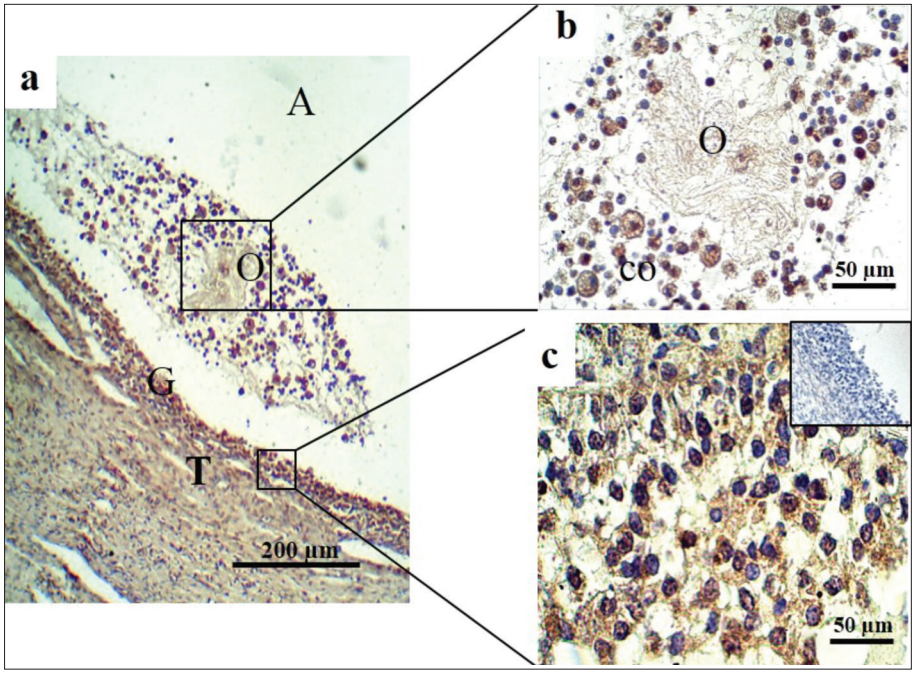

Fig 9. Immunolocalization of active caspase-3 in atretic antral follicles in Bedouin goat ovaries. No immunostaining was observed in negative controls (inset). (a) Antral atretic follicle; (b) oocyte surrounded by the cumulus oophorus cells; (c) granulosa layer; A: Antrum; Co: Cumulus oophorus; G: Granulosa; O: Oocyte, T: Theca

authors suggest that the half-life of active caspase-3 may be shorter to be detected, or that the atresia of small follicles was very rapid [25]. According to Berardinelli et al.[10], we hypothesize that apoptosis was a phenomenon taking place in time; the early expression of active caspase-3 in these follicles, primarily, recruits multiple molecular signaling inducing morphological modifications at the cellular and the follicular scales. Moreover, atresia of the antral follicle was a gonadotropin dependent process related to the sex steroid hormones. Particularly, the androgens were atretogenic to ovarian follicles and caused an elevation in morphological signs of atresia by increasing number of pycnotic granulosa cells [38]. The folliculogenesis study in the Bedouin goat during non-breeding season showed an intense immunolabeling to androgen receptors in the granulosa cells of atretic antral follicles [39]. Our study revealed that atresia process occurs principally in the antral stage; this result is in agreement with those reported in mice [40].

In conclusion, in Bedouin goat, morphological changes reach atretic follicles by apoptosis via caspase-3 signaling pathway in breeding and non-breeding seasons with a high proportion in antral stage.

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Research Article

Determination of ANAE and ACP-ase Positive Lymphocytes of Peripheral Blood and Endometrium Tissues in Experimental Hypothyroidism-Induced Rats [1]

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Abstract

This study was aimed to provide information about the status of the immune system by revealing changes in peripheral blood leukocyte (PBL) percentages, ANAE- and ACP-ase(+) lymphocyte rates in peripheral blood (PB) and endometrium tissues of experimental hypothyroidism-induced rats. In this study, 15 healthy female Wistar Albino rats were used. Rats were fed through 4 weeks. The Group E (experimental, n=9) is group that were made hypothyroidism by intraperitoneal methimazole enjection for 2 weeks. Rats of the Group C (control, n=6) were untreated. In the 2nd and 4th weeks, ANAE- and ACP-ase(+) lymphocyte rates of the Group E were higher than Group C in PB. Excepting eosinophil and basophil leukocyte rates, there was no statistical difference in the other PBL percentages in the both of weeks. In PB, while lymphocyte rate of the 4th week was no statistically different (P>0.05), it was found lower in Group C. There was no alteration in ANAE- and ACP-ase(+) lymphocyte rates of uterine tissue. As a result, whereas hypothyroidism caused significant alterations in PBL and T lymphocyte rates, the any marked changes was not observed in the uterine tissue.

Keywords: *ACP-ase, ANAE, Endometrium, Hypothyroidism, Methimazole*

Deneysel Hipotiroidizm Oluşturulan Ratlardaki Periferik Kan ve Endometriyum Dokularında ANAE ve ACP-az Pozitif Lenfositlerin Belirlenmesi

Öz

Bu çalışma, deneysel hipotiroidi oluşturulan ratların periferal kan lökosit (PKL) yüzdelerindeki, periferal kan ve endometriyum dokularındaki ANAE ve ACP-az (+) lenfosit oranlarındaki değişiklikleri açığa çıkararak bağışıklık sisteminin durumu hakkında bilgi vermeyi amaçlamaktadır. Çalışmada 15 sağlıklı dişi Wistar Albino rat kullanıldı. Ratlar 4 hafta boyunca beslendi. Grup D (deneysel, n=9) 2 hafta boyunca intraperitoneal methimazol enjeksiyonu ile hipotiroidizm oluşturulan gruptur. Grup K'nın (kontrol, n=6) ratları normal beslendi. İkinci ve dördüncü haftalarda, Grup D'nin periferal kandaki ANAE- ve ACP-az(+) lenfosit oranları Grup K'den yüksekti. Eozinofil ve bazofil lökosit oranları hariç olmak üzere, her iki haftada da diğer PKL oranlarında istatistiksel bir fark yoktu. 4. haftadaki periferal kan lenfosit oranı istatistiksel olarak farklı bulunmazken (P>0.05), Grup K'de daha düşük bulundu. Uterus dokusunda ANAE- ve ACP-az(+) lenfosit oranlarında değişiklik görülmedi. Sonuç olarak, hipotiroidizmin PKL ve T lenfosit oranlarında önemli değişikliklere neden olurken uterus dokusunda herhangi belirgin bir değişikliğe yol açmadığı gözlendi.

Anahtar sözcükler: *ACP-az, ANAE, Endometriyum, Hipotiroidizm, Methimazol*

INTRODUCTION

Thyroid hormones play an important role not only in metabolic disorders [1], but also in the development and function of the immune and reproductive systems [2]. Hypothyroidism, one of the most common thyroid disorders

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in humans, is insufficient production of thyroid hormones by the thyroid gland [3]. This disorder can be arised as a consequence of thyroid disfunction, impedement in mechanism that control thyroid function, or complication during treatment of hyperthyroidism [4]. In studies showing the effects of thyroid hormones on adaptive immunity have been seen that human hypothyroidism, as well as in rodents hypothyroidism induced pharmacologically and surgical, is associated with a decrease in thymic activity. Low concentrations of 3,3´,5-triiodo-L-thyronine (T3) and L-thyroxine (T4) can stimulate T cell proliferation including cell-mediated immunity [5,6]. Activation of T lymphocyte subtypes occur in severe hypothyroidism [7]. In clinical cases of hypothyroidism the spontaneous migration of polymorph nuclear leukocytes (PMNL) was found to be impaired [8]. Furthermore, the effects of hypothyroidism are directly on gonadotropin and steroid hormones [9]. Hypothyroidism is one of the most obvious causes of infertility, menstrual disturbance, spontaneous recurrent abortion, and of stillbirths [10]. The effect of hypothyroidism is more marked on the endometrium [11]. One of the most complex tissues is endometrium. Because it undergoes many dynamic changes, such as cytokines, growth factors, hormones and adhesion molecules [12,13]. Martinez et al.[14] reported that T lymphocyte was major class of lymphocytes in uterine.

Alpha-naphthyl acetate esterase (ANAE) demonstration is a method, which is used distinct from each other of T lymphocytes, B lymphocyte and monocytes [15] Acid phosphatase (ACP-ase) demonstration is also a method and specific for cell populations in which the majority of the T lymphocytes are formed in mammals [16].

This study was planned to provide information about the immune system by revealing changes in peripheral blood leukocyte (PBL) percentages, ANAE- and ACP-ase(+) lymphocyte rates in PB and endometrium tissues of experimentally hypothyroidised rats.

MATERIAL and METHODS

Research Material

Ethic approval was obtained from Selcuk University Experimental Medical Practice and Research Center (SUDAM) Animal Experiments Ethics Committee (2016/13). In this study, it was used 15 healthy female Wistar Albino rats (198-250 g), 12-14 weeks of age. Rats were caged individually on 12:12 h light-dark schedule at the room temperature (22±1°C), and fed with commercial rat food and water which were available *ad bilitum*.

Experimental Procedures

The rats were divided into two groups: animals from the first group (n=9) were made hypothyroid by intraperitoneal (IP) methimazole enjection (10 mg/kg/day) for 2 weeks as per methods of Parija et al.[17] and Swann [18]. Animals in the other group (n=6) were untreated control (C). In the 2nd week, total serum T3 and T4 concentrations in PB which was taken from the lateral tail veins were determined using the ADVIA Centaur CP Immunoassay System (detection kits provided by Siemens). To see chronic effects on the tissues, the both of groups were made normal feeding for 2 weeks. In the 4th week, the all rats were sacrificed by cervical dislocation under general anesthesia with ketamin (10 mg/kg, IM) and ksilazin (5 mg/kg, IM).

Collection and Processing of Tissue Samples

In the 2nd and 4th weeks, from each blood samples, six blood smears were prepared and fixed in a gluteraldehyde-acetone solution. Two smears were stained for each PBL formula, ANAE and ACP-ase demonstrations [15]. Uterine samples were fixed in formol calcium solution and the samples were incubated in 22 h formal sucrose solution and kept in 22 h Holt's solution for enzyme demonstrations. Then, cryostat sections (12 µm) were taken from samples. These preparations were stained for ANAE and ACP-ase demonstrations [19]. Both PB and uterine tissue samples were stained with 1% methyl green (Merck) for counterstain. For PBL formula, blood smears were stained with May Grünwald-Giemsa staining method [20].

Evaluation of the Stained Tissue Samples

Respectively, as shown in *Fig. 1* and *Fig. 2*, in the PB smears and uterine preparations which were made ANAE demonstration, there were dot-like reddish brown granules of ANAE (+) lymphocytes/T lymphocytes. Respectively, as shown in *Fig. 3* and *Fig. 4*, in PB smears and uterine preparations lymphocytes containing one to three pinkish red cytoplasmic granules were considered to be ACP-ase (+). In each of the PB smears stained for ANAE and ACP-ase activity, 200 lymphocytes were counted and positivity rates were expressed as the percentage of counted cells. For PBL counts, 100 leukocytes were counted in a light microscope, and leukocyte formula were calculated *(Fig. 5)*. In the uterine preparations, ANAE- and ACP-ase (+) lymphocytes were counted in the total 0.1 mm^2 area from 10 different uterine areas.

Statistical Analysis

Comparison of parameters between groups of the 2nd and 4th weeks was analysed using Independent-Samples T test. Differences in parameters between the 2nd and 4th weeks within the groups was drawn using the Paired-Samples T test. Significance was set at $P<0.05$ [21].

RESULTS

Changes in T3 and T4 levels of groups after methimazole treatment were given *Table 1*. In the 2nd and 4th weeks, T3 and T4 hormone levels were statistically lower in the

Table 1. *Serum T_3 (pg/ml) ve T_4 (ng/dl) levels* ±SE

Groups	FT3(2)	FT4(2)	FT3(4)	FT4(4)
Control (n=6)	3.22±0.13[a]	2.35±0.06[a]	3.35±0.18[a]	2.39±0.14[a]
Experimental (n=9)	2.83±0.21[b]	1.61±0.19[b]	2.12±0.15[b]	1.72±0.25[b]

[a,b] Values within a column with no common superscripts are significantly (P<0.05) different; FT3(2): 2nd week serum T_3, FT4(2): 2nd week serum T_4, FT3(4): 4th week serum T_3, FT4(4): 4th week serum T_4

Table 2. *Differences in parameters between the 2nd and 4th weeks within the groups (%)*±SE

Week	FT3	FT4
2nd	2.99±0.15[a]	1.90±0.15
4th	3.27±0.12[b]	2.00±0.14

[a,b] Values within a column with no common superscripts are significantly (P<0.05) different

Table 3. *The proportions of peripheral bood lymphocyte and leukocyte in different weeks (%)*±SE

Week	Groups	ANAE(+)	ACP-ase(+)	Lymphocyte	Neutrophil	Monocyte	Eosinophil	Basophil
2nd	Control (n=6)	21.17±4.68[b]	37.17±9.25[b]	80.17±3.71	18.00±4.26	1.17±0.48	0.67±0.21	0.00±0.00
	Experimental (n=9)	74.67±2.52[a]	49.56±5.81[a]	73.22±2.85	21.33±3.24	2.11±0.51	1.11±0.39	0.00±0.00
4th	Control (n=6)	29.17±4.37[b]	38.50±8.42[b]	74.17±3.77	21.00±3.70	2.50±0.89	2.00±0.58[a]	0.33±0.21[a]
	Experimental (n=9)	68.78±2.20[a]	47.08±5.68[a]	78.22±3.53	19.33±3.48	2.33±0.37	0.11±0.11[b]	0.01±0.00[b]

[a,b] Values within a column with no common superscripts are significantly (P<0.05) different

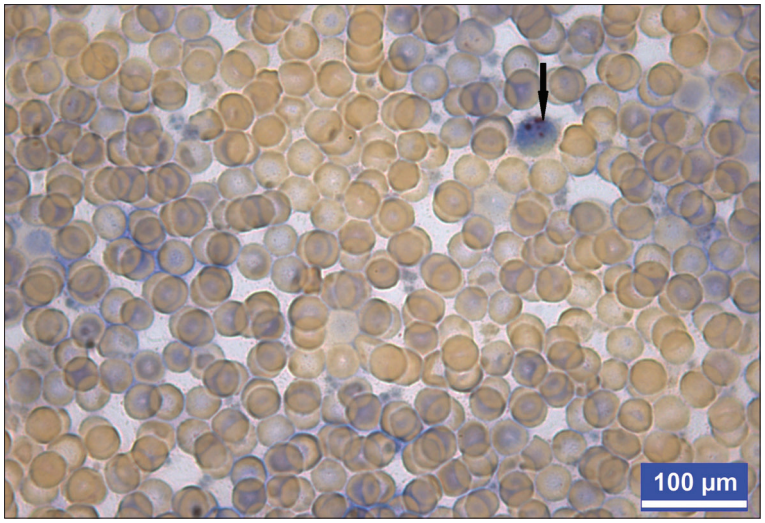


Fig 1. A peripheral blood T-lymphocyte in Group E. ANAE demonstration. *Arrow:* ANAE (+) lymphocyte/T lymphocyte. Bar: 20 µm

Group E when compared with Group C (P<0.05). According to the weeks within groups, it was statistically seen that T3 level of 2nd week was lower than that of it in the 4th week (P<0.05) *(Table 2)*. As shown in *Table 3*, in the 2nd and 4th weeks, the highest peripheral blood ANAE- and ACP-ase (+) lymphocyte *(Fig. 1* and *Fig. 3*, respectively) rates were determined in the Group E (P<0.05). In the 4th week, according to the PBL percentages *(Fig. 5)*, the eosinophil and basophil leukocyte rates were statistically lower in the Group E than that of the Group C (P<0.05). No statistically significant, it was observed that the Group E had higher lymphocyte rate than the Group C (P>0.05). For both of weeks, there was no statistical difference in the other leukocytes between the groups (P>0.05). No the differences between two weeks within the groups are statistically significant *(Table 4)*. In the endometrium, as shown in *Table 5*, while the ANAE(+) lymphocyte *(Fig. 2)* number in the Group C was 19.33/0.1 mm^2, this rate was found 20.00/0.1 mm^2 in the Group E. There was no statistical difference between the groups (P>0.05). When the ACP-ase(+) lymphocyte *(Fig. 4)* number of the Groups C and E were examined, it was noted that there was no statistical difference between the groups (P>0.05).

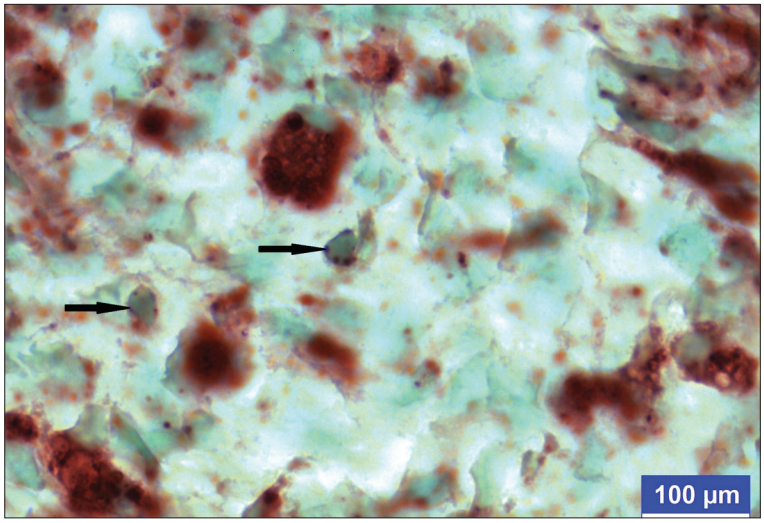
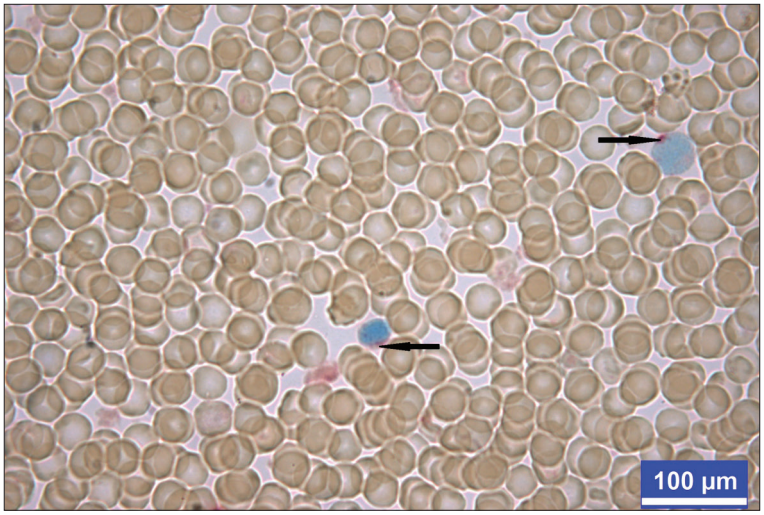


Fig 2. T-lymphocytes in endometrium basalis from Group E. ANAE demonstration. *Arrows:* ANAE (+) lymphocyte/T lymphocyte. Bar: 100 µm

Fig 3. An ACP-ase (+) peripheral blood lymphocyte in a rat from Group E. ACP-ase demonstration. *Arrows:* ACP-ase (+) lymphocytes. Bar: 20 µm



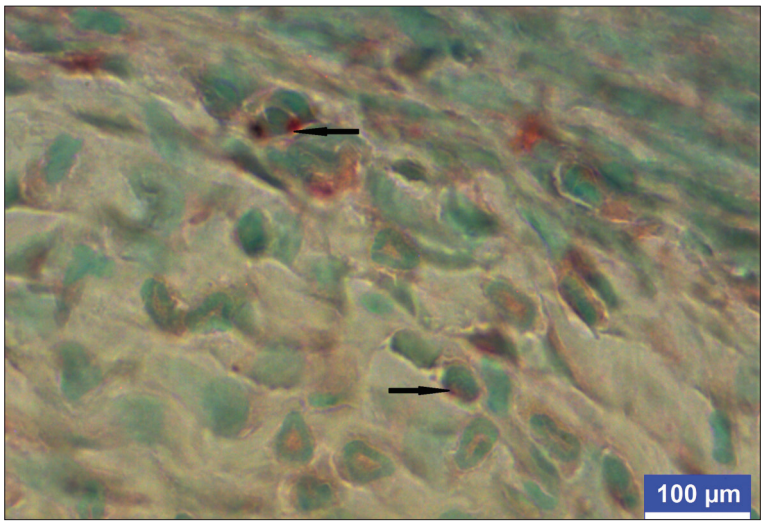


Fig 4. An ACP-ase (+) lymphocyte in endometrium basalis from Group C. ACP-ase demonstration. *Arrows:* ACP-ase (+) lymphocyte. Bar: 100 µm

DISCUSSION

T3 and T4 hormones secreted by the thyroid gland are formed from a large prohormone molecule called as thyroglobulin and enter the cells by binding to the thyroid hormone receptor α and β [22]. These thyroid hormones which is necessary for sexual development and life regulate the steroid hormones secretion. Findings such as lipidode deficiency, impotence, menopause, excessive bleeding and menstrual irregularities are observed in

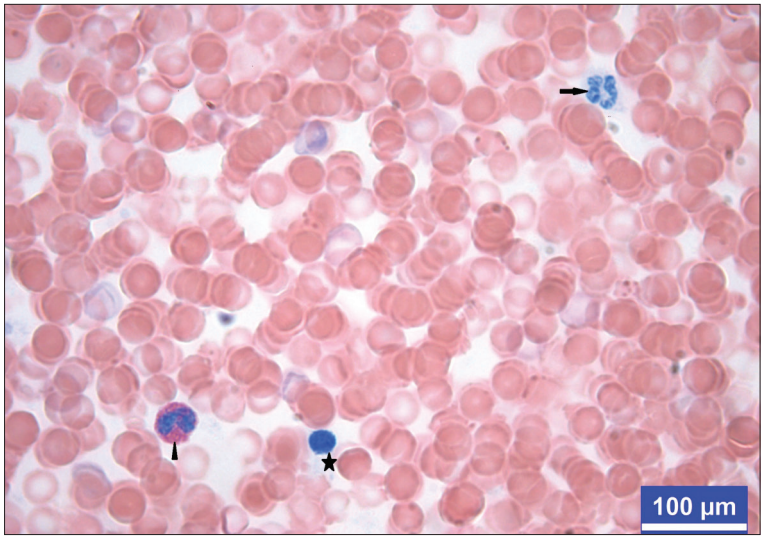


Fig 5. Peripheral blood smear in a rat from Group E. May Grünwald-Giemsa staining. *Arrow:* Neutrophil, *Arrowhead:* Eosinophil, *Asterisk:* Lymphocyte. Bar: 20 µm

Table 4. *Values of parameters between the 2nd and 4th weeks within the groups (%)*±SE

Week	ANAE(+)	ACP-ase(+)	Lymphocyte	Neutrophil	Monocyte	Eosinophil	Basophil
2nd	53.27±7.38	84.60±12.47	76.00±2.36	20.00±2.53	1.73±0.37	0.93±0.25	0.00±0.00
4th	52.93±5.59	89.60±4.61	76.60±2.57	20.00±2.48	2.40±0.40	0.87±0.34	0.13±0.91

No the differences between two weeks within the groups are statistically significant (P>0.05)

Table 5. *The proportions of the ANAE(+) ve ACP-ase (+) lymphocytes in endometrium basalis region of uterine (%)* ± SE (number/0.1 mm²)

Week	Groups	ANAE(+)	ACP-ase(+)
4th	Control (n=6)	19.33±0.80	8.50±0.62
	Experimental (n=9)	20.00±0.71	8.00±0.44

No the differences between the groups are statistically significant (P>0.05)

hypothyrodism. Although the most synthesized hormone in the thyroid gland is T4, the most effective is the T3 [23].

In humans, one of the most common thyroid disorders is hypothyroidism, which is defined as low levels of thyroid hormones in the blood [3]. Deficiency of thyroid hormone production causes serious abnormalities [1]. For establishing hypothyroidism in experimental animals, methimazole is frequently used in the treatment of human hyperthyroidism [24]. In this study, the methimazole dose and form of implementation given to the Group E was determined from the results of previous studies [17,18].

ANAE is an enzyme that demonstrates T lymphocytes. It is also known that dot-like positivity is specific for T lymphocytes [15]. ACP-ase is one of the lysosomal enzymes in lymphocytes. Some investigators have demonstrated ACP-ase reactivity in human peripheral blood T lymphocytes [15,16]. In the direction of this information, we found that T lymphocyte and ACP-ase (+) lymphocyte rates were higher in the Group E (P<0.05). This situation was explained that low serum T3 and T4 concentrations can stimulate T lymphocyte proliferation [7].

Although we did not statistically find any change in the other PBL excluding eosinophil and basophil leukocytes, we was observed that the lymphocyte ratio was higher in the Group E. This suggests that thyroid hormones have regulatory effects on immunological activity at the cellular level [25]. There is also information that thyroid hormones modify lymphocyte activity [26,27]. In severe hypothyroidism, activation of T lymphocyte subclasses, reduction of natural killer cells and reduction of CD4 T lymphocyte responses are seen [7]. Compared with healthy subjects, hypothyroidism has been found to be impaired in the spontaneous migrations of polymorphonuclear leukocytes in clinical cases [8]. Hypothyroidism is accompanied by spleen and lymph node involution and decreases in humoral and cellular immune responses [28].

As in other systemic mucosal tissues, uterine mucosa normally contains T and B lymphocytes [29]. However, the uterine tissue under the influence of hormonal changes is rearranged with the sexual cycle or the pregnancy. Karaca et al.[30] reported that the distribution of ANAE (+) T lymphocyte numbers in uterine tissues of pre-implantation period of goats was lower than that of non-pregnant animals. Akbulut et al.[15] reported significant reductions in ANAE- and ACP-ase (+) lymphocyte numbers throughout the entire pregnancy in the decidua basalis region of endometrium. In the direction of the data obtained from this study, there was no change in terms of both ANAE- and ACP-ase(+) lymphocyte numbers and distribution in endometrium basalis. We can say that hypothyroidism has no any an effect on uterine in terms of lymphocyte number and distribution.

Prevention of mental and developmental disorders is possible with a good understanding of the interaction between the endocrine and immune systems. The immune system is under the influences of many hormones. Routine control and treatment of thyroid gland functions at pre-pregnancy and throughout pregnancy is an important factor for a healthy pregnancy. As a result of this study, hypothyroidism made significant changes both the peripheral blood T lymphocyte and leukocytes counts. Obtained findings may help doctors to evaluate the immunological status of hypothyroid women. Since the these techniques are simple, much cheaper, less time consuming applications, we suggest that it can be given as a laboratory service to assist of women in the early diagnosis of some gestational disorders. However, further studies should be planned in terms of our understanding more detailed of relationship between thyroid gland diseases and uterine.

As a result, whereas hypothyroidism caused significant alterations in PBL and T lymphocyte rates, the any marked changes was not observed in the uterine tissue.

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Research Article

Investigation of the Relationships Between Wool Quality and Microsatellite in Hybrids of Australian Merino and Chinese Merino

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Abstract

This work was carried out to analyze of effects of twelve microsatellite loci and genetic heterozygosity on wool fineness and natural length, which are important indicators for evaluating of wool quality. 131 individuals from F2 and F3 generations of Australian Merino and Chinese Merino sheep (Xinjiang military reclamation type) mating were used as experimental subjects. Five loci on chromosome 1 and seven loci on chromosome 6 were determined as related with fineness and natural length of wool. The results showed that 3 loci significantly associated with wool fiber diameter (WFD), and 4 loci were significantly related to wool natural length (WNL). WFD increased by approximately 0.2%-2.5%, and WNL decreased by 2%-10.93%, and also heterozygosity increased by 0.05 in the range of 0.5 to 1.0. These results could partially explain the molecular mechanism of heterosis for sheep wool quality. Provide theoretical support for the effective exploitation and utilization of this precious resource.

Keywords: *Wool fiber diameter, Wool natural length, Microsatellite, Heterozygosity, Sheep*

Avustralya Merinosu ve Çin Merinosu Hibritlerinde Yün Miktarı İle Mikrosatellit Arasındaki İlişkinin Araştırılması

Öz

Bu çalışma, yün kalitesini değerlendirmede önemli belirteçler olan yün inceliği ve doğal uzunluğu üzerine on iki mikrosatellit bölgenin ve genetik heterozigotluğun etkilerini incelemek amacıyla yapılmıştır. Avustralya Merinosu ve Çin Merinosu eşleşmesinden (Xinjiang askeri ıslah tipi) F2 ve F3 jenerasyonlarından 131 koyun deneysel materyal olarak kullanıldı. Kromozom 1'de beş bölge ve kromozom 6'da yedi bölge yün inceliği ve doğal uzunluğuyla ilişkili olarak belirlendi. Elde edilen sonuçlar 3 bölgenin anlamlı derecede yün teli çapı ile 4 bölgenin de anlamlı derecede yün doğal uzunluğu ile ilişkili olduğunu gösterdi. Yün teli çapı yaklaşık %0.2-%2.5 artarken ve yün doğal uzunluğu %2-%10.93 azaldı ve heterozigotluk 0.5 ile 1.0 aralığında 0.05 kadar arttı. Bu sonuçlar koyun yün kalitesinde heterozisin moleküler mekanizmasını kısmen açıklamaktadır. Çalışma ile bu değerli kaynağın etkili yayılımı ve kullanımı konusunda teorik destek sağlanmıştır.

Anahtar sözcükler: *Yün lifi çapı, Yün doğal uzunluğu, Mikrosatellit, Heterozigotluk, Koyun*

INTRODUCTION

Xinjiang is an important sheep production base in China, and the fine-wool sheep number ranks first in the country. China Merino sheep (Xinjiang Army-type) has also become one of the most famous fine wool sheep in the world [1]. In the breeding system of fine-wool sheep, when the wool has a fineness of more than 80, it can be grouped into the same type for breeding [2,3]. However, in the present situation, it is difficult to improve or maintain the wool quality of existing high-quality fine wool sheep, either in normal breeding or in the application of high-quality new breeding technology [4]. The key problem is that there are few studies on the genetic methods of controlling sheep wool fiber traits. Without understanding this genetic relationships, improving and enhancing high-quality fine-wool sheep can easily be counterproductive, leading to quality degradation [5,6]. To improve this imminent problem, it is necessary to study the genetic relationships related to the wool production traits of Chinese Merino sheep.

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Microsatellites (simple repeat sequences or short simple tandem sequences) are a very popular molecular genetic marker [7]. They consist of a core sequence of 1-6 bp and a flanking sequence specifically located at a certain position on a chromosome. Studies have shown that microsatellites have been widely used in paternity testing of large domestic animals, such as pigs [8,9], horses [10], sheep [11] and cattle [12], and in poultry [13] and fish [14]. Because microsatellites are highly polymorphic and are widely used in the construction of genetic linkage maps [15]. In marker-assisted selection of livestock, microsatellites inferred genotypic values from phenotypic values by changing the population level and using the established sheep resource families to locate quantitative trait loci (QTL) related to reproduction [16], carcass [17], meat quality [18], etc., near some microsatellites. The studies have shown that WFD and WNL is an important indicator of economic value. Its variation accounts for 61% of total wool profit [19]. However, it is necessary to study the molecular genetic basis of wool characteristics, which provide theoretical support for the effective exploitation and utilization of this precious resource.

Wool fineness and WNL are important indices for evaluating wool quality [20]. That, this study analyzed the effects of twelve microsatellite loci and genetic heterozygosity on wool fineness and natural length to provide a theoretical basis in sheep breeding.

MATERIAL and METHODS

Experimental Animal Establishment of Reference Families and Sample Collection

All procedures involving animals were approved by the Animal Care and Use Committee of Shihezi University, Shihezi, Xinjiang. All animal procedures and experiments were conducted based on the guidelines approved by the Committee of Animal Care and Use at Shihezi University, China. All of the feeding, experimental management and sample collection were conducted by professionals who were highly experienced in conducting these activities to avoid human error and relieve the suffering of the animals.

Chinese Merino sheep (Xinjiang Army) were selected from the Ziniquan breeding sheep farm in Xinjiang, China. All test wool and blood samples were obtained from the Ziniquan breeding sheep farm. To eliminate the influence of other factors, the unified management system of the field was adopted in the study farm feeding and stabling.

Six Australia merino rams were selected according to WFD for 70, 66 and 58 yams and crossed with 160 Chinese Merino ewes having WFD of 70, 66, 64, 60 and 56 yams (diameter 19.57-27.56 μm). The F1 progeny were mated with half-siblings to obtain 131 F2 generation ewes with trait records. Then, from the F2 generation, 6 rams (fineness-selective traits) similar to the fineness of the ancestral rams were selected for semi-sibling mating. Thirteen mating combinations were produced *(Table 1)*, and 70 F3 individuals with trait records were obtained, of which 12 pairs were double lambs.

Wool Fibre Diameter (WFD) and Wool Nature Length (WNL) Assay Method

Three small bundles of wool were randomly taken from a different part of a bundle of hair samples and combined into a bundle. The 0.3 mm wool fiber fragments were cut by a slicer in the middle of the hair bundle, and the process was repeated three times. The chips were made into wool, and the diameter of the fibers was measured under a microscope to at least 300 μm, with a weighted average of wool fineness, that is WFD. Two hundred wool fibers were randomly selected from a bundle of wool samples, and the length in the natural state was measured with a ruler; and the average length of the wool was obtained by averaging the WNL [21].

Microsatellite Genotyping

Blood samples from a total of 173 sheep (6 Australia merino rams, 160 Chinese Merino ewes, and their 7 offspring) were collected from the jugular vein using vacutainer tubes containing EDTA as an anticoagulant. DNA isolation kit (Tiangen, China) was used to extract genomic DNA from blood samples.

Twelve candidate microsatellite loci were selected: BM6506, BM1824, BM6438, ILSTS004, OarDB6 from chromosome 1 and OarAE101, BM 415, BM1329, BM4621, OarHH55, BM143, OarJMP8 from chromosome 6. Primers were synthesized by Shanghai Biotechnology Co., Ltd. and designed according to the literature and Australian Sheep Gene Mapping Web Site *(http://rubens.its. unimelb.edu.cn/-jillm/jill.htm)* [22,23].

Polymerase Chain Reaction (PCR) was performed in a 25 μL

Table 1. *Sheet of thirteen kinds of matiny compose*

Ram (♂) / Ewe (♀)	70 yams (18.78-20 μm)			66 yams (20.22 μm)		580 yams (25.75 μm)	
	n	♀	F3	♀	F3	♀	F3
70 yams (18.78-20.02 μm)	9	3	3 (17.55-18.73 μm)	3	3 (19.09-22.46 μm)	3	3 (15.08-18.91 μm)
66 yams (20.09-21.47 μm)	21	16	16 (17.81-23.58 μm)	3	3 (19.28-21.06 μm)	2	2 (16.88-21.12 μm)
64 yams (21.15-23.01 μm)	19	13	14 (15.99-20.07 μm)	4	4 (18.76-20.59 μm)	2	2 (20.62-22.08 μm)
60 yams (23.10-24.09 μm)	15	9	9 (17.90-21.78 μm)	4	5 (18.59-22.04 μm)	2	2 (25.06-23.66 μm)
56 yams (27.10-28.11 μm)	4			4	4 (21.92-22.64 μm)		

reaction volume containing 2 μL of DNA (50 ng), 1 μL of each primer, 12.5 μL of PCR Mix, and 8.5 μL of water. Loci were amplified using standard PCR cycling conditions of 95°C for 2 min, 36 cycles of 30 s at 94°C, 30 s at the primer sequences and annealing temperatures (T1), and 45 s at 72°C. PCR products were resolved by electrophoresis on standard 10% polyacrylamide sequencing gels and detected by silver staining [11]. The gels were stained with silver to visualize microsatellite loci and allelic patterns and were analyzed by software. T1 of all markers are presented in *Table 2*.

Statistical Method

Population gene heterozygosity (Heterozygosity, H) is calculated using Nei [24]:

$$H = 1 - \sum_{i=1}^{n} P_i^2$$

Calculation of individual gene heterozygosity Hi: If an individual has m loci, let n be a sample of m loci. In the sampled n individual microsatellite loci, the ratio H1 of the number of heterozygous seats H1 to the number of sampled seats n is the individual gene heterozygosity, and the formula is as follows:

$$H_i = H_l / n$$

Individuals were divided into 10 heterozygosity grades in steps of 0.05 based on individual gene heterozygosity, according to whether the microsatellite locus and WFD are related to the genetic heterozygosity: individual heterozygosity calculated in all 12 loci, 3 individual heterozygosities calculated in relation to the fineness of wool, 9 individuals calculated in neutral seats heterozygosity, 4 individual heterogeneities calculated with WNL, and 8 neutral loci with individual heterozygosity. Classification criteria and classification results are shown in *Table 3, Table 4*.

Table 2. *Primer sequence of nine microsatellite markers and reference annealing*

Gene Name	Primer Sequence	T1/°C
BM6506	GCACGTGGTAAAGAGATGGC AGCAACTTGAGCATGGCAC	50
BM1824	GAGCAAGGTGTTTTTCCAATC CATTCTCCAACTGCTTCCTTG	47
BM6483	TTGAGCACAGACACAGACTGG ACTGAATGCCTCCTTTGTGC	58
ILSTS004	CTTAAAATCTGTCTTTCTTCC TAGTGTGTATTAGGTTTCTCC	54
OarDB6	GACATGACTAAAGCAATTTAGCATGC TGGACTACAGTCCATAGCTCTC	57.5
OarAE101	TAAGAAATATATTTGAAAAAACTGTATCTCCC TTCTTATAGATGCACTCAAGCATGG	63
BM415	GCTACAGCCCTTCTGGTTTG GAGCTAATCACCAACAGCAAG	52
BM1329	TTGTTTAGGCAAGTCCAAAGTC AACACCACAGCTTCATCC	64
BM4621	CAAATTGACTTATCCTTGGCTG TGTAACATATGGGCTGCATC	51
OarHH55	GTTATTCCATATTCTTTCCTCCATCATAAGC CCACACAGAGCAACTAAACCCAGC	66.5
BM143	ACCTGGGAAGCCTCCATATC CTGCAGGCAGATTCTTTATCG	68
OarJMP8	CGGGATGATCTTCTGTCCAAATATGC CATTTGCTTTGGCTTCAGAACCAGAG	67

Polymorphic information content (PIC) is calculated according to the formula provided by Botstein [25]:

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2(P_i P_j)^2$$

Pi and Pj are the i-th and j-th allele frequencies in the population, and n is the number of alleles.

The phenotypic mean of microsatellites was analyzed by the generalized linear model (GLM) of SAS (V6.12) software, and multiple comparisons between genotypes were performed using the least significant difference method (LSD) [26].

RESULTS

Wool Phenotypic Traits and Microsatellite Locus Heterozygosity

WFD: 20.96±0.2008 μm, WFL: 10.784±0.8907 cm.

The heterozygosity and PIC of 12 microsatellite loci on chromosomes 1 and 6 are shown in *Table 5*. The heterozygosity ranged from 0.6069 to 0.7965, and the PIC ranged from 0.5258 to 0.8008. This result demonstrates that heterozygosity significantly associates with PIC. The number of alleles reported in the literature and Australian Sheep Gene Mapping Web Site *(http://rubens.its.unimelb.edu.cn/-jillm/jill.htm)* were not fully detected in this study.

Microsatellite Heterozygosity Associations with WFD and WNL

BM1824, BM6438, BM6506 alleles at 12 microsatellite loci indicate significant association with WFD, but the remaining 9 loci do not (P<0.05, *Table 6)*. Chr1:ILSTS004 and Chr6:OARAE101, OARJMP8, BM143 alleles at 12 microsatellite loci manifest significant relevance to WNL (P<0.05, *Table 7)*.

Correlation Analysis Between Heterozygosity with WFD and WNL

The experimental sheep were divided into 10 levels according to the level of heterozygosity. The sample in each of the levels were similar *(Table 3, Table 4)*. In terms of heterozygosity classified by counting 12 microsatellite loci, analysis showed that the value of WFD was significantly different (P<0.05) among 10 heterozygosity levels. Heterozygosity and WFD were directly proportional *(Table 8, Fig. 1)*. The WFD of level 2 (average heterozygosity 0.5450) had the highest diameter at 19.90 μm. In terms of heterozygosity

Table 3. *Mean heterozygosity and number of individuals at 10 levels in different classifications*

Level	Classified by 12 Loci		Classified by 3 Loci Associated with Wool Fiber Diameter		Classified by 9 Loci Unassociated with Wool Fiber Diameter	
	Heterozygosity Range/ (mean)	Observe Number/(%)	Heterozygosity Range/ (mean)	Observe Number/ (%)	Heterozygosity Range/ (mean)	Observe Number/ (%)
1	h≤0.5 (0.4605)	6 (4.58)	H≤0.5 (0.4826)	13 (9.93)	H≤0.5 (0.4210)	11 (8.40)
2	0.5<h≤0.55 (0.5450)	7 (5.34)	0.5<h≤0.55		0.5<h≤0.55	
3	0.55<h≤0.6 (0.5886)	10 (7.63)	0.55<h≤0.6		0.55<h≤0.6 (0.5600)	11 (8.40)
4	0.6<h≤0.65 (0.6360)	9 (6.87)	0.6<h≤0.65		0.6<h≤0.65 (0.6250)	7 (5.34)
5	0.65<h≤0.7 (0.6730)	11 (8.40)	0.65<h≤0.7 (0.6639)	66 (50.38)	0.65<h≤0.7 (0.6670)	13 (9.92)
6	0.7<h≤0.75 (0.7340)	24 (18.32)	0.7<h≤0.75		0.7<h≤0.75 (0.7370)	16 (12.21)
7	0.75<h≤0.8 (0.7930)	10 (7.63)	0.75<h≤0.8		0.75<h≤0.8 (0.7790)	16 (12.21)
8	0.8<h≤0.85 (0.8290)	25 (19.08)	0.8<h≤0.85		0.8<h≤0.85 (0.8330)	1 (0.76)
9	0.85<h≤0.9 (0.8810)	8 (6.11)	0.85<h≤0.9		0.85<h≤0.9 (0.8790)	28 (21.37)
10	h>0.9 (0.9580)	21 (16.04)	h>0.9 (0.9226)	52 (39.69)	h>0.9 (1.000)	26 (19.85)

Heterozygosity range/mean and observation number/(%) for ten heterozygosity levels classified by 12 loci, by 3 loci associated with wool diameter or by 9 loci unassociated with wool fiber diameter

Table 4. *Mean heterozygosity and number of individuals at 10 levels in different classifications*

Level	Classified by 12 Loci		Classified by 3 Loci Associated with Wool Natural Length		Classified by 9 Loci Unassociated with Wool Natural Length	
	Heterozygosity Range/ (mean)	Observe Number/ (%)	Heterozygosity Range/ (mean)	Observe Number/ (%)	Heterozygosity Range/ (mean)	Observe Number/ (%)
1	h≤0.5 (0.4605)	6 (4.58)	H≤0.5 (0.3960)	42 (32.06)	H≤0.5 (0.4620)	14 (10.69)
2	0.5<h≤0.55 (0.5450)	7 (5.34)	0.5<h≤0.55		0.5<h≤0.55	
3	0.55<h≤0.6 (0.5886)	10 (7.63)	0.55<h≤0.6		0.55<h≤0.6 (0.5760)	6 (4.58)
4	0.6<h≤0.65 (0.6360)	9 (6.87)	0.6<h≤0.65		0.6<h≤0.65 (0.6250)	12 (9.16)
5	0.65<h≤0.7 (0.6730)	11 (8.40)	0.65<h≤0.7 (0.6670)	8 (6.11)	0.65<h≤0.7 (0.6670)	1 (0.76)
6	0.7<h≤0.75 (0.7343)	24 (18.32)	0.7<h≤0.75 (0.7500)	40 (30.53)	0.7<h≤0.75 (0.7330)	32 (24.43)
7	0.75<h≤0.8 (0.7934)	10 (7.63)	0.75<h≤0.8		0.75<h≤0.8 (0.8000)	4 (3.05)
8	0.8<h≤0.85 (0.8288)	25 (19.08)	0.8<h≤0.85		0.8<h≤0.85 (0.8370)	11 (8.40)
9	0.85<h≤0.9 (0.8785)	8 (6.11)	0.85<h≤0.9		0.85<h≤0.9 (0.8680)	23 (18.32)
10	h>0.9 (0.9578)	21 (16.03)	h>0.9 (1.0000)	41 (31.30)	h>0.9 (1.000)	27 (20.61)

Heterozygosity range/mean and observe number/(%) for ten heterozygosity levels classified by 12 loci, by 4 loci associated with wool natural length or by 8 loci unassociated with wool natural length

Table 5. *Locus number, gene frequency, heterozygosity and polymorphism information content(PIC)*

Chromosome	Microsatellite	Allele Number	Gene Frequency	Heterozygosity	PIC
1	BM1824	4	0.1311, 0.0410, 0.0074, 0.0164	0.7377	0.7877
1	BM6438	5	0.1271, 0.0085, 0.0593, 0.0254, 0.0085	0.7712	0.8008
1	BM6506	4	0.1951, 0.0488, 0.0244, 0.0325	0.6992	0.6762
1	ILSTS004	5	0.0708, 0.0177, 0.0177, 0.0354, 0.0619	0.7965	0.7646
1	OarDB6	6	0.0964, 0.0175, 0.0175, 0.0175, 0.0351, 0.0088	0.7472	0.7185
6	OarAE101	5	0.1774, 0.0081, 0.0242, 0.0403, 0.0161	0.7339	0.7274
6	OarHH55	5	0.0645, 0.0645, 0.0242, 0.0161, 0.0403	0.7955	0.7769
6	OarJMP8	4	0.1145, 0.1985, 0.0153, 0.0076	0.6641	0.6123
6	BM4621	5	0.0756, 0.0672, 0.0756, 0.0252, 0.0084	0.7480	0.7234
6	BM143	3	0.1536, 0.1536, 0.0859	0.6069	0.5258
6	BM415	5	0.1111, 0.0171, 0.0171, 0.0256, 0.0513	0.7778	0.7699
6	BM1329	4	0.1750, 0.0250, 0.0167, 0.0583	0.7250	0.6097

classed by arithmetic heterozygosis of 12 microsatellite loci, analysis indicated that the value of WNL was significantly different (P<0.05) among 10 heterozygosity levels. Heterozygosity and WNL were inversely proportional *(Table 9, Fig. 2)*. The WNL of level 7 (average heterozygosity 0.7934) was the longest, at 11.588 cm.

Table 6. *The genotypic variance analysis of WFD at three microsatellite loci*

Loci	BM1824		BM65106		BM6438	
Genetype	n	Fiber Diameter (X±Sx)	n	Fiber Diameter (X±Sx)	n	Fiber Diameter (X±Sx)
AA	15	19.76±0.5605^{BCc}	23	20.48±0.4235^{A}	14	20.87±0.5385Aab
AB	0		3	20.53±0.4265^{A}	11	18.72±0.5399Bc
AC	44	20.96±0.3130^{ABb}	67	20.83±0.2465^{A}	42	20.53±0.2806Ab
AD	11	19.16±0.5402^{CDcd}	2	20.73±1.5350^{A}	10	20.49±0.8028Ab
BB	5	18.18±0.8821^{Dd}	1	24.46±0.0000	1	24.72±0.0000
BC	12	20.98±0.4683^{ABb}	0		0	
BD	5	22.26±0.7865^{Aa}	0		3	20.99±1.1875Aab
BE	0		0		2	21.99±0.4100Aa
CC	7	20.83±0.5417^{ABb}	2	17.17±0.3900^{B}	5	21.83±1.2218Aa
CD	2	20.38±3.7950^{ABbc}	4	17.12±0.9159^{B}	10	20.61±0.4772Aab
DD	1	21.47±0.0000	4	20.41±1.5077^{A}	3	21.79±1.6404Aa
EE	0		0		1	24.90±0.0000

Significant differences (P<0.05 or P<0.01) are shown using different letters in columns, as shown in Table 5

Table 7. *The genotypic variance analysis of WNL at four microsatellite loci*

Loci	OarAE101		OarJMP8		BM143		ILSTS004	
Genetype	n	Natural Length (X±Sx)	n	Natural Length (X±Sx)	n	Natural Length (X±Sx)	n	Natural Length (X±Sx)
AA	19	11.54±0.5533^{ACa}	14	13.00±0.6404^{Aab}	15	11.13±0.3637^{ABa}	8	10.76±0.6760^{ABb}
AB	12	9.97±0.5425^{BCb}	57	10.83±0.2192^{ABb}	39	10.67±0.3088^{ABab}	22	10.49±0.2988^{ABb}
AC	0		7	13.14±0.3221^{Aa}	13	11.61±0.5283^{A}	19	11.69±0.3259^{Aab}
AD	34	10.46±0.2837^{ABb}	5	10.51±0.8864^{ABb}	0		9	11.86±0.4773^{Aab}
AE	13	10.48±0.5106^{ABb}	0		0		12	11.33±0.6007^{Aab}
BB	1	8.22±0.0000	21	9.89±0.4174^{BC}	19	11.29±0.5018^{A}	1	6.66±0.0000
BC	2	11.73±1.2700^{ACa}	0		15	9.62±0.4129^{Bb}	2	8.52±1.9800^{Cd}
BD	3	12.50±0.8660^{ACa}	6	11.30±0.5626^{ABab}	0		1	7.89±0.0000
BE	1	11.05±0.0000	0		0		1	7.87±0.0000
CC	2	7.94±1.3950^{Bb}	2	9.28±1.3350^{BC}	10	11.66±0.4039^{A}	2	9.16±1.9750^{BCcd}
CD	7	10.81±0.7633^{ABab}	0		0		3	9.39±0.7285^{BCcd}
CE	5	12.60±0.1000^{Aa}	0		0		1	11.28±0.0000
DD	1	12.00±0.0000	1	11.51±0.0000	0		4	12.25±0.5951^{Aa}
DE	7	11.93±0.5051^{ACac}	0		0		8	10.23±0.7222^{ABb}
EE	2	10.36±0.9350^{ABab}	0		0		3	10.21±1.6647^{ABbc}

Table 8. *Effect of genetic heterozygosity on wool fineness in sheep*

Heterozygosity Level	Classified by 12 Loci		Classified by 3 Loci Associated with Wool Fiber Diameter	Classified by 9 Loci Unassociated with Wool Fiber Diameter
	Mean±Std[1]	PWD[2]	Mean±Std	Mean±Std
1	20.79±0.8853^{abc}	20.2128	20.6200±0.5897	20.79±0.8853^{abc}
2	19.90±0.3651^{c}	20.2847		19.90±0.3651^{c}
3	20.43±0.7009^{ab}	20.4734		20.43±0.7009^{ab}
4	20.65±0.3794^{ab}	20.5698		20.65±0.3794^{ab}
5	21.04±0.4657^{ab}	20.6451	21.1500±1.1254	21.04±0.4657^{ab}
6	19.93±0.3017^{c}	20.7692		19.93±0.3017^{c}
7	21.17±0.9182^{ab}	20.8893		21.17±0.9182^{ab}
8	20.21±0.2217^{bc}	20.9625		20.21±0.2217^{bc}
9	21.45±0.7090^{a}	21.0683		21.45±0.7090^{a}
10	21.63±0.8499^{a}	21.2250	21.4800±0.8033	21.63±0.8499^{a}

Sheep wool fiber diameter and their regression value at 10 levels or gene heterozygosity. Different superscripts in the same column differ significantly (P<0.05)

Table 9. *Effect of genetic heterozygosity on wool fineness in sheep*

Heterozygosity Level	Classified by 12 Loci		Classified by 3 Loci Associated with Wool Natural Length	Classified by 9 Loci Unassociated with Wool Natural Length
	Mean±Std	PWNL	Mean±Std	Mean±Std
1	11.074±0.2859^{ab}	11.4662	11.307±0.9637	11.074±0.2859^{ab}
2	11.243±0.8660^{ab}	11.2639		11.243±0.8660^{ab}
3	11.373±0.1000^{ab}	11.1595		11.373±0.1000^{ab}
4	10.836±0.6532^{ab}	11.0460		10.836±0.6532^{ab}
5	11.505±0.4562^{a}	10.9573	10.573±0.8548	11.505±0.4562^{a}
6	10.326±0.8652^{bc}	10.8105	10.678±0.3629	10.326±0.8652^{bc}
7	11.588±0.6521^{a}	10.6690		11.588±0.6521^{a}
8	11.133±0.4516^{ab}	10.5842		11.133±0.4516^{ab}
9	9.492±0.9530^{c}	10.4652		9.492±0.9530^{c}
10	10.127±0.4870^{bc}	10.2753	10.499±0.6591	10.127±0.4870^{bc}

Sheep wool natural length and their regression value at 4 levels for gene heterozygosity. Different superscripts in the same column differ significantly (P<0.05)

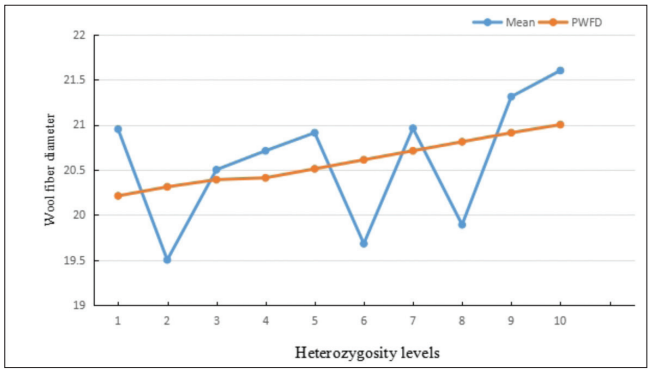

Fig 1. Wool fiber diameter increased as gene heterozygosity increased

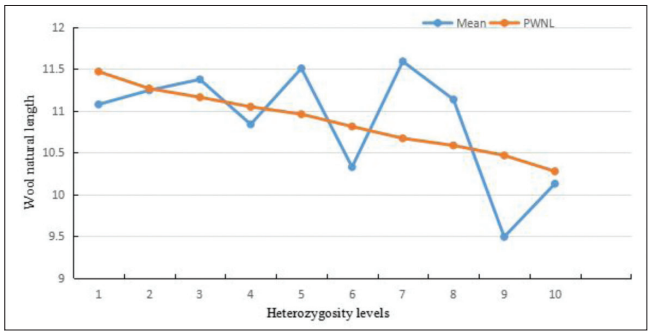

Fig 2. Wool natural length decreased as gene heterozygosity increased

All levels of WFD and WNL between the average heterozygosity of each level were analyzed by regression. The results showed that the regression relation was significant. Regression models were WFD=19.2758+2.3646×Hi (P<0.05), R^2=0.8070, harmony R^2=0.7175.

WNL=12.5691-2.3949×Hi(P<0.05), R^2=0.8592, harmony R^2=0.7725

The average value of WFD was 1.73 μm, which is the difference between the highest value and the lowest value. With heterozygosity increasing by 0.05, the average value of WFD will increase approximately 0.04-0.53 μm, which is 0.2-0.5% of the group WFD average 20.96 μm, except for the 1, 6, and 9 heterozygosity levels. PWFD by the regression model was 1.0122 μm, which is the difference between the highest value and the lowest value. The average value of WNL was 2.096 cm, which was the difference between the highest value and the lowest value. With an increase in heterozygosity of 0.05, the average value of WNL will decrease approximately 0.13-1.179 cm, which is 1.2-10.93% of the group WNL average of 10.784 cm, except for the 7 and 8 heterozygosity levels. The PWNL by regression model is 1.1909 cm, which is the difference between the highest value and the lowest value.

WFD was classified by 3 significantly connected loci and showed heterozygosity mainly distributed in the 1 (h≤0.5), 5 (0.65<h≤0.7), and 10 (h>0.9) levels, and average heterozygosities were 0.4826, 0.6639 and 0.9226, respectively, corresponding to WFD values of 20.62, 21.15 and 21.48 μm, showing that the trend of wool fineness significantly increases with increasing heterozygosity. The difference between the highest point and the lowest was 0.84 cm, which is very close to the difference estimated by all seats.

WNL was classified by 4 significantly connected loci and showed heterozygosity mainly distributed in the 1 (h≤0.5), 6 (0.65<h≤0.7), and 10 (h>0.9) levels, and the average heterozygosities were 0.3960, 0.7500, and 1.0000, respectively, and corresponding WNLs were 11.307, 10.678 and 10.499 cm, respectively. The results clearly show that with the increase in heterozygosity, the WNLs decreased. The difference between the highest value and the lowest value was 0.808 cm, which closely approaches the estimated difference of all loci. The analysis of 9 and 8 neutral loci also showed that the WFD and WNL increased or decreased with increasing heterozygosity.

DISCUSSION

China is one of the most developed countries in the world's

textile industry. The demand for wool is very high. Fine wool is the preferred raw material for ideal worsted processing because of its unique advantages in insulation, moisture absorption, elasticity, antistatic and antiwrinkle properties, and so on. Wool traits are an important economic trait in fine wool sheep [27]. The use of molecular markers has become an important phenotypic method, and it have been widely used in genetics and breeding [28]. Wool fineness and the natural length of wool are important factors in determining the economic value of wool. Therefore, it is necessary to understand the effects of genetic heterozygosity on wool fineness and WNL among different microsatellite loci of sheep, laying a foundation for improving the fineness and natural length of wool in sheep breeding.

By analyzing the relevance of 5 loci on chromosome 1 and 7 loci in chromosome 6 to WFD and WNL in sheep, the conclusion that 3 loc: BM1824, BM6506, BM6438 had a significant relation($P<0.05$) with WFD, which suggested that chromosome 1 was an important link block and that the genes or QTL had a greater contribution to WFD. There were 4 loci: ILSTS004 on chromosome 1 and OarAE101, OarJMP8, BM143 on chromosome 6, that had a remarkable connection to WNL. The results showed that the genes or QTLs have key genetic effects on WNL.

The relationship between gene heterozygosity and fiber diameter was analyzed using classification by 12 loci, 3 loci and another 9 loci associated with WFD. The results showed that while the gene heterozygosity increased, the fiber diameter increased. In addition, the gene heterozygosity increased by 0.05, and the diameter increased by 0.2%-2.5% of the average diameter of the group. When the relationship between gene heterozygosity and natural length was analyzed using classification by 12 loci, 3 loci and another 8 loci were associated with WNL, suggesting that while the gene heterozygosity increased, the natural length decreased. When the gene heterozygosity increased by 0.05, the natural length decreased by 1.2%-10.93% of the average length (10.784 μm) of the group. The phenomenon is coincident with heterozygosity involved with traits of the plant. In the heterotic utilization of sheep, it is necessary that the filial generation have proper gene heterozygosity through the appropriate choice of parents. The results showed that of all 12 loci, 3 and 4 loci had similar results. It is more convenient to attain results when using loci associated with traits that analyze the relationship between WFD, WNL and gene heterozygosity [29]. This result could partially explain the molecular mechanism of heterosis of for sheep wool quality.

At present, such as DNA fingerprinting, rapid DNA and protein polymorphism, have been used to explore the heterosis of animals [30,31]. The relationship of gene heterozygosity with heterosis has been studied in wildlife and livestock [33-35]. These studies are based on the group level, the use of heterosis is performed by specified male and female animals in practice. That these studies based on the group level instruct the practical use of heterosis [36]. Gene heterozygosity results in heterosis, the law of which would be easy to analyze at the gene level when the effect of gene heterozygosity on production quality was studied directly [37].

This paper evaluated the gene heterozygosity of the individual and analyzed the wool traits, especially the fiber diameter and natural length of gene heterozygosity of different individuals, The results showed that the gene heterozygosity of the individual would keep some main characteristics that are best suited to make main breeding characteristics by economy of sheep [38]. For example, the sheep had the smallest diameter when the gene heterozygosity was 2 (0.5450) and 6 (0.7340). If the largest diameter was the primary goal of breeding, the gene heterozygosity can be used to help in breeding. We can select a proper ram and ewe according to the individual gene heterozygosity and then obtain sheep of individual gene heterozygosity of 0.5 or 0.7, which would ensure the best heterozygosis effect. This study focused on the analysis the effects of gene heterozygosity on WFD and WNL between different microsatellite marker loci in sheep. Indirect selection and breeding of wool economic traits can be carried out using molecular markers linked to wool traits to improve the accuracy of line selection in sheep breeding. To lay the foundation for (MAS) selection of Chinese Merino (Xinjiang Army) breeding marker-assisted selection, and to accelerate the breeding, genetic analysis and genetic breeding speed of ultrafine lines. It lays a foundation for the separation and cloning of wool fiber trait control genes in the future and has important local characteristics, research value and significance.

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Research Article

Evaluation of Some Biological Effects of *Incarvillea emodi* (Royle ex Lindl.) Chatterjee and Determination of Its Active Constituents

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Abstract

This present study was an evaluation of the antioxidant and cytotoxic activities of *Incarvillea emodi* (Bignoniaceae). The aqueous extracts of different parts of *Incarvillea emodi,* collected from different places in Pakistan, were tested for radical scavenging effects on 2,2-diphenyl-1-picrylhydrazil (DPPH), nitric oxide (NO), superoxide (SO) and [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] ($ABTS^{\cdot+}$) radicals. DPPH radical scavenging effects of three polyamide column fractions of one extract were also tested. The cytotoxicity of the extracts were tested against Hep-2 (human larynx epidermoid carcinoma) cancer cell line by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. In addition, an HPLC-DAD system was used to show the presence of phenylethanoid glycosides in the most active polyamide column fraction. The free radical scavenging effects of the extracts were found comparable to that of reference antioxidants, 3-*t*-butyl-4-hydroxyanizole (BHA), quercetin and ascorbic acid (AA). Concentration dependent cytotoxic activity was observed against Hep-2 cancer cell line. Two phenylethanoid glycosides, acteoside and leucosceptoside A, were identified in the active polyamide column fraction at HPLC-DAD system. The presence of phenylethanoid glycosides in *Incarvillea emodi* was shown for the first time with this study. Antioxidant and cytotoxic activity researches are important for developing new drugs. Our results supported to use of *Incarvillea emodi* as folk medicine due to several biological effects in Pakistan. Moreover, the use of *Incarvillea* genus as a traditional ethnoveterinary medicine in dyspepsia and internal diseases were reported in previous studies.

Keywords: *Incarvillea emodi, Bignoniaceae, Radical scavenging effect, Cytotoxic activity, HPLC, Phenylethanoid glycosides*

Incarvillea emodi (Royle ex Lindl.) Chatterjee'nin Bazı Biyolojik Etkilerinin Değerlendirilmesi ve Aktif Bileşiklerinin Tayini

Öz

Bu çalışmada, *Incarvillea emodi*'nin antioksidan ve sitotoksik etkileri araştırılmıştır. Pakistan'ın farklı bölgelerinden toplanan *Incarvillea emodi*'nin farklı kısımlarından hazırlanan sulu ekstrelerin radikal süpürücü etkileri, 2,2-difenil-1-pikrilhidrazil (DPPH), nitrik oksit (NO), süperoksit (SO) ve 2,2'-azino-bis 3-etilbenzotiyazolin-6-sülfonik asit ($ABTS^{\cdot+}$) radikallerine karşı incelenmiştir. Bir ekstrenin üç poliamit kolon fraksiyonunun DPPH radikal süpürücü etkileri de araştırılmıştır. Ekstrelerin sitotoksik aktiviteleri, MTT [3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromür] yöntemi kullanılarak Hep-2 (insan larinks epidermoit karsinoma) kanser hücre dizisine karşı saptanmıştır. Ayrıca, en etkili poliamit kolon fraksiyonunda, feniletanoit glikozitlerinin varlığını göstermek için bir HPLC-DAD sistemi geliştirilmiştir. Ekstrelerin serbest radikal süpürücü etkileri, referans antioksidanlar, 3-*t*-butil-4-hidroksianizol (BHA), kersetin ve askorbik asit (AA) ile karşılaştırılabilir seviyede bulunmuştur. Hep-2 kanser hücre dizisine karşı konsantrasyona bağlı sitotoksik aktivite gözlenmiştir. HPLC-DAD sistemine uygulanmış etkili poliamit kolon fraksiyonunda, akteozit ve lökoseptozit A isimli 2 feniletanoit glikozitinin bulunduğu tespit edilmiştir. *Incarvillea emodi*'de feniletanoit glikozitlerinin varlığı ilk kez bu çalışma ile gösterilmiştir. Antioksidan ve sitotoksik aktivite araştırmaları ilaç geliştirme çalışmalarında önemlidir. Çalışmamızın sonuçları, *Incarvillea emodi*'nin, Pakistan'da çeşitli biyolojik etkileri nedeniyle halk arasında kullanılışını desteklemektedir. Ayrıca, *Incarvillea* cinsinin dispepsi ve iç hastalıklarda geleneksel etnoveteriner ilaç olarak kullanımı da daha önceki çalışmalarda belirtilmektedir.

Anahtar sözcükler: *Incarvillea emodi, Bignoniaceae, Radikal süpürücü etki, Sitotoksik aktivite, HPLC, Feniletanoit glikozitleri*

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INTRODUCTION

Plants have been used as medicines by human for almost 60.000 years [1]. Different parts of plants and plant derived constituents have been used as a medicine for prevention and therapy of the diseases. For these purposes biologically active constituents that are mainly alkaloids, flavonoids, tannins and other phenolic compounds are used [2,3]. The anticancer potential of plant derived nutritive and non-nutritive constituents have been proved in different *in vitro* and *in vivo* models [4-6].

The genus *Incarvillea* is represented by 16 species in the family Bignoniaceae. *Incarvillea* species are spreading out in Central and Eastern Asia. The majority of these plants are growing in higher altitudes of the Himalaya and Tibet. Unlike the majority, other members of Bignoniaceae, which are mostly tropical woody plants, species of *Incarvillea* are herbs from temperate regions [7]. *Incarvillea* genus have been used for the treatment of hepatitis, diarrhea, febrifuge, dyspepsia and contagious diseases [8]. As well as therapeutical use of plants for human health, plants are source for the treatment of diseases in animals. Ethnoveterinary studies report the use of *Incarvillea* genus as a traditional ethnoveterinary medicine. In Ruoergai region, Sichuan province, China, *Incarvillea compacta* was reported as a medicine in dyspepsia and internal diseases of animals [9].

Herbal medicines derived from genus *Incarvillea* are used for the treatment of rheumatism and relieve pain. Previous studies on the *Incarvilllea* resulted determination of its neurotrophic, antiinflammatory, antinociceptive and anti-hepatitis activities. On this genus, isolation studies resulted with the determination of some secondary metabolites alkaloids, ceramides, iridoids, flavonoids and triterpenes [10].

Incarvillea genus is represented by 16 species of family Bignoniaceae and *I. emodi* is a rare wild attractive plant of family, distributed in Afghanistan, Pakistan, Nepal and India [11]. Bignoniaceae family is placed in super order Lamiae which are very well known for the synthesis of iridoids. In a study according to Rana *et al.*[12] two major compounds with a structure of iridoid glycoside have been isolated from the aerial parts of *I. emodi*. One of the compounds was plantarenaloside with neurotrophic effect and other one boschnaloside with an antibacterial activity [13,14]. There are only a few studies on titled plant.

The aim of this study was to determine antioxidant potential against different free radicals and cytotoxicity through human larynx epidermoid carcinoma (Hep-2) cancer cells, along with HPLC-DAD analysis of active fractions obtained from *I. emodi*.

Collection of the plant material was done from two different regions, Abbottabad and Kashmir in Pakistan. Methanolic extracts of the aerial parts (ABD-Ap) and the roots of plant (ABD-Rt) collected from Abbottabad and the aerial parts of plant collected from Muzaffarabad-Azad Jammu & Kashmir (AJK-Ap) were prepared separately. Aqueous part of each methanolic extract and polyamide column fractions of ABD-Ap were examined for their radical scavenging effects using 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO), super oxide (SO) and 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$) radicals spectroscopically. To determine cytotoxicity ABD-Ap and K-Ap were evaluated against Hep-2 cells. The most active fraction of polyamide column were compared by HPLC-DAD system with authentic compounds, this comparison gave information about the phenylethanoid glycoside contents.

MATERIAL and METHODS

Plant Material

Incarvillea emodi (Royle ex Lindl.) Chatterjee was collected from Himalayan regions i.e. in village Silhad-Abbottabad (ABD) and Muzaffarabad-Azad Jammu & Kashmir (AJK), Pakistan. Identification of the plant was done by Dr. Uzma Khan (Hazara University, Pakistan) and each of the plant samples were deposited at the Herbarium of Hazara University, Mansehra, Pakistan [Vouchers No. HUBOT 04707 and HUBOT 04708].

Whole plant was collected from village Silhad-Abbottabad in flowering period and seperated to aerial parts (ABD-Ap) and roots (ABD-Rt). The aerial parts were collected from Muzaffarabad-AJK in fruiting period (AJK-Ap). The plant parts were dried, powdered and then methanolic extracts were prepared.

Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitro blue tetrazolium (NBT), ascorbic acid were obtained from Sigma-Aldrich Chem Co (St. Louis, MO). 3-*t*-butyl-4-hydroxyanizole (BHA) was purchased from Nacalai Tesque Co. (Kyoto, Japan), polyamide (50-160 µm) from Fluka (Seelze, Germany), TLC plates (60 F_{254}) from Merck (Darmstadt, Germany). Fetal bovine serum (FBS), minimal essential medium with Earl's salts (MEM-EARLE) with nonessential amino acids, 1% antibiotic solution (penicillin and streptomycin) and trypsin solution (1:250) were purchased from Biochrom AG (Berlin, Germany). Hep-2 (human larynx epidermoid carcinoma) cell line was kindly provided by Refik Saydam Hygiene Center, Virology Laboratory, Ankara, Turkey.

Extraction of Plant Material

Extraction was done by maceration of 400 g powdered plant material in methanol and keeping this material at room temperature for seven days, followed by filtration. The residues were again macerated in methanol for

additional seven days and filtered thereafter. All collected filtrates were evaporated at 40°C under vacuum and got a greenish thick syrup material termed as "methanolic extract". Weights were noted for ABD-Ap=144.3 g, ABD-Rt=84.6 g and AJK-Ap=47.6 g (Yields: 36%, 21.1%, and 11.9%, respectively).

Preparation of Aqueous Extract

After complete drying in rotary evaporator, dissolution of crude methanolic extract carried out in distilled water (100 mL). After complete mixing, first the plant extract was filtered by cotton and then equal volume of petroleum ether was used to remove chlorophyll (3-4 times). The aqueous extract was evaporated to dryness and then lyophilized to give dry aqueous extract. Lyophilized weights of ABD-Ap, ABD-Rt and AJK-Ap were noted as 41.5 g, 68.4 g and 21.8 g respectively.

DPPH Radical Scavenging Effect

The DPPH radical scavenging effect was evaluated by the discoloration of methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) spectroscopically; 3-*t*-butil-4-hidroksianizol (BHA) and ascorbic acid (AA) were used as reference compounds. DPPH (50 µL) solution was added to methanol solution (200 µL) of the extracts and fractions with a concentration range of 5-200 µg/mL. After 30 min incubation the absorbance of mixture was measured at 520 nm [15,16]. Results were calculated using the following formula to give inhibition percentage.

% Radical scavenging effect = [(blank abs. - sample abs.)/ blank abs.] ×100

NO Radical Scavenging Effect

For the determination of nitric oxide (NO) radical scavenging effect of the aqueous extract, 60 µL of sample (50-1000 µg/mL) and 60 µL of 10 mM sodium nitroprusside, dissolved in phosphate buffered saline (PBS), were added to each well of the 96 well plate and incubated under light at room temperature for 150 min. To detect the nitrite content an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphtyl ethylene diamine dihydrochloride, 2.5% H_3PO_4) was added. After 10 min, absorbance was measured at 577 nm [17,18]. AA and quercetin were used as references.

SO Radical Scavenging Effect

The method of Elizabeth and Rao [19] was slightly modified by us for the assessment of superoxide radical scavenging effect of samples. Concentrations were used in the range of 10-800 µg/mL. 10 µL of nitro blue tetrazolium (NBT) (1 mg/mL solution in DMSO) and 30 µL DMSO dissolution of the samples were mixed. 100 µL of alkaline DMSO (1 mL DMSO containing 5 mM NaOH in 0.1 mL water) was added and the absorbance was measured at 560 nm. BHA and quercetin were used as references.

$ABTS^{\bullet+}$ Radical Scavenging Effect

The method of Re *et al.*[20] was used to determine trolox equivalent antioxidant capacity by using $ABTS^{\bullet+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)], with slight modification. Briefly, $ABTS^{\bullet+}$ radical cation was produced by a reaction of 7 mM ABTS with 2.45 mM potassium persulphate. The mixture was left in the dark for 16 h at room temperature to carry out the reaction and the final product was used within 48 h. Dilutions of $ABTS^{\bullet+}$ solution was continued with ethanol till to achieve the absorbance of 0.700±0.050 at 734 nm. A mixture of fifty microliters of sample and 1.9 mL of diluted $ABTS^{\bullet+}$ solution was prepared and was left at room temperature for 6 min and decolorization of $ABTS^{\bullet+}$ was measured spectrophotometrically at 734 nm. As a reference, trolox solution (0-200 µg/mL) was used. The results were given as mg Trolox/g dry weight of extract. The TEAC value (Trolox equivalent antioxidant capacity) was calculated from the standard curve of trolox.

Cytotoxic Activity

100 µL of cells (6 x 10^4 cells/mL) were cultured into 96-multi-well plates and cultured for 24 h (in a humidified 5% CO_2 incubator at 37°C) in MEM supplemented with 10% FBS, 1% penicillin-streptomycin solution. Different concentrations of the extract (0-800 µg/mL) was applied to cells and incubated for 48 h. After that, each well were washed and changed by fresh medium. 10 µL of MTT solution (5 mg/mL in PBS) was added and left to stand for 4 h. After that to dissolve formazan crystals, 100 µL of 10% SDS (Sodium dodecyl sulfate) was added to each well. The absorbance was measured at 577/655 nm. Cell viability was calculated using the comparison between the absorbance of treated and nontreated cells (Ratio expressed as percentage) [21]. Nontreated cells were accepted as negative control.

Polyamide Column Chromatography

150 g of polyamide was suspended in 1000 mL of distilled water in a large beaker and was added into the column gently. Aqueous extract of ABD-Ap (41.5 g, dry weight), the richest one in secondary metabolites among the tested extracts, was applied to polyamide column chromatography for fractionation using rising concentrations of methanol in water (0-25-50-75-100%). The fraction eluted with 50% methanol, rich in phenylethanoid glycosides (PC. Fr. 33-38) was applied to HPLC.

High Performance Liquid Chromatography (HPLC)

HPLC was used for analytical purposes. Polyamide column fraction rich in phenylethanoid glycosides (PC. Fr. 33-38) was selected for HPLC studies. For stock solution, 3 mg of fraction was taken in small bottles and dissolved in 3 mL of methanol.

HPLC-DAD system was equipped with Dionex P680 HPLC pump, Dionex ASI-100-automated sample injector, Dionex thermostatted column compartment TCC-100. Analyses were performed on Hichrom-Nucleosil 100-5 C18, 25 cm x 4.6 mm column. Detection was from 200 to 600 nm. A gradient program was used as follows: 35% B in the first 4 min, 30% B during 4-25 min, 45% B at 35 min, then B held at 45% for 20 min. The flow rate was 1 mL/min and the injection volume was 20 µL. Column temperature was at room temperature. UV chromatogram was screened at 330 nm.

Statistical Analysis

Statistical analyses have been computed by IBM SPSS Statistic 23 software. The statistical significance was determined by one-way ANOVA post hoc Dunnett's test. Results of experiments are expressed as mean±standard deviation. The inhibition values were determined using at least three independent experiments.

RESULTS

Three aqueous crude extracts of *I. emodi*, ABD-Ap, ABD-Rt and AJK-Ap were studied for DPPH radical scavenging effect using BHA and ascorbic acid as reference compounds. For extracts and references, concentrations were used in the range 5-200 µg/mL. In comparison, ABD-Ap and AJK -Ap showed 88.3% and 90.5% inhibition respectively while ABD-Rt gave 67.8% at 100 µg/mL concentration *(Table 1)*.

In view of the fact that aqueous extracts showed strong antioxidant activity, the same effects of the three polyamide fractions, rich in phenolics, were also bring into test against DPPH free radicals. At 50 µg/mL concentration, all tested fractions showed strong activity in a range of 87.3-90.2% inhibition *(Table 2)*.

Almost all aqueous extracts of *I. emodi* were showed concentration dependent nitric oxide radical scavenging effect. At 750 µg/mL, ABD-Rt extract showed 71.7% effect which is the highest activity as compared to ABD-Ap extract (63.5%) and AJK-Ap extract (33.1%) *(Table 3)*.

To determine the super oxide radical scavenging effect of *I. emodi*, the same three crude extracts of the plant were used. In this experiment, at 800 µg/mL ABD-Ap extract showed 88.0% inhibition which is the highest activity comparable to the other two extracts of the plant that is ABD-Rt (80.0%) and AJK-Ap (76.7%). All extracts showed concentration dependent radical scavenging effect in the range i.e. 10-800 µg/mL of concentrations *(Table 4)*.

Aqueous extracts of *I. emodi* were tested against $ABTS^{\cdot+}$ radical. The given results have been shown as TEAC i.e.

Table 1. *Comparative DPPH radical scavenging effect of aqueous extracts. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/mL)	Plant Extracts[a] % Inhibition			Control % Inhibition	
	ABD-Ap	ABD-Rt	AJK-Ap	AA	BHA
5	7.38±1.88	13.12±0.99	-	40.38±4.67	39.41±2.27
10	11.99±2.06	17.42±5.45	18.70±1.93	86.97±3.14	63.71±0.41
25	31.38±4.34	18.74±3.31	26.41±3.65	91.06±0.49	86.96±0.92
50	53.00±5.81	37.16±6.69	49.08±5.42	90.97±0.37	89.71±0.42
100	88.27±4.56	67.79±6.75	90.47±1.30	91.47±0.38	89.36±0.72
200	92.15±1.03[b]	90.55±1.32	91.92±0.78[b]	91.17±0.71	89.87±0.33

[a] ABD-Ap: aerial parts of I. emodi collected from Abbottabad, ABD-Rt: roots of I. emodi collected from Abbottabad, AJK-Ap: aerial parts of I. emodi collected from Muzaffarabad-Azad Jammu & Kashmir; [b] P<0.05 as compared to positive control 3-t-butyl-4-hydroxyanizole (BHA)

Table 2. *Comparative DPPH radical scavenging effect of selected polyamide column fractions from ABD-Ap[a]. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/mL)	Polyamide Column Fractions % Inhibition			Control % Inhibition	
	PC. Fr. 19-27	PC. Fr. 33-38	PC. Fr. 44-46	AA	BHA
5	29.46±4.83	18.90±1.76	17.16±3.07	40.39±4.67	39.41±2.27
10	40.20±3.18	36.63±2.23	27.17±3.99	86.97±3.14	63.71±0.41
25	84.14±4.44	83.96±5.87	57.47±5.80	91.06±0.49	86.96±0.92
50	90.20±0.68	89.96±0.92	87.31±0.95	90.97±0.37	89.71±0.42
100	90.39±1.10[b]	89.68±0.37	89.00±0.44	91.47±0.38	89.36±0.72
200	90.09±0.72	90.10±0.54	89.35±0.67	91.17±0.71	89.87±0.33

[a] ABD-Ap: aerial parts of I. emodi collected from Abbottabad; [b] P<0.05 as compared to positive control 3-t-butyl-4-hydroxyanizole (BHA)

Table 3. *NO radical scavenging effect of aqueous extracts. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/mL)	Plant Extracts[a] % Inhibition			Control % Inhibition	
	ABD-Ap	ABD-Rt	AJK-Ap	AA	Quercetin
50	12.24±5.04	22.05±3.32[b]	8.26±3.54	14.78±3.84	16.74±2.62
100	11.76±4.36	22.89±3.58[b]	23.59±5.37[b]	10.92±2.86	18.59±3.49
250	30.66±3.47[b]	36.70±3.55[b]	24.98±5.37[b]	15.74±2.56	41.80±6.27
500	35.52±4.63[b]	64.07±3.76[b]	36.59±5.02[b]	23.49±1.81	48.71±7.25
750	63.47±3.37[b]	71.69±0.58[b]	33.11±2.14[b]	24.25±2.20	46.71±5.37
1000	69.83±1.06[b]	72.37±0.38[b]	49.82±3.59[b]	28.08±6.55	63.63±1.84

[a] ABD-Ap: aerial parts of I. emodi collected from Abbottabad, ABD-Rt: roots of I. emodi collected from Abbottabad, AJK-Ap: aerial parts of I. emodi collected from Muzaffarabad-Azad Jammu & Kashmir; [b] P<0.05 as compared to positive control ascorbic acid (AA)

Table 4. *SO radical scavenging effect of aqueous extracts. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/ mL)	Plant Extracts[a] % Inhibition			Control % Inhibition	
	ABD-Ap	ABD-Rt	AJK-Ap	BHA	Quercetin
10	21.38±1.56	16.08±0.83	6.16±4.15	47.59±3.06	55.13±3.09
25	58.30±7.22	31.85±2.68	24.60±8.22	50.25±7.92	64.40±2.85
50	56.19±6.84	45.78±5.99	33.00±2.99	55.75±4.71	73.19±2.68
100	69.59±3.19	58.93±5.57	46.10±0.74	62.60±3.32	81.14±2.82
200	78.08±1.55[b]	66.28±5.92	56.17±0.88	63.71±5.70	87.55±1.33
400	84.51±3.05[b]	75.47±6.19[b]	64.97±0.63	63.96±4.06	91.49±1.70
800	88.02±1.99[b]	79.98±7.46[b]	76.70±1.89	69.98±3.75	91.52±1.61

[a] ABD-Ap: aerial parts of I. emodi collected from Abbottabad, ABD-Rt: roots of I. emodi collected from Abbottabad, AJK-Ap: aerial parts of I. emodi collected from Muzaffarabad-Azad Jammu & Kashmir; [b] P<0.05 as compared to positive control 3-t-butyl-4-hydroxyanizole (BHA)

Table 5. *Comparative ABTS radical scavenging effect of aqueous extracts. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/mL)	Plant Extracts[a] % Inhibition		
	ABD-Ap	ABD-Rt	AJK-Ap
25	1.70±0.43	6.34±0.46	0.24±0.92
50	5.16±0.30	6.49±1.05	2.94±0.43
200	18.99±1.10	21.31±1.26	10.09±0.62
400	41.62±0.86	45.79±2.11	20.03±0.86

[a] ABD-Ap: aerial parts of I. emodi collected from Abbottabad, ABD-Rt: roots of I. emodi collected from Abbottabad, AJK-Ap: aerial parts of I. emodi collected from Muzaffarabad-Azad Jammu & Kashmir

Table 6. *Comparative cytotoxic activity of aqueous extracts against Hep-2 cell line. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/mL)	Plant Extracts[a] % Inhibition	
	ABD-Ap	AJK-Ap
20	10.44±7.78	9.18±3.78
50	12.31±10.77	15.56±4.56[b]
100	55.87±8.79[b]	12.94±8.35
200	95.77±0.74[b]	61.15±7.82[b]
400	96.12±0.69[b]	95.17±0.33[b]
800	95.62±0.40[b]	95.07±0.39[b]

[a] The aerial parts collected from Abbottabad (ABD-Ap) and Muzaffarabad-Azad Jammu & Kashmir (AJK-Ap); [b] P<0.05 as compared to negative control

Trolox Equivalent Antioxidant Capacity (mg Trolox equivalent in 1 g of extract). TEAC value was calculated for all the three aqueous extracts of the plant. ABD-Rt extract had high TEAC value and showed strong $ABTS^{\cdot+}$ radical scavenging effect as comparable to ABD-Ap extract *(Table 5)*. TEAC values of ABD-Ap, ABD-Rt and AJK-Ap were 244.5, 269.4, 115.2, respectively.

Seven concentrations of each crude extract (0-800 µg/mL) were tested for cytotoxicity against cancer cell line (Hep-2). The results of MTT assay were expressed as percent inhibition and IC_{50} values. The results indicated that the plant is significantly active against Hep-2 cell line and the activity was seen as concentration dependent manner. The IC_{50} values of ABD-Ap and AJK-Ap were found as 101.3 and 199.7 µg/mL, respectively. At 200 µg/mL, maximum inhibition calculated for ABD-Ap and AJK-Ap were 95.8% and 61.2% respectively *(Table 6)*.

Polyamide column fraction 33-38 (PC.Fr. 33-38) of aqueous

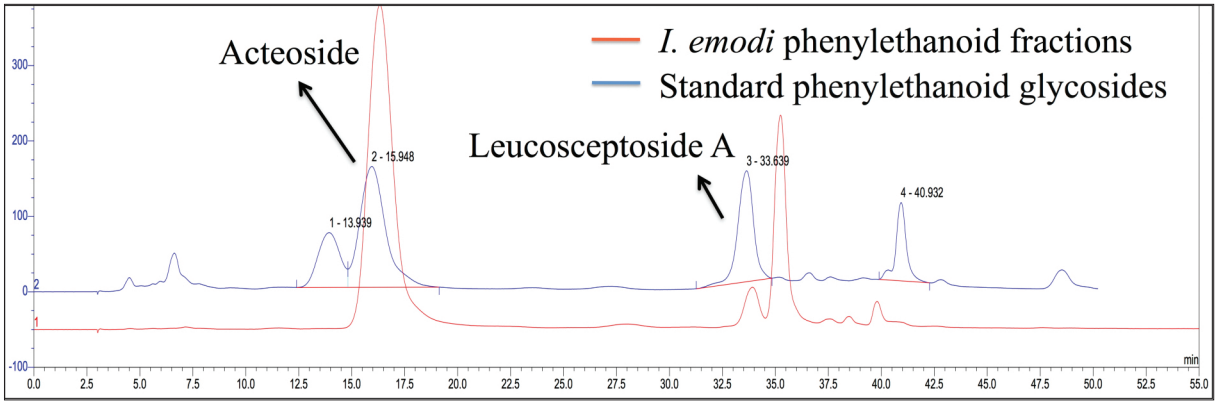

Fig 1. HPLC chromatogram of polyamide column Fr. 33-38 of *Incarvillea emodi* at 276 nm

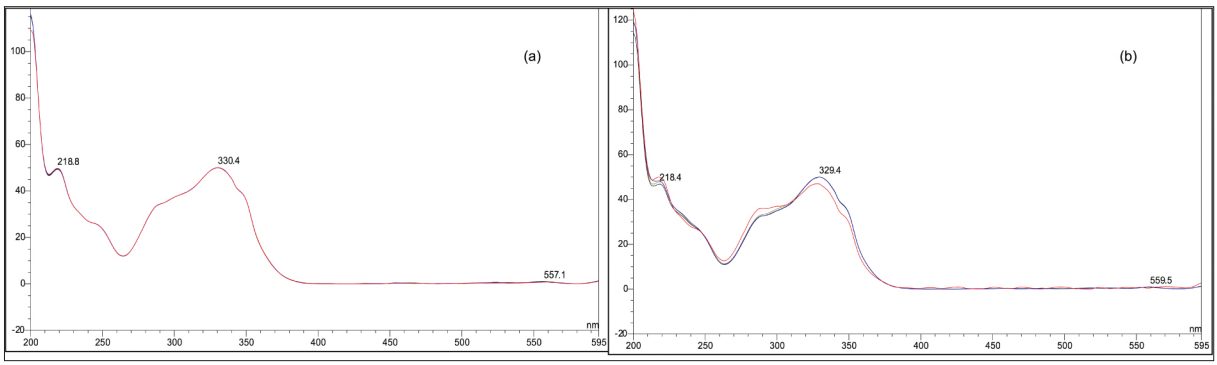

Fig 2. UV spectra of acteoside (a) and leucosceptoside A (b) of PC.Fr. 33-38 and standard compounds (Red Line: Compound of PC.Fr.33-38, Blue Line: Standard compound)

I. emodi extract was tested for the presence of phenylethanoid glycosides using HPLC-DAD system. On application of PC.Fr. 33-38 to HPLC-DAD system, 4 phenylethanoid glycosides were determined in the fraction. In comparison of phenylethanoid glycosides to the standard phenylethanoid glycosides, 2 of them were identified as acteoside and leucosceptoside A. *Fig. 1* depicts HPLC chromatogram of PC.Fr. 33-38 of *I. emodi* at 276 nm showing the presence of both compounds while *Fig. 2* showed UV spectra of acteoside and leucosceptoside A.

DISCUSSION

In countless contrasting diseases, contribution of the free radical intervened cell damage has directed us to establish the antioxidant activity of aq. extracts and three selected polyamide column fractions of *I. emodi* together with its HPLC studies. Aq. extracts were tested against DPPH, NO, SO and $ABTS^{\cdot+}$ radicals. Aerial parts of *I. emodi* aqueous extracts (ABD-Ap and AJK-Ap) were bring into being to be evidence for concentration reliant significant DPPH radical scavenging capacity, where given results were observed extremely close to that of standards (BHA and AA) at 200 µg/mL ($P<0.05$, compared to BHA). Nitric oxide (NO), and super oxide (SO) scavenging effects of aq. extracts were tested in the assortment of 50-1000 µg/mL and 10-800 µg/mL concentration respectively. ABD-Rt crude extract has given stronger NO radical scavenging effect than the standard compounds ($P<0.05$, compared to AA in all tested concentrations). However, SO scavenging effect of ABD-Ap was in much accordance to that of quercetin at 800 µg/mL while BHA showed weakest SO scavenging effect at the same concentration ($P<0.05$, compared to BHA in 200, 400 and 800 µg/mL). TEAC value was also determined for all the three crude aqueous extracts in the range of 25-400 µg/mL and ABD-Rt gave the maximum TEAC value i.e. 269.4 mg trolox/g extract. In comparison of all aqueous extracts, ABD-Ap showed the highest radical scavenging activity against DPPH and SO radicals and highest cytotoxicity. Therefore, aqueous extract of ABD-Ap was choosen for fractionation by polyamide column for further phytochemical investigations. Three polyamide column fractions (PC. Fr. 19-27 25% MeOH, PC.Fr. 33-38 50% MeOH and PC. Fr. 44-46 75% MeOH) were also bring into test of 5-200 µg/mL where PC.Fr. 19-27 and 33-38 were found to show the strongest effect (90.2% and 90.0% inhibition at 50 µg/mL, respectively) against DPPH radical. Rana *et al.*[22], performed their studies on various parts (shoots, roots and flowers) of *I. emodi* using ethyl acetate, butanol and aqueous crude extracts. Higher total antioxidant potential (253.0-384.6 mg/g as trolox equivalent) was recorded for ethyl acetate fraction in all parts which is similar to our results [22].

Cancer is still a frightful disease due to inadequate availability of efficient drugs in cancer therapy. Limitations linked with the present-day chemotherapeutic agents to treat cancer are that they are highly expensive, mutagenic and sometimes even carcinogenic. Their applications are limited [23]. Therefore, efforts are made to isolate and identify anticarcinogens that are naturally present in plants, which can effectively be used to prevent, slow or reverse cancer development. Regarding the cytotoxic activity of two aqueous crude extracts of the plant, ABD-Ap gave comparatively higher percentage growth inhibition (PGI) i.e. 95.8% at 200 µg/mL concentration ($P<0.05$, compared to negative control).

In a previous study, according to Rana *et al.*[22], *in vitro* cytotoxic effects of pure isolated iridoids- plantarenaloside and boschnaloside from *I. emodi* were studied against HCT-15 (colon), Hela (cervix), THP-1 (leukaemia), A549 (lung) and PC-3 (prostate) using sulphorhodamine B (SRB) assay. From the results, it is quite obvious that plantarenaloside is active only against THP-1 (leukaemia) cancer cells, while boschnaloside was found active against THP-1, A-549 and PC-3 [24]. This study supports our present study as we also isolated plantarenaloside from the aq. crude extract of aerial parts of *I. emodi* [24].

In the present study, polyamide column fraction 33-38 (PC. Fr. 33-38) was tested for the presence of phenylethanoid glycosides using HPLC-DAD. Two phenylethanoid glycosides, acteoside and leucosceptoside A, were detected in fraction when compared to the standard phenylethanoid glycosides. There were seen two more phenylethanoid glycosides different from acteoside and leucosceptoside A which could not be identified in our HPLC system. This is the first report for the occurrence of phenylethanoid glycosides in *I. emodi.*

Antioxidant and cytotoxic activity study results are an important basis for drug development studies. The results of our study also clearly highlight the free radical scavenging and cytotoxic secondary metabolites and supports to use of *I. emodi* as folk medicine due to several biological effects in Pakistan.

The presence of phenylethanoid glycosides in *I. emodi* was shown for the first time with this study. Biological activities of the plant may be attributed to the phenylethanoid glycosides such as acteoside and leucosceptoside A, shown in HPLC-DAD analysis.

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Conflict of Interest

No conflict of interest was declared by the authors.

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Research Article

The Effect of Rocuronium and Sugammadex on Progesterone Levels in Pregnant Rabbits Under General Anesthesia [1] [2]

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Abstract

The purpose of this study was to investigate the effect of rocuronium and sugammadex on progesterone (P4) levels in pregnant rabbits under general anesthesia. Twenty-one pregnant New Zealand rabbits were used in the study. After the rabbits were divided into three groups of three (Control, Group I and Group II), each animal was given 0.5 mg/kg midazolam and 6 mg/kg propofol and then put under general anesthesia with sevoflurane on the 21st day of pregnancy. No procedure was performed on the control group apart from anesthesia. Rocuronium was administered to GI at the onset of anesthesia, and in GII, sugammadex was administered 60 min after general anesthesia + rocuronium. All of the rabbits were monitored during the anesthesia procedure. A sample of venous blood was taken and biochemically analyzed to test P4 levels. The administration of rocuronium was determined to have caused an increase in the serum progesterone level in all recorded min. Sugammadex was found to cause a quantitative decrease in the level of progesterone. In conclusion, it was found out that rocuronium and sugammadex administration did not have a negative effect on progesterone levels in pregnant rabbits receiving general anesthesia.

Keywords: *Pregnant, Progesterone level, Rabbit, Sugammadex, Rocuronium*

Genel Anestezi Altındaki Gebe Tavşanlarda Rocuronium ve Sugammadex'in Progesteron Düzeyine Etkisi

Öz

Sunulan çalışmada, genel anestezi uygulanan gebe tavşanlarda, rocuronium ve sugammadex uygulamasının progesteron düzeyine etkisinin saptanması amaçlandı. Çalışmada, 21 adet gebe Yeni Zelanda tavşanı kullanıldı. Her grupta 7 tavşan olacak şekilde üç gruba ayrıldı (Kontrol, Grup I, Grup II). Gebeliğin 21. gününde bulunan tüm tavşanlara 0.5 mg/kg midazolam ve 6 mg/kg propofol verildi. Daha sonra Sevofluran ile genel anesteziye alındı. Kontrol (C; n=7) grubundaki tavşanlar ise yalnızca genel anesteziye alındı. Grup I'deki tavşanlara genel anestezi başladığında roküronyum verildi. Grup II'de yer alan tavşanlara ise, genel anestezi ve rocuronium uygulamasından 60 dk sonra sugammadex verildi. Tüm tavşanlar genel anestezi süresince monitorize edildi. Tüm tavşanlardan venöz kan örneği alındı ve biyokimyasal olarak progesteron düzeyi analiz edildi. Rocuronium uygulamasının serum progesteron düzeyini tüm kaydedilen dakikalarda artışa neden olduğu belirlendi. Sugammadex uygulamasının ise, progesteron düzeyinde sayısal bir azalmaya neden olduğu belirlendi. Sonuç olarak, genel anesteziye alınan gebe tavşanlarda, rocuronium ve sugammadex uygulamasının progesteron düzeyi üzerine olumsuz bir etkisinin olmadığı saptandı.

Anahtar sözcükler: *Gebe, Progesteron düzeyi, Tavşan, Sugammadex, Rocuronium*

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INTRODUCTION

In addition to events that occur in pregnancy like appendicitis, ovarian diseases and trauma [1], surgical operations are performed in intensive care units [2] for various indications. These surgical operations are usually performed under general anesthesia.

Rocuronium is a one of the steroid-type non-depolarizing neuromuscular blocker muscle relaxants with a short duration of action [3]. Sugammadex, on the other hand, is a new generation reversal agent used to terminate the effect of neuromuscular blockers (vercuronium and rocuronium). The mechanism of action is that it creates a complex with the circulating muscle relaxant at the nerve juncture to terminate its effect [4]. The sugammadex-rocuronium interaction reduces the amount of free rocuronium in plasma, thus altering rocuronium in plasma by significantly reducing the levels of rocuronium at the neuromuscular juncture. This ensures that muscle activity resumes because it quickly releases the acetylcholine receptors [5]. However, there have not been enough studies conducted on rocuronium and/or sugammadex's effect on pregnancy [6].

Progesterone prevents the endometrium from breaking down in pregnancy and suppresses the stimulating effect of estrogen on uterine contractions to ensure that pregnancy continues [7]. The decline in P4 levels causes the relaxing effect on the uterus to disappear, thus terminating pregnancy [8,9].

The aim of this study was to demonstrate how the use of rocuronium, an effective neuromuscular blocker, and its antagonist sugammadex in pregnant rabbits given general anesthesia affects P4 levels at different times in pregnancy.

MATERIAL and METHODS

Ethics Approval

This study was conducted after obtaining approval from the Kafkas University Local Experimental Animals Ethics Committee (Approval no: KAÜ-HADYEK: 2016-096).

Animals

This study used twenty-one pregnant New Zealand rabbits with an average weight of 2.7-3.3 kg obtained from the Fırat University Experimental Research Center. The veterinarian was delivered with a referral report. The rabbits were transported in a single cage and vented for 10 min per hour by transit minibus. Rabbits that were bred on the same day were included in the study, and the day of mating was considered day 0. Rabbits were housed in individual cages where they received 12 h of sunlight and 12 h of darkness. Rabbits were fed *ad-libitum* with a daily average of 250 g of pellet feed and 100 mL of water.

Ultrasonography Examination

Pregnancy was confirmed with an ultrasound examination immediately before anesthesia was administered 21 days after mating. A maximum of four at least one fetus was detected in the ultrasound examination of rabbits The rabbits were shaved up to their rib cage and a trans-abdominal examination was performed on the abdominal region of the rabbits as they were held on their back. The ultrasound device used for the ultrasonography procedure was a B Mode real-time device with a 7.5 mHz linear probe (DRAMINSKI iScan, Poland).

Anesthesia Procedure

All of the rabbits were sedated with 0.5 mg/kg midazolam (Zolamid®, 5 mg/5 mL, Defarma Pharmaceutical Industry and Trade Company., Turkey). Propofol İntravenous (IV) (Propofol-Lipuro 10 mg/mL 20 mL, Braun Pharmaceuticals, Germany) was administered slowly at a dose of 6 mg/kg. Anesthesia maintenance was started in the first five min with a mixture of sevoflurane (AbbVie Pharmaceutical Industry and Trade Company, Turkey) 3-4% + oxygen 4 L/min. Because the surgical procedure would not be performed five min later, inhalation anesthesia was reduced and continued with a mixture of sevoflurane 2% + oxygen 4 L/min. All the rabbits were provided with respiration support via ventilation mask. Spontaneous breathing was blocked in the group, which had been given rocuronium, due to the medication. Breathing was administered in head extension via mask ventilation. No respiratory arrest or a complication occurred in any of the groups.

Sevoflurane was cut off sixty min later. All of the rabbits were monitored prior to anesthesia 0 and at 5, 30, 60 and 90 min after the onset of anesthesia to measure physiological parameters. Furthermore, venous blood was collected from the marginal ear vein at each of these time intervals.

Experimental Groups

The anesthesia procedures were performed on pregnant rabbits 21 day after mating.

Control Group: The rabbits in the control (C: n=7) group were only given general anesthesia.

Group I: The rabbits in group 1 (GI: n=7) were given rocuronium (Esmeron®, 50 mg/5 mL, Merck Sharpoo Dohme (MSD) Pharmaceuticals Ltd., Germany) at a intravenous dose of 0.6 mg/kg.

Group II: Unlike the rabbits in Group I, the rabbits in this group were given sugammadex (Bridion® 200 mg/2 mL, Merck Sharpoo Dohme (MSD) Pharmaceuticals Ltd., The Netherlands) at the 60th min mark via IV.

Measurements

Pulse (P), systolic (SAP), diastolic (DAP) and median artery pressure (MAP), oxygen saturation (SPO_2), respiration (R)

and body temperature (T) were monitored using a multi-parametric monitor (Veteriner Monitör, MMED6000DP S6-V). This parameters were recorded for all rabbits at 0, 5, 30, 60 and 90 min.

In Group I and Group II, the Train of Four (TOF) values for the rabbits (TOF time to zero and TOF time to return to 100) were recorded. Firstly the hair on the foreleg of the rabbit was shaved. Since the weight of the rabbit was equal to a baby's, two pediatric electrodes were placed on the ulnar nerve trace of the foreleg. TOF device was switched to pediatric mode and the device's connection clips were mounted on the electrodes. Heat probe was placed on the first phalanges of the extremity with electrodes. Then, transducer was assembled on the phalanges with heat probe. Induction agents were injected as the device was turned on. Finally, electrical stimulation was applied at 10 sec intervals on pediatric mode and TOF values on the device were monitored and recorded.

At the same time that the vital signs were checked, blood was collected from the marginal ear vein with a yellow intravenous catheter and stored in an eppedorf tube. These samples were centrifuged at 1200 rpm for 10 min and stored at -20°C until the tests were performed. The P4 levels were determined using the Rabbit Progesterone ELISA (ABIN365369, Antibodyonline, USA) kit.

Statistical Analysis

Statistical analyses were performed using the SPSS® (SPSS 20, IL, USA) software program. The data was analyzed with the Shapiro Wilk normality test. Paired comparisons were examined with the Mann Whitney U test. The internal differences on day 21 of pregnancy were established with Friedman's test. The results were analyzed as mean ±S.D. A P value of <0.05 was considered statistically significant.

RESULTS

Signs of sedation were observed in all of the individuals given midazolam, and the animals were then given propofol for induction, followed by problem-free general anesthesia for 60 min with sevoflurane.

On day 21 after mating, there was no statistically significant difference among the groups in terms of (P/min, SAP/mmHg, DAP/mmHg, MAP/mmHg) and (sPO_2/%) values (P>0.05).

On day 21 of pregnancy, respiratory values of control group at the 5th, 30th and 60th min were found out to be significantly lower than those of Group II (P<0.05). Respiratory values of both the Control Group and Group II were also significantly lower at the 90th min than they were at the 5th, 30th and 60th min (P<0.05) *(Table 1)*.

There was a statistically significantly difference in body temperature between the control group and Group I on day 21 of pregnancy (P<0.05). There was a statistically significant difference within the control group between min 5 and 60 (P<0.05). There was a statistically significant difference within Group I between baseline and 60 min and 0 and 5 min (P<0.05) *(Table 1)*.

In Group I, it was observed that TOF went down to zero approximately 45.4 sec after administering rocuronium. In Group II, TOF went down to zero 43.2 sec after the same process and it took TOF an average of 73.6 sec following sugammadex administration to reach 100%.

P4 levels (ng/mL) at 60 and 90 min in Group I were significantly higher than in Group II (P<0.05). Although there was a statistically significant difference in Group I between 0 and 5 min with 30 and 60 min (P<0.05), in Group II, P4 levels at 30 min were significantly higher than at 60 and 90 min (P<0.05) *(Table 2)*. None of the groups in the study experienced abortus, early birth or still birth. The births took place when due. Each rabbit delivered 4 to 6 babies.

DISCUSSION

It is a known fact that the rate of non-obstetrical operations in pregnancy is quite high [10]. Anaesthesia means, loss of sensation in the entire body or any part of the body [11]. Modern anesthesia methods are used in many of the stated operations. One of the most important aspects of modern anesthesia methods is the use of neuromuscular blocker agents. These agents create better intubation conditions by preventing voluntary and/or reflexive muscle movements. As a result, muscle relaxation sufficient for the operation is achieved using fewer less anesthetic. If the neuromuscular junction does not adequately recover after anesthesia, post-operative pulmonary complications may develop, and mortality rates can rise [10]. Therefore, sugammadex, a neuromuscular blocker antagonist has been used in recent years to eliminate residual muscle relaxation [12].

It has been established that the TOF ratio, which is a neuromuscular recovery parameter in the adductor pollicis muscle, must be higher than 0.90 to lower the risk of aspiration after intubation and to avoid postoperative atelectasis and pneumonia [13,14]. However, Eriksson et al.[14] demonstrated that upper esophageal sphincter tonus decreased noticeably in 14 conscious patients when the TOF ratio was less than 0.90, and that muscle coordination also decreased when the TOF ratio was less than 0.60. Studies with humans found that it took 70 sec for TOF to reach 80% after administering sugammadex 2 mg/kg to reverse neuromuscular block induced with rocuronium (0.5 mg/kg) [15]. A study conducted on rabbits found that it took an average of 123 sec for TOF to reach 90% after administration of sugammadex 2 mg/kg to reverse neuromuscular block induced with rocuronium (0.6 mg/kg) [16]. It has been determined that TOF was 100% after approximately 73.6 sec following the administration of

Table 1. *Values for parameters measured on day 21 after mating*

Groups	Values Measured	Time					Statistical Measurements
		0th min.	5th min.	30th min.	60th min.	90th min.	
		Mean.±S.D.	Mean.±S.D.	Mean.±S.D.	Mean.±S.D.	Mean.±S.D.	P[b]
Control	P (min)	242.57±39.93	228±38.39	254±23.58	232.86±26.67	256.29±16.72	ns
	SAP (mmHg)	157.57±45.85	145.86±36.07	179.57±46.72	129±7.85	152.57±30.66	ns
	DAP (mmHg)	130.86±48.21	143.71±51.57	107.86±41.25	84.57±12.58	124±36.31	ns
	MAP (mmHg)	137.57±45.37	154.57±47.73	126.86±39.77	99.71±12.49	132.71±30.96	ns
	sPO_2 (%)	93.43±2.99	94.14±3.8	95.71±3.25	96.14±2.48	94.86±3.13	ns
	R (min)	18.86±4.56[a]	29.57±8.18[a]	31.86±6.41[a]	32.29±7.13[abc]	15.57±3.41[b]	a:b:0.000
	T (°C)	38.13±0.48[abx]	38.36±0.39[a]	37.83±0.56[ab]	37.66±0.59[b]	37.81±0.38[ab]	a:b:0.018
Group I	P (min)	258.29±31.9	248±25.92	248.57±20.15	241.86±8.51	237.57±27.64	ns
	SAP (mmHg)	144.86±23.86	154±36.1	152.14±25.63	134±27.17	157.14±23.41	ns
	DAP (mmHg)	120.29±29.49	112.86±37.48	115.43±48.17	88.71±34.09	124.43±29.63	ns
	MAP (mmHg)	128.57±22.11	125.57±30.22	130.29±46.95	103.14±32.06	133±26.2	ns
	sPO_2 (%)	95.71±2.29	96±2.94	95.71±2.69	95.86±2.61	94.43±2.76	ns
	R (min)	25±10.5	35.71±8.4[xy]	33.6±6.8[xy]	34.2±7.69[xy]	18.29±8.06	ns
	T (°C)	38.87±0.39[ay]	38.66±0.99[a]	38.36±0.57[ab]	37.94±0.53[b]	38.01±0.61[ab]	a:b:0.002
Group II	P (min)	250.86±16.07	264±31.3	239.71±17.17	230.71±11.57	261.86±36.29	ns
	SAP (mmHg)	123.43±19.05	147.43±22.31	155.71±31.53	131.43±22.91	161±35.57	ns
	DAP (mmHg)	100.86±16.47	110.71±43.34	105.86±25.96	94.86±31	134.14±36.24	ns
	MAP (mmHg)	112.57±18.95	129±34.18	105.29±47.11	105.29±28.34	138.86±35.8	ns
	sPO_2 (%)	95±1.63	94.71±1.5	94.14±2.73	95.86±2.67	93.14±2.48	ns
	R (min)	23.71±15.68[a]	43.14±6.6[ay]	41.71±0.49[a]	41.43±2.82[a]	14.14±3.72[b]	a:b:0.002
	T (°C)	38.56±0.49	38.63±0.83	38.5±0.76	38.31±0.6	38.3±0.43	ns
P[a]		x:y:0.027 (T)	x:y:0.043	x:y:0.029	x:y:0.041	ns	ns

P[a]: *Refers to comparisons between groups;* ***P[b]:*** *Refers to comparisons within groups. Pulse (P), Systolic Arterial Pressure (SAP), Diastolic Arterial Pressure (DAP), Median Arterial Pressure (MAP), Saturation (SPO_2), Respiratory Rate (R), Body Temperature/Centigrade degree (T/°C);* [a,b,c] *Refers to the statistical difference on each row;* ***ns:*** *non significant*

Table 2. *Distribution of progesterone levels on day 21 after mating*

Groups	Time					P
	0 min.	5th min.	30th min.	60th min.	90th min.	
	Mean.±S.D. (ng /mL)	Mean.±S.D. (ng /mL)	Mean.±S.D. (ng /mL)	Mean.±S.D. (ng /mL)	Mean.±S.D. (ng/mL)	
Control	5.77±0.17	5.72±0.36	5.76±0.38[a]	5.74±0.33[a]	5.79±0.23[a]	ns
Group I	5.51±0.14[x]	5.67±0.2[x]	6.71±0.32[by]	6.67±0.33[aby]	6.64±0.22[abxy]	x:y:0.001
Group II	5.66±0.37[xy]	5.61±0.19[xy]	6.59±0.29[bx]	4.62±0.23[acy]	4.59±0.2[acy]	x:y: 0.001
P	ns	ns	a:b: 0.003	b:c:0.001	b:c: 0.001	ns

[a,b,c] *Refers to the statistical difference in each column (P=0.001);* [x,y] *Refers to the statistical difference on each row (P=0.001);* ***ns:*** *non significant*

sugammadex (2 mg/kg), which was used in the process of reversing the neuromuscular block induced with rocuronium in Group II. It is considered that the fact that it takes less time for TOF to get to 100% might correlate with the dose of rocuronium.

All animal studies have shown that sugammadex effectively antagonizes the neuromuscular blockage caused by rocuronium without having any significant effect on arterial blood pressure or heart rate [5,17-19]. One study found that systolic, diastolic and mean arterial pressures and heart

rate were lower in the sugammadex group than in the neostigmine group, and the difference was statistically significant [20]. Our study did not find significant differences within groups with regard to pulse and systolic or diastolic arterial pressures on day 21 after mating. This suggests that rocuronium and sugammadex do not affect these parameters. The differences observed in parameters such as median arterial pressure, sPO_2, respiration values and body temperature are thought to be associated with changes expected in anesthesia.

It has been shown that the use of sugammadex (4 mg/kg) for contraceptive purposes in women reduces the amount of P4 by 34% and reduces its efficacy [21]. Progesterone is very important to pregnancy and continuation of pregnancy [8,9]. It ensures that desidual tissues develop, and it facilitates implantation following fertilization. Furthermore, it stimulates uterine growth and prevents the activity of factors that cause myometrial contractions [22]. If there is a significant decline in P4 levels, the pregnancy will be terminated [8,9]. Progesterone levels in rabbits gradually increase starting three days after mating. They peak in the middle of pregnancy and slowly decline towards the end [23,24]. Different results have been reported for P4 levels during pregnancy in rabbits. A study that investigated P4 levels in rabbits using the radioimmunoassay method found that the average was 5.3 ng/mL 3 days after mating and an average of 17-19 ng/mL on days 12-15 [23], but another study which used the chemiluminescent enzyme immunoassay method found P4 was 8.6 ng/mL on day six after mating, 15 ng/mL on day thirteen, 9.8 ng/mL on day 18 and 11.7 ng/mL on day 25 [25]. In our study, the average P4 level on day 21 after mating was 5.65 ng/mL. This difference in P4 levels is thought to be due to the day of pregnancy, as well as the brand of kit and the measuring technique that were used.

In recent years, there has been a significant increase in sedation performed on pregnant women in anesthesia procedures outside of the operating room [26]. It is not known whether or not rocuronium, which is used as a neuromuscular relaxant, poses a risk to the fetus during pregnancy [27]. No complication has been reported in an infant born during the normal gestational week to a woman who was given rocuronium for neuromuscular blockage when she was known to be pregnant [26]. Insufficient data are available from animal studies [27]. In our study, the administration of rocuronium at 21 day after mating was found to significantly increase P4 levels compared to the other groups (control, Group II). This finding is the most important conclusion of our study. These results suggest that in pregnant rabbits rocuronium supports the corpus luteum that secretes P4.

Serious complications can result due to the risk of residual curarization. Sugammadex is widely used to eliminate residual neuromuscular muscle relaxation [12]. Studies have reported that sugammadex interacts with and binds to externally administered medicinal products, thus reducing the medicines' effect [12,28]. It is not recommended for use in women during pregnancy because there is no clinical data and minimal placental transfer is possible [29]. Animal studies have demonstrated that the use of sugammadex has no harmful effect, either directly or indirectly, on pregnancy, embryonic/fetal development and birth or during the post-natal period [21].

A study by Et et al.[6] investigated the effect of administering rocuronium and sugammadex on P4 levels in rats on day 12 of pregnancy. They found that administering rocuronium followed by sugammadex did not change P4 levels, and that administration of sugammadex only lowered serum P4 levels in rats numerically. However, this decline was not statistically significant. The fact that pregnancy continued to develop normally without abortus or stillbirth suggests that sugammadex can be used safely on day 12 of pregnancy. A different study, however, reported that administration of sugammadex had no effect on serum P4 levels [12]. Because endogenous steroids similar to P4, like rocuronium, do not contain the ammonium compounds found in steroidal neuromuscular blockers, sugammadex reportedly demonstrates low affinity for these steroids [30,31]. Furthermore, steroidal hormones contribute to this low affinity because they bind strongly to special transporter proteins [12,31]. In our study, on day 21 of pregnancy, there was a significant decline in serum P4 levels at 60 and 90 min in Group II compared to levels prior to sugammadex administration. Gunduz Gul et al.[12] found that there was a temporary decline in P4 levels after using sugammadex, but that levels rose again when measured 4 h later. None of the rabbits in our study experienced abortus or stillbirth during pregnancy in spite of the decline in P4 levels. This suggests that the P4 levels rose again some h later.

In conclusion, our study did not find that the use of the neuromuscular blocker rocuronium and its antagonist sugammadex in rabbits on day 21 of pregnancy had a negative effect on serum P4 levels during pregnancy.

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Research Article

The Effects of Zinc Methionine Chelate and $ZnSO_4$ on the Growth Performance and Immune Function of the Weaned Piglets and on IPEC-J2 Cell Immune Function

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Abstract

Zinc Methionine chelate (Met-Zn) shows a better palatability, stability, and bioactivity than traditional zinc preparations, therefore this study evaluated the effect on the growth performance and immunologic functions of the weaned piglets. Two *in vivo* tests were conducted: (I) cross-breeding piglets [duroc × (landrace × large white pigs)] were fed 80 mg/kg $ZnSO_4$ or 20, 40, 60, or 80 mg/kg Met-Zn after the weaning on day 21.The content of serum globulin and lymphocyte transformation rate were measured on days 21, 35, 45 and 60; (II) another group of piglets weaned on day 28 days were fed 80 mg/kg $ZnSO_4$ or 80 mg/kg Met-Zn after orally administrated of *Escherichia coli.* The levels of some immune factors in the small intestine were measured after the feeding for one month. An *in vitro* experiment studied the expression of some immune factors and zinc transporters in the porcine small intestinal epithelial cells (IPEC-J2) after treatments with $ZnSO_4$+LPS and Met-Zn+LPS. Both Met-Zn and $ZnSO_4$ increased the lymphocyte transformation rate and the content of serum globulin. But, Met-Zn showed better effect than ZnSO4 in improving the growth performance, particularly the average daily gain, after *E. coli* insults. With *E. coli* insults, Met-Zn promoted the expression of TNF-α and IL-6 in the anterior and middle segments of the small intestine respectively, but inhibited the expression of IL-8 in the middle segment. $ZnSO_4$ promoted the expression of IL-6 in the posterior segment of the small intestine, but inhibited the expression of TNF-α in the middle segment. Both Met-Zn and $ZnSO_4$ dose-dependently increased the expression levels of TNF-α, IL-6, and IL-8 in IPEC-J2 cells after the LPS stimulation. In summary, Met-Zn improved the growth performance of piglets and changed the immunologic functions.

Keywords: *Met-Zn, Piglets, Growth performance, Intestinal tract, Immunity*

Çinko Metionin Şalat ve $ZnSO_4$'ın Sütten Kesilmiş Domuz Yavrularında Büyüme Performansı ve Bağışıklık İle IPEC-J2 Hücre İmmun Fonksiyonları Üzerine Etkileri

Öz

Çinko metionin şalatı geleneksel çinko preprasyonları ile karşılaştırıldığında daha lezzetli, stabil ve bioaktiftir. Bu çalışmada sütten kesilmiş domuz yavrularında çinko metionin şalatın büyüme performansı ve immunolojik fonksiyonlar üzerine etkisi çalışılmıştır. İki *in vivo* test uygulanmıştır: (I) Melez domuz yavruları [Duroc x (Landrace x Büyük Beyaz domuz)] 80 mg/kg $ZnSO_4$, veya 20, 40, 60 ve 80 mg/kg Met-Zn ile 21 gün süresince sütten kesme sonrasında beslendi. Serum globülin miktarı ve lenfosit transformasyon oranı 21, 35, 45 ve 60. günlerde ölçüldü. (II) 28. Günde sütten kesilmiş olan ve ağız yoluyla *Escherichia coli* uygulanan domuz yavruları 80 mg/kg $ZnSO_4$ veya 80 mg/kg Met-Zn ile beslendi. Bir aylık besleme sonrasında, ince barsaklarda bazı immun faktörlerin seviyeleri ölçüldü. $ZnSO_4$+LPS ve Met-Zn+LPS uygulaması sonrasında domuz ince barsak epitel hücrelerinde (IPEC-J2) bazı immun faktörler ve çinko transporterlerinin ekspresyonu *in vitro* olarak araştırıldı. Hem Met-Zn hem de $ZnSO_4$ lenfosit transformasyon oranı ve serum globülin miktarını artırdı. Met-Zn büyüme performansını iyileştirmede, özellikle de *E. coli* maruziyeti sonrasında ortalama günlük kilo kazanımınd $ZnSO_4$'tan daha iyi etki gösterdi. *E. coli* maruziyetinde, Met-Zn ince barsakların anterior ve orta bölümde sırasıyla TNF-α ve IL-6 ekspresyonlarını uyarırken orta bölümde IL-8 ekspresyonunu inhibe etti. $ZnSO_4$ ince barsakların posterior bölümünde IL-6 ekspresyonlarını uyarırken orta bölümünde TNF-α ekspresyonunu inhibe etti. Hem Met-Zn hem de ZnSO4 LPS stimulasyonu sonrasında IPEC-J2 hücrelerinde TNF-α, IL-6 ve IL-8 ekspresyon seviyelerinde doza bağlı artmeya neden oldu. Özet olarak, Met-Zn domuz yavrularında büyüme performansında iyileşmeye ve immunolojik fonksiyonlarda değişime neden oldu.

Anahtar sözcükler: *Met-Zn, Domuz yavrusu, Büyüme performansı, İntestinal kanal, İmmunite*

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INTRODUCTION

Zinc is an essential microelement in organisms, which adjusts multiple physiological functions, especially the immunological functions. Zinc maintains the development of immune organs and modulates cellular immunity and humoral immunity functions. Lack of zinc hinders the development of thymus gland and spleen of animals, and even results in the atrophy [1,2]. Zinc also has an influence on the number, transformation rate, phagocytosis and secretion function of immune cells. Lack of zinc significantly affects the number and functions of these cells, and reduces cell-mediated immune response, therefore influences the immune function of animals [3]. In the animal production industry, zinc can be added to diet in three manners, that is, inorganic zinc, simple organic zinc and zinc amino acid chelate. Although inorganic zinc is cheap, it shows a series of problems in the actual production. For example, the absorption and utilization efficiency of inorganic zinc is generally low, leading to the excessive supplementation and the environmental pollution resulted from unabsorbed zinc [4]. Besides, it is easy for inorganic zinc to influence the absorption and utilization of other nutritional factors [5]. In contrast, zinc amino acid chelates, such as Zinc Methionine chelate (Met-Zn) and Zinc glycine chelate, show many advantages. First, the palatability of inorganic zinc, similar to other inorganic salt, is poor due to the metallic taste. However, the smell of zinc amino acid chelates is close to the amino acid, and its palatability is better to promote animals to eat [6-8]. Moreover, zinc ion interacts with amino acid to form a five-membered (or six-membered) ring through the strong coordination bond in zinc amino acid chelates. Therefore, zinc ions are completely bound to the chelate ring, and the internal charge of zinc amino acid chelates tends to be neutral, resulting in a very table chemical structure. However, inorganic zinc and simple organic zinc show a poor stability because only simple ionic bond is formed in these chemical compounds, thus they easily interact with other nutrient substances [9,10]. The stable chemical structure of Met-Zn prevents Met-Zn from the combination with anti-nutritional factors, such as phytic acids forming insoluble compounds, and from the impairment of gastric acid. Consequently, metal trace elements smoothly enter the absorption site, which guarantees a high-efficient absorption [11]. Met-Zn and other trace element amino acid chelates generally show higher biological effects than the trace element sand amino acids, as well as exert many special physiological actions, such as improving the utilization rate of protein and vitamin, participating in intracellular redox reactions and modulating enzyme activities in organisms [8]. These effects are considered to improve the activity of immune cells and immune response, whereby enhancing the effects of cellular and humoral immunity of animals [12]. At present, early weaning of piglets is widely adopted to increase production efficiency. Early weaning influences the immune system of piglets in several aspects, especially hindering piglets to obtain maternal immune factors, which reduces the levels of antibodies in piglets. Maternal passive immunity plays a critical role in immune response of piglets, before the immune function of piglets gradually develop after the 4-5 weeks old. Therefore, the earlier the weaning time, the greater influence on the immunity. Early weaning also causes some stress reactions in piglets, resulting in the immunosuppression [6]. The intestinal tract is the largest digestive and absorption organ and functions as a protective screen against foreign harmful bacteria. However, the intestinal absorption and immunity are interfered by a series of physical, and environmental changes after the early weaning, which reduce the nutrient absorbing ability, makes the intestinal tract easy to be infected by pathogenic bacteria, and finally influence the growth of piglets [13].

The present study aimed to compare the effects of Met-Zn and zinc sulfate ($ZnSO_4$) on improving the growth performance and immune functions of early weaned piglets. The *in vivo* test initially evaluated the effect of Met-Zn at different dosages on the content of serum globulin and lymphocyte transformation rate of the early weaned piglets. Then the effects of Met-Zn and $ZnSO_4$ on the growth performance and immune functions were investigated in the early weaned piglets that were subjected to *Escherichia coli*. The *in vitro* test was performed in the porcine small intestinal epithelial cells (IPEC-J2) after the stimulation of LPS. Met-Zn and $ZnSO_4$ were added to the cells to evaluate their influence on the expression of immune factors and zinc transporters.

MATERIAL and METHODS

Ethics Statement

All animal work was approved by the University of Hunan Agricultural Animal Care Committee (Changsha, Hunan Province, China; Permit Number: 27-2956; Date: 2017. 6. 5). The experimental procedures were conducted in accordance with the Chinese guidelines for animal welfare.

The In Vivo Test

Two *in vivo* tests were conducted. In the first, a total of 288 duroc × (landrace × large white pig, DLY) three-way cross-breeding piglets weaned at the age of 21 days were divided into six groups: control group (basal diet group), $ZnSO_4$ group (adding 80 mg/kg $ZnSO_4$ to the diet) and four Met-Zn groups (respectively adding 20, 40, 60, and 80 mg/kg Met-Zn to the diet). Met-Zn and $ZnSO_4$ were purchased from Xingjia Bio-Engineering Co., Ltd. Each group included 8 repetitions and each repetition included 6 piglets. The test was carried out in two periods: period one: 21 - 35 days old; period two: 35 - 60 days old. The selected piglets were fed with different basal diets in the two periods *(Table 1)*, and the nutritional level was designed according to the

Table 1. *Composition and nutrient levels of basal diets*

Ingredients	The First Stage (%)	The Second Stage (%)
Corn	54.00	60.00
Dehulled soybean meal	8.00	12.00
Extruded Soybean	8.00	8.00
Whey powder	5.00	0.00
Fish meal	0.00	2.50
Fermented soybean meal	10.00	6.00
Glucose	2.50	2.50
Plasma protein	3.50	0.00
Soybean protein concentrate	0.00	2.00
Soybean oil	2.00	2.00
$CaHPO_4$	0.70	0.70
Limestone	0.70	0.70
Citric acid	1.30	1.30
L-Lyso•HCl	0.30	0.30
Premix[1]	4.00	2.00
Nutrient Levels[2]		
DE (MJ/kg)	14.65	14.48
CP (%)	20.5	20.00
TP (%)	0.60	0.60
Ca (%)	0.70	0.70
Lys (%)	1.45	1.30
Met (%)	0.48	0.44
Thr (%)	0.95	0.84
Try (%)	0.29	0.26
Zn (mg/kg)	22	22

[1] The full price of feed per kilogram of premix provided: Vit. A, 1500 IU; Vit. D_3, 200 IU; Vit. E, 85 IU; D-pantothenic acid, 35 mg; Vit. B_2, 12 mg; Folic acid, 1.5 mg; Nicotinic acid, 35 mg; Vit. B_1, 3.5 mg; Vit. B_6, 2.5 mg; Biotin, 0.2 mg; Vit. B_{12}, 0.05 mg; Cu (as copper sulfate), 15 mg; Fe (as ferrous sulfate), 100 mg; Mn, 20 mg; I (as calcium iodate), 1 mg; Se (as sodium selenite), 0.35 mg; Co (as cobalt sulfate) 0.2 mg; Cr (as chromium picolinate), 0.2 mg
[2] DE, CP, and TP are measured values. Other nutrient levels are calculated value

standard of NRC (2012) [14]. In the second, a total of 144 DLY three-way cross-breeding piglets weaned at the age of 28 days were administrated orally with 3×10^9 colony-forming units of *Escherichia coli* (O157: H7 strain) to induce immunological stress. *Escherichia coli* (O157: H7) strain was gifted by Prof. Tan (Key Laboratory of Agro-Ecological Processes in Subtropical Region, Hunan Research Center of Livestock & Poultry Sciences, South-Central Experimental Station of Animal Nutrition and Feed Science in Ministry of Agriculture, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, China). Then the piglets were divided into three treatments: control group (basal diet group); $ZnSO_4$ group (basal diet + 80 mg/kg $ZnSO_4$); Met-Zn group (basal diet + 80 mg/kg Met-Zn). Each treatment included 8 repetitions, and each repetition included 6 piglets. The preliminary trial period was 3 days, and formal trial period was 30 days (that is, at the age of 28~58 days). The level of basal diet referred to the nutrient level and composition of diet in the second period in *Table 1*. Body weight (BW) of pigs was measured at the beginning and end of the experiment, and the feed consumption of each group was monitored every day during the experiment period.

Sample Collection and Tests

In the first part of the test, a male and a female piglet were randomly selected in each repetition. 5 mL blood was collected from the precaval vein after 12 h of fasting at the age of 21, 35, 45, and 60 days. Lymphocyte was isolated from the blood to evaluate the transformation rate of peripheral blood lymphocyte. The transformation rate of peripheral blood lymphocyte was measured with methyl thiazolyl tetrazolium (MTT) colorimetric method [15]. The serum was obtained by the centrifuge and used to inspect the serum globulin content. To test the serum globulins, the contents of total protein and albumin were measured. The contents of total protein minus that of albumin content was globulin content. The total protein was measured with the biuret method, and albumin was measured with Bromcresol green dye method [16]. In the second part of the test, a male and a female piglet were selected and euthanized in each repetition. The small intestine was cleaned with physiological saline and cut to three segments: the anterior segment included the duodenum and jejunum; the middle segment included the anterior and middle part of ileum; the posterior segment included the posterior part of ileum. Each segment of the small intestine was sampled and frozen in liquid nitrogen for long-term preservation.

Enzyme-Linked Immunosorbent Assay (ELISA)

The C3 and C4 complements in the small intestine were determined by the commercially available ELISA kits (Sigma, St. Louis, MO, USA) following the manufacture protocols. Briefly, a 96 well coated with one specific antibody at bottom was incubated at room temperature. After washing, suitably diluted samples were added to designated wells with subsequent addition of secondary antibodies and orth-ophenylenediamine (OPD). Following an incubation period, the reaction was stopped with H_2SO_4 and the absorbance was determined at 492 nm in Multiskan ELISA plate reader (Thermo lab systems, Finland).

Real-time PCR (RT-PCR)

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific Inc., MA, USA), and then reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (Takara Biotechnology (Dalian) CO., LTD., Dalian, China). Primers for RT-PCR were presented in *italic*. qPCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems, MA, USA), and data were analyzed with the 2-ΔΔCT method. GAPDH expression was used as an internal control to calculate the relative expression levels of targeted genes *(Table 2)*.

The In Vitro Test

Porcine intestinal epithelial IPEC-J2 cell line was gifted by Prof. Tan (Key Laboratory of Agro-Ecological Processes in Subtropical Region, Hunan Research Center of Livestock & Poultry Sciences, South-Central Experimental Station of Animal Nutrition and Feed Science in Ministry of Agriculture, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, China). Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 [1:1] (Hyclone; Logan, Utah, USA) supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen; Carlsbad, CA, USA), 1% insulin-transferrin-selenium (Invitrogen), and 1% glutamine (Sigma; St Louis, MO, USA). Cells were cultured at 37°C in a 95% air-5% CO_2 atmosphere and passaged every 72 h. IPEC-J2 cells were incubated with different doses of Met-Zn (25, 50, 75, 100 and 125 μM) or $ZnSO_4$ (25, 50, 75, 100 and 125 μM) for 24 h after the stimulation with 1 μg/mL LPS (Sigma; St Louis, MO, USA). The cells were harvested to test the expression levels of some immune factors and zinc transporters using RT-PCR.

Table 2. *Forward and reverse primers sequences of each gene*

Genes	Primer Sequence	Size
TNF-α	F:5′-TTCGGGGTGATCGGTCCCAA-3′	157
	R:5′-AGCATCTCGTGTGTTTCTGA-3′	
IL-6	F:5′-CCTGAACGACCCTACCAAG-3′	242
	R:5′- AGGCTCCATAAATGAAAGA-3′	
IL-8	F:5′-CCTGAAGACCCTACCAAG-3′	238
	R:5′-AGGCTCCATAAATGAAAGA-3′	
ZIP4	F:5′-CTGCACACACATGATGGGGA -3′	103
	R:5′-GGTTGAAAAGGCTCTCGAACA-3′	
ZIP5	F:5′-CGAGGGAACAGGACAACCA-3′	154
	R:5′-CCTATCGCCAGTCCGTCAG-3′	
ZnT1	F:5′-GAATCATTGCCACTGCTCACA-3′	115
	R:5′-GGTTGAATGGTGGTAGCGTG-3′	
GAPDH	F:5′-CTTCCTGGGCATGGAGTCCT-3′	107
	R:5′CGTGTTGGCGTAGAGGTCCTT-3′	

Statistical Analysis

Data from these experiments were analyzed by SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by a Tukey's test. Significant differences were considered with values of $P<0.05$.

RESULTS

As shown in *Table 3*, no significant difference in the lymphocyte transformation rate was observed among groups at the age of 21 days. The lymphocyte transformation rate was increased by 80 mg/kg $ZnSO_4$ and 80 mg/kg Met-Zn at the age of 35, 45 and 60 days, compared to control ($P<0.05$). But, feeding lower dosages of Met-Zn, except that 60 mg/kg Met-Zn group showed increased lymphocyte transformation rate only at the age of 35 days, had no promoting effect on the lymphocyte transformation rate. At the age of 21 and 35 days, there was no significant difference in the contents of serum globulin among groups *(Table 4)*. Feeding 80 mg/kg $ZnSO_4$ and 80 mg/kg Met-Zn to the piglets increased serum globulin content at the age of 45 and 60 days ($P<0.05$). Besides, 60 mg/kg Met-Zn increased serum globulin content at the age of 60 days ($P<0.05$).

Table 3. *Effects of Met-Zn and $ZnSO_4$ on the transformation rate of peripheral lymphocytes in weaned piglets*

Time	Control	$ZnSO_4$ 80 (mg/kg)	Met-Zn (mg/kg)				*P* Value
			20	40	60	80	
21 d	1.42±0.17	1.57±0.13	1.47±0.11	1.54±0.08	1.50±0.07	1.52±0.06	0.18
35 d	1.33±0.08^{b}	1.67±0.09^{a}	1.55±0.17^{ab}	1.53±0.13^{b}	1.56±0.14^{a}	1.57±0.12^{a}	0.02
45 d	1.35±0.06^{b}	1.45±0.07^{a}	1.31±0.03^{b}	1.35±0.11^{b}	1.37±0.08^{b}	1.47±0.04^{a}	0.03
60 d	1.37±0.12^{b}	1.65±0.13^{a}	1.36±0.06^{b}	1.37±0.23^{b}	1.43±0.17^{b}	1.68±0.10^{a}	0.04

^{a,b} values with different superscripts in the same raw are significantly different ($P<0.05$)

Table 4. *Effects of Met-Zn and $ZnSO_4$ on globulin content of weaned piglets*

Time	Control	$ZnSO_4$ (80 mg/kg)	Met-Zn (mg/kg)				*P* Value
			20	40	60	80	
21 d (g/L)	10.90±0.93	13.07±0.99	12.36±0.66	11.65±0.78	12.12±1.13	12.09±1.05	0.75
35 d (g/L)	12.30±1.13	14.87±1.37	13.15±0.88	13.56±0.93	13.62±0.84	13.57±0.73	1.01
45 d (g/L)	19.95±1.78^{b}	23.45±2.02^{a}	18.31±1.45^{b}	21.35±1.66^{ab}	22.54±1.83^{ab}	25.52±1.72^{a}	0.02
60 d (g/L)	15.67±1.24^{b}	18.65±1.19^{a}	18.36±1.72^{ab}	16.37±1.06^{b}	18.43±1.54^{a}	18.88±1.63^{a}	0.02

^{a,b} values with different superscripts in the same raw are significantly different ($P<0.05$)

Table 5. *Effects of Met-Zn and $ZnSO_4$ on the growth performance of the weaned piglets*

Items	Control	$ZnSO_4$ group	Met-Zn group	P value
Initial BW (kg)	8.52±0.04	8.52±0.09	8.53±0.06	0.95
Final BW (kg)	20.68±0.24^{b}	22.07±0.15^{a}	23.29±0.54^{a}	0.04
ADG (g)	405.33±13.73^{c}	451.51±5.53^{b}	492.13±17.26^{a}	0.03
ADFI (g)	767.26±34.27	750.26±19.10	793.60±23.34	0.12
F/G	1.89±0.03^{a}	1.66±0.03^{b}	1.62±0.02^{b}	0.21
DR (%)	4.71±0.95^{a}	1.11±0.50^{b}	2.69±1.23^{b}	0.01

^{a,b} values with different superscripts in the same raw are significantly different (P<0.05)
BW, ADFI, ADG, and F/G mean body weight, average daily feed intake, average daily gain, and feed conversion ratio, respectively

Table 6. *Effects of different dosages of Met-Zn on the expression of TNF-α, IL-6 and IL-8 in IPEC-J2 cells under the LPS stimulation*

Genes	Control	Met-Zn (μM)				
		25	50	75	100	125
TNF-α	1.00±0.12	1.75±0.28*	1.91±0.24*	2.01±0.15*	2.51±0.29**	2.56±0.21**
IL-6	1.00±0.21	1.21±0.19*	1.27±0.09*	2.03±0.18*	2.53±0.23**	2.59±0.32**
IL-8	1.00±0.09	1.97±0.31*	2.03±0.21*	2.31±0.31**	2.99±0.34**	3,27±0.18**

** P<0.05 and ** P<0.01vs. control in the same row*

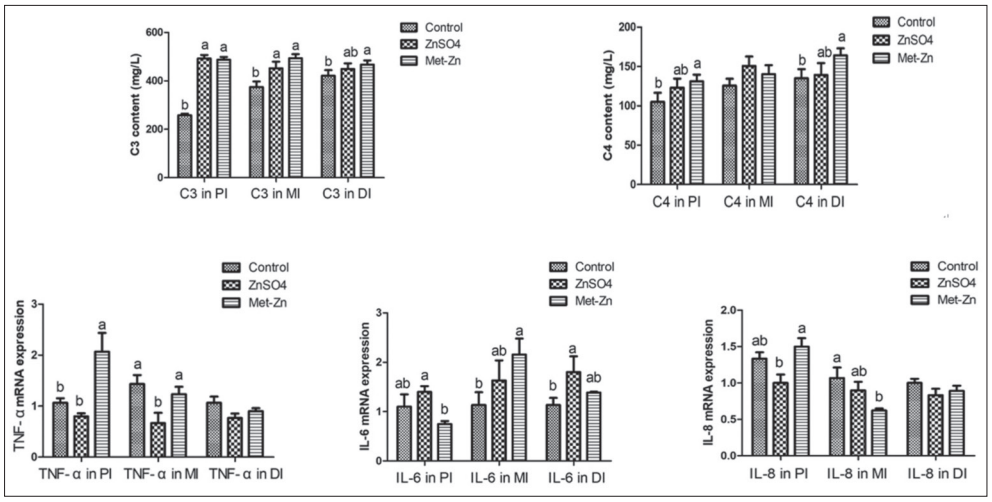

Fig 1. Effects of Met-Zn and $ZnSO_4$ on the immune-related factorsin the small intestine; ^{a,b} valueswith different superscripts in the bar are significantly different (P<0.05)

As shown in *Table 5*, the final BW and ADG were higher in the Met-Zn and $ZnSO_4$ groups than the control group (P<0.05). In addition, Met-Zn groups showed higher ADG than $ZnSO_4$ group (P<0.05). Met-Zn and $ZnSO_4$ groups showed decreased F/G (P<0.05) and DR (P<0.01) compared to control group.

Feeding Met-Zn and $ZnSO_4$ increased C3 concentrations in the anterior and middle segments of the small intestine (P<0.01, *Fig. 1)*. In addition, Met-Zn increased C3 concentrations in the posterior segment of the small intestine (P<0.01). Feeding $ZnSO_4$ showed no effect on C4 concentrations in the small intestine. However, Met-Zn increased C4 concentrations in the anterior and posterior segments of the small intestine (P<0.01). Met-Zn promoted the expression of TNF-α and IL-6 in the anterior and middle segments of the small intestine respectively (P<0.05), but inhibited the expression of IL-8 in the middle segment (P<0.05). $ZnSO_4$ promoted the expression of IL-6 in the posterior segment of the small intestine (P<0.05), but inhibited the expression of TNF-α in the middle segment (P<0.05). Under the LPS stimulation, Met-Zn and $ZnSO_4$ significantly increased the mRNA expression levels of TNF-α, IL-6 and IL-8 in IPEC-J2 in dose-dependent manners *(Table 6* and *Table 7)*

Under the LPS stimulation, Met-Zn dose-dependently increased the mRNA expression of *ZnT1* (P<0.05 or P<0.01, *Table 8)*. Met-Zn at low dosage (25 μM and 50 μM, P<0.05) and high dosage (125 μM, P<0.01) inhibited the mRNA expression of *ZIP4*. Met-Zn at dosages of 25, 50, 75, and 100 μM inhibited the expression of *ZIP5* (P<0.05), 125 μM Met-Zn promoted *ZIP5* expression (P<0.05). $ZnSO_4$ dose-dependently increased the *ZnT1*mRNA expression, but

Table 7. *Effects of different dosages of $ZnSO_4$ on the expression of TNF-α, IL-6 and IL-8 in IPEC-J2 cells under the LPS stimulation*

Genes	Control	$ZnSO_4$ (μM)				
		25	50	75	100	125
TNF-α	1.00±0.11	1.49±0.16*	1.91±0.21*	2.02±0.21*	2.76±0.24**	2.96±0.39**
IL-6	1.00±0.08	1.42±0.19*	1.25±0.17	2.24±0.27**	3.01±0.48**	3.09±0.52**
IL-8	1.00±0.15	1.46±0.20*	1.22±0.31	2.53±0.30**	2.89±0.39**	3.18±0.37**

** P<0.05 and ** P<0.01 vs. control in the same row*

Table 8. *Relative expression of target of ZnT1, ZIP4 and ZIP5 genes in the IPECs by different dosage of Met-Zn in IPECs*

Genes	Control	Met-Zn (μM)				
		25	50	75	100	125
ZnT1	1.00±0.24	1.98±0.21*	2.52±0.42*	2.48±0.23*	4.08±1.04*	7.86±2.32**
ZIP4	1.00±0.38	0.63±0.08*	0.65±0.09*	1.05±0.18	1.97±0.74	0.18±0.02**
ZIP5	1.00±0.27	0.31±0.09*	0.43±0.12*	0.24±0.11*	0.25±0.09*	2.03±0.53*

** P<0.05 and ** P<0.01 vs. control in the same row*

Table 9. *Relative expression of target of ZnT1, ZIP4 and ZIP5 genes in the IPECs by different dosage of $ZnSO_4$ in IPECs*

Genes	Control	$ZnSO_4$ (μM)				
		25	50	75	100	125
ZnT1	1.00±0.23	1.15±0.22	2.52±0.63*	3.21±1.01*	4.11±2.03*	5.42±2.83**
ZIP4	1.00±0.31	0.88±0.14	0.53±0.07*	0.33±0.09*	0.23±0.05*	0.21±0.05*
ZIP5	1.00±0.06	0.21±0.03*	0.41±0.03*	1.19±0.18	2.18±0.11*	2.67±0.14*

** P<0.05 and ** P<0.01 vs. control in the same row*

inhibited the*ZIP4* mRNA expression in a dose-dependent manner (P<0.05 or P<0.01, *Table 9)*. Low dosages of $ZnSO_4$ (25 μM and 50 μM, P<0.05) inhibited the expression of *ZIP5*, but high dosages of $ZnSO_4$ (100 μM and 125 μM, P<0.05) promoted the expression of *ZIP5*.

DISCUSSION

There is close correlation between zinc and the immune function of animals. The supplementation with zinc increased the transformation rate of peripheral lympho-cytes of piglets that lack zinc [17]. A study reported that the lack of zinc decreased the level of corticosterone in animal blood and thus influenced the immune function [18]. In this experiment, both Met-Zn and $ZnSO_4$ increased the transformation rate of peripheral lymphocytes and the content of immune globulin in piglets, which indicated that Met-Zn and $ZnSO_4$ enhance the immune function. These results are consistent with the relevant report [19], that is, adding 200 mg/kg Met-Zn in the feed increased the concentration of IgG in the piglets. Some studies also reported that zinc oxide and Met-Zn significantly increased the transformation rate of lymphocytes of piglets [20,21].

Early-weaned piglets are vulnerable to foreign pathogenic bacterium, due to a poorly developed immune function. *Escherichia coli* is such bacterium, usually causing severe diarrhea, retarded growth and even the death in early-weaned piglets, which significantly reduced the growth performance of early-weaned piglets. Complement is a group of glycoproteins that positively regulate the activity of phagocytes to eliminate pathogenic microorganism [22]. The study showed that feeding Met-Zn significantly increased the contents of C3 and C4 in the small intestine, suggesting that Met-Zn can improve the immune function of the piglets. Although feeding $ZnSO_4$ also increased C3 in the small intestine, it had no effect on the content of C4. Therefore, $ZnSO_4$ may be less effectively than Met-Zn in promoting the immune function. In line with the speculation, Met-Zn more effectively improved the growth performance, particularly the average daily gain, of the *Escherichia coli*-infected piglets than $ZnSO_4$.

Preinflammatory cytokines, including IL-6 and IL-8, play an important role in the initiation of the immune response after infection.IL-6 and IL-8 can induce the activation of macrophage, B cell and neutrophil, and the activated macrophage is able to generate TNF-α, mediating the following immune responses [23,24]. After the stimulation by the LPS of *Escherichia coli*, IL-6 and IL-8 are rapidly released, which is conducive to activating the immune system of piglets [23,24]. However, the excessive release of IL-6 and IL-8 affects the permeability of intestinal epithelium and the height of intestinal villi, leading to the down-

regulation of ion transport and digestive enzyme activity, and consequently inhibiting the digestive and absorption function. Moreover, a large amount of nutrients used for the immune response following the immune stimulation to some extent reduces the amounts of nutrients used for the growth performance. Therefore, the generation of preinflammatory cytokines should be precisely controlled to balance their effects on immune functions and growth performance. In the present study, Met-Zn and $ZnSO_4$ on the one hand stimulated the expression of some preinflammatory cytokines in some segments of small intestine; on the other hand inhibited their expression on other segments of small intestine. In addition, feeding Met-Zn and $ZnSO_4$ showed many differences in the expression of IL-6, IL-8 and TNF-α in various segments of the small intestine. These results suggested that Met-Zn and $ZnSO_4$ exerted complicated effects on the immune functions.

LPS, as the main toxic ingredient of Gram-negative bacterium, is able to induce the release of multiple inflammatory mediators from the small intestine epithelial cells [25]. In addition, the increase of zinc in the cell culture medium also promotes the expression of TNF-α, IL-6 and IL-8 [26]. In the study, under the LPS stimulation, Met-Zn and $ZnSO_4$ significantly enhanced the expression of TNF-α, IL-6 and IL-8ina dose-dependent manner. But these data were different from the results of the *in vivo* test. Zinc enters the blood circulation through intestinal mucosa, which meets the requirement of all the immune organs and thus affects the immune response throughout the body. However, in the *in vitro* test, zinc was absorbed through the zinc transporters at the cell membranes, which only affected the immune response of IPEC-J2 [19]. This likely results in the difference in the expression of TNF-α, IL-6 and IL-8 between *in vivo* and *in vitro* tests.

Immune response is accompanied with the change in the content of zinc in bodies [27-29]. Lack of zinc in cytoplasm hinders the development of precursor cells of B and T lymphocytes, reducing the immune function [30], and a high concentration of zinc in cytoplasm is highly toxic to cells [31]. Zinc transporters play important role in maintaining a reasonable level of zinc in cells and the body [32]. ZnT1 is mainly distributed in the jejunum and regulates the excretion of zinc from cells [33]. When zinc is high in surrounding environment, intestinal cells transport zinc out of cells or transport zinc to intracellular vesicles by up-regulating the mRNA expression of ZnTl, so as to reduce zinc content in the cytoplasm [34]. In this study, both Met-Zn and $ZnSO_4$ promoted the mRNA expression of ZnT1 in LPS-treated IPEC-J2 cells, which likely accelerates the efflux of zinc.

ZIP4 is located at the apical membrane of intestinal epithelial cells and mainly responsible for zinc absorption in cells. Met-Zn at low and high dosages significantly down-regulated the mRNA expression of ZIP4 in the cells under the LPS stimulation. Besides, high dosages of $ZnSO_4$ also down-regulated the ZIP4 mRNA expression. Down-regulated ZIP4 may reduce the absorption of zinc by cells and prevent toxic effect of zinc accumulation on cells. Gui et al.[35] also found that the mRNA expression of ZIP4 in IPEC-J2 cells was decreased dose-dependently by $ZnSO_4$. Han et al.[36] found that adding zinc lactate down-regulated the mRNA expression of ZIP4, and promote the proliferation of IPEC-J2. However, feeding glycine zinc to rats increased the mRNA expression level of ZIP4 in the duodenum [37].

ZIP5 is located at the basolateral side of intestinal cells and may be responsible for transferring zinc from the serosal layer to the mucous layer. Therefore, it can transport the zinc from the body to intestinal cells and finally secreted zinc from intestinal cells [38]. After ZIP5 gene is knocked out, the content of zinc in the liver of mice fed with high zinc is increased and the function of pancreas to store zinc is influenced [39]. In addition, the loss of the function of ZIP5 of intestinal cells significantly increases the content of zinc in pancreas of mice fed with high zinc and up-regulate the mRNA expression of ZIP4 in the intestinal tract [40]. In this experiment, low dosage of Met-Zn and $ZnSO_4$ down-regulated ZIP5 in IPEC-J2 cells, while high dosages of Met-Zn and $ZnSO_4$ up-regulated ZIP5.This suggests that lake of zinc in diet likely inhibits the outflow of zinc from body, while the sufficient zinc in diet likely promote the excretion of zinc from bodies.

In summary, Met-Zn showed better effects on the improvement of the growth performance and immunologic functions of early-weaned piglets than $ZnSO_4$. Met-Zn and $ZnSO_4$ showed different effect on the expression of preinflammatory cytokines in small intestine, but most similar effects on the expression of preinflammatory cytokines and zinc transporters in LPS-treated IPEC-J2 cells.

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Research Article

Evaluation of the Accelerator Effect of Coral and Platelet Rich Fibrin on Bone Healing [1]

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Abstract

This experiment was conducted to investigate the accelerator effect of coral and platelet rich fibrin (PRF) on bone healing in rabbits (n=12) with clinically, radiologically, and histologically. The rabbits were divided randomly into two groups. There were two bone defects (3.5 mm diameter) created bilaterally on the proximal part of the tibia of rabbits. In control group, the defects were left empty. The other defects (twelve defects in each group) were filled with coral, PRF and coral plus PRF. Postoperatively, on the 30th and 60th days, clinical, radiographic and histologic examinations were performed. On the radiological examinations, bone healing was better seen in the grafted groups than in the control group (P<0.01). During the histological examinations on the 30th day, differences between groups were not important. On the 60th day, bone healing was found to be better in the coral, PRF and coral plus PRF groups than in the control group (P<0.01). However, the best bone healing was observed in the PRF group (P<0.01). In conclusion, applications of coral, PRF and coral plus PRF (especially PRF) are significantly effective for bone healing.

Keywords: *Coral, Platelet rich fibrin, Bone graft, Rabbit*

Mercan ve Trombositten Zengin Fibrinin Kemik İyileşmesi Üzerindeki Hızlandırıcı Etkisinin Değerlendirilmesi

Öz

Bu deney, tavşanlarda (n=12) mercan ve trombosit açısından zengin fibrinin (TZF) kemik iyileşmesi üzerindeki hızlandırıcı etkisini klinik, radyolojik ve histolojik olarak araştırmak amacıyla yapıldı. Tavşanlar rastgele iki gruba ayrıldı. Tavşanların tibialarının proksimalinde bilateral olarak iki kemik defekti (3.5 mm çapında) oluşturuldu. Kontrol grubundaki defektler boş bırakıldı. Diğer defektler (her grupta oniki defekt) mercan, TZF ve mercan ve TZF ile dolduruldu. Operasyon sonrası, 30 ve 60. günlerde klinik, radyografik ve histolojik muayeneler yapıldı. Radyolojik incelemelerde greftli gruplarda kemik iyileşmesi kontrol grubuna göre daha iyi olduğu gözlendi (P<0.01). 30. günde yapılan histolojik incelemelerde gruplar arasındaki farklar önemli değildi. 60. günde, kemik iyileşmesinin, kontrol grubuna göre mercan, TZF ve mercan artı TZF gruplarında daha iyi olduğu bulundu (P<0.01). Bununla birlikte, en iyi kemik iyileşmesi TZF grubunda gözlendi (P<0.01). Sonuç olarak, mercan, TZF ve mercan artı TZF (özellikle TZF) uygulamaları kemik iyileşmesinde önemli derecede etkilidir..

Anahtar sözcükler: *Mercan, Trombositten zengin fibrin, Kemik grefti, Tavşan*

INTRODUCTION

Bone defects due to trauma, resection of bone cysts and bone tumours, congenital defects or corrective osteotomies are a worldwide problem. Autogenous, allogeneic, xenogeneic and alloplastic bone graft materials are usually used to treat bone defects [1,2]. Autogenous bone grafts have been widely used as the gold standard for accelerating bone healing. However, it is known that there is donor site morbidity, and limited sources of autogenous bone grafts are an important problem. Disadvantages of using allografts include the risk of disease transfer and non-union [3,4]. Alternative options are attractive and continue to be sought [5,6].

Coral, as marine invertebrates, have been widely used

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for orthopaedics [4]. It is reported that the exoskeleton of corals is a good biomaterial that exhibits porosity very similar to that of human cancellous bone, with suitable mechanical, natural resorbable and osteoconductive properties. Many investigators have reported that coral has a high compressive breaking stress, interconnected porous structure, and good resorbability and biocompatibility [1,5].

Many factors, such as hormones and growth factors, are effective on increasing bone development and on accelerating bone healing. Platelet rich fibrin (PRF) is an autogenous fibrin matrix used to improve bone formation [7-12]. Platelets produce cytokine-like transforming growth factor beta, platelet-derived growth factor, insulin-like growth factor and vascular endothelial growth factor. The cytokines and growth factors in the fibrin network are released slowly [13,14]. Kang et al.[15] reported the influences of PRF in bone healing, either alone or in combination with other graft materials.

In light of the above information, we hypothesised that the PRF (osteoinductive graft material, containing cytokines and growth factors), and coral (osteoconductive graft material) would be accelerate bone healing alone or in combination. The establishment of therapeutic condition of PRF and coral would aid greatly in guiding clinicians in treatments of bone healing. Ideally these would be under animal studies that would help the analysis of bone regeneration capacity. The findings obtained from this study will be useful in the treatment of similar cases in domestic animals. For this purpose, clinical, radiological and histological comparisons of the effects of coral, PRF and coral plus PRF on bone healing were investigated. The purpose of this experimental study was to appraise the efficiency of coral and PRF (alone and in combination) on bone healing together in terms of clinical, radiological, and histological results.

MATERIAL and METHODS

The experiment was performed on twelve male New Zealand rabbits (5-6 months old, weighing 2.5-3 kg) in the Firat University Experimental Research Centre. The rabbits were fed standard rabbit food and allowed to move freely. This study was confirmed by The Animal Experiment Ethics Committee of the Firat University (Number: 102, 14.10.2010 dated).

Madreporaria sp. coral was used in this study. A coral branch was divided into small granules, and the granules were sterilized by gamma irradiation.

PRF was prepared using a previously described technique [16]. Venous blood samples (8 mL) were collected from vena jugularis (into a tube without an anticoagulant) and then centrifuged at 400 *g* for 10 min. The centrifuged product had three layers: platelet poor plasma at the upper of the tube, PRF in the middle and a red blood cell layer at the lower part. The PRF clot (2-3 mL) was removed from the tube, and it was isolated from the residual blood components. The PRF clots were kept in sterile petri dishes until they were grafted.

For coral plus PRF, the PRF clot was cut into small pieces, and then equal amounts of PRF and coral granules were mixed.

Surgical Procedure

Anaesthesia was performed using xylazine HCl (5 mg/kg, IM, Rompun, 23.32 mg/mL, Bayer) and ketamine HCl (35 mg/kg, IM, Ketalar 50 mg/mL, Parke-Davis). Both hind limbs of the rabbits were shaved. Operation regions were prepared with 1% povidone iodine. A skin incision (2 cm length) was made on the medial surface of the tibia. Totally forty-eight unicortical bone defects (3.5 mm diameter, two defects in the proximal part of each tibia,1 cm distance between two defects) were created on rabbits. Twelve defects of the 48 bone defects were left empty (Control group) *(Fig. 1A)*. The other defects were filled with coral granules *(Fig. 1B)*, PRF *(Fig. 1C)* or coral granules plus PRF *(Fig. 1D)* (12 defects in each group). After the bone defects were filled with grafts, the subcutaneous connective tissue was sutured with 3/0 chromic catgut, and the skin was sutured with 2/0 silk. The rabbits were administered intramuscularly with procaine penicillin (400.000 IU, IM, Iecilline, I.E. Ulagay) and metamizol sodium (25 mg/kg, IM, Novalgine 1000 mg/mL, Sanofi) for postoperative 5 days.

Radiographic Evaluations

Radiographic images were taken to evaluate new bone formation in the defects (after surgery, on the 30th and 60th days). Radiographic images were evaluated according to the modified Lane and Sandhu [17] scoring method. Each radiograph was given a score of 0-4: 0, no sign of bone formation; 1, 25% bone formation filling the defect; 2, 50% bone formation filling the defect; 3, 75% bone formation filling the defect; and 4, 100% bone formation filling the defect.

Histological Examinations

Rabbits were sacrificed 30 (n=6) and 60 days (n=6) after the surgery. Bone samples including grafts were resected, and bone samples were fixed in 10% buffered formalin. These samples were decalcified in nitric acid solution and processed for histological examination. Histological sections (5 µm thick) were prepared from the centre of each sample, were stained with haematoxylin and eosin, and were evaluated using a light microscope.

The histological scores were assigned in a blinded manner, as described previously [18]. Each specimen was given a score of 1-7: 1, only fibrous tissue; 2, more fibrous tissue than cartilage tissue; 3, more cartilage tissue than fibrous tissue; 4, more cartilage tissue than trabecular bone tissue;

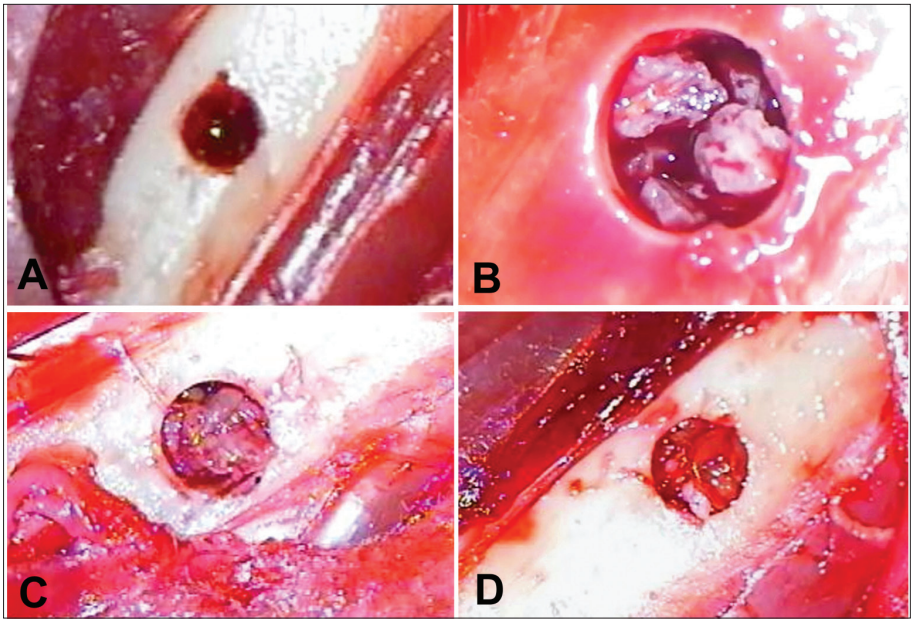

Fig 1. Postoperative appearance of the bone defects. **A-** Control, **B-** Coral, **C-** Platelet rich fibrin, **D-** Coral plus platelet rich fibrin

5, same amount of immature bone tissue and cartilage tissue; 6, more trabecular bone tissue than cartilage tissue; and 7, compact bone tissue.

Statistical Analysis

SPSS (22.0 version) was used for statistical evaluations. The non-parametric Kruskal-Wallis H test were used to identify differences among the groups. The non-parametric Mann-Whitney-U test was used to compare significant differences between the results on the 30th and 60th days within each group. Values are presented as the means±SEM. Differences were considered statistically significant when $P<0.05$.

RESULTS

The surgical procedures were well tolerated by all rabbits. Surgical regions healed uneventfully. The rabbits completed the study without death or surgical complications. During the study, adverse effects related to graft materials on health were not observed.

The radiological appearances of the bone defects are given in *Fig. 2*. Radiologic evaluation of the bone formation according to the modified Lane and Sandhu [17] radiologic scoring method is given in *Table 1*.

In postoperative radiographs, bone defects and coral granules were observed clearly. The PRF showed decreasing radiolucent contrast. On the 30th and 60th days, in the coral and coral plus PRF groups, non-resorbed small coral particles were found. Migration of placed graft materials was not observed. When the comparison between groups was made, bone healing was found to be worse in the control group compared to in the other groups ($P<0.01$).

Microscopic images are shown in *Fig. 3*. According to the modified histological scoring criteria [18], the evaluations of bone healing on the 30th and 60th days are summarised in *Table 2*.

On the 30th day, in the control group, the defects were filled with neoformed granulation tissue and capillary vessels. Minor cartilage tissue formation was observed. The defects were partially filled with newly formed granulation tissue in the coral group. New bone formation was started between coral grafts and the host bone. In the PRF group, the defects were completely filled with a collagen-rich fibrous callus. In addition to the formation of fibrous tissue and cartilage tissue, osteoblastic activity was evident. Spicular primary bone areas incorporated with each other, and some of the structures transformed to trabecular structures. In addition, bone marrow formation began within defects. In the coral plus PRF group, defects were partially filled with fibrous callus, but the edges of the defects did not have union. Areas of cartilage tissue separate from connective tissue were evident. New trabecular bone formation around the coral grafts was observed. However, differences between groups on the 30th day were not important.

On the 60th day, in the control group, it was observed that a small amount of cartilage tissue formed within the capillary vessels-rich fibrous callus. In addition, spicular primary bone tissue formation was present. In the coral group, the defects were fully filled with cartilage and primer bone tissue. Trabecular bone formation was detected, and osteoblastic activity was evident. Osteoclasts in contact with new bone formation were observed. It was determined that the defects were fully filled by fine primary and secondary bone formations in the PRF group. It was also determined that haversian canals were formed. Cartilage and fibrous tissue formations were not found. Osteoblastic activity was on the bone marrow defect side. In the coral plus PRF

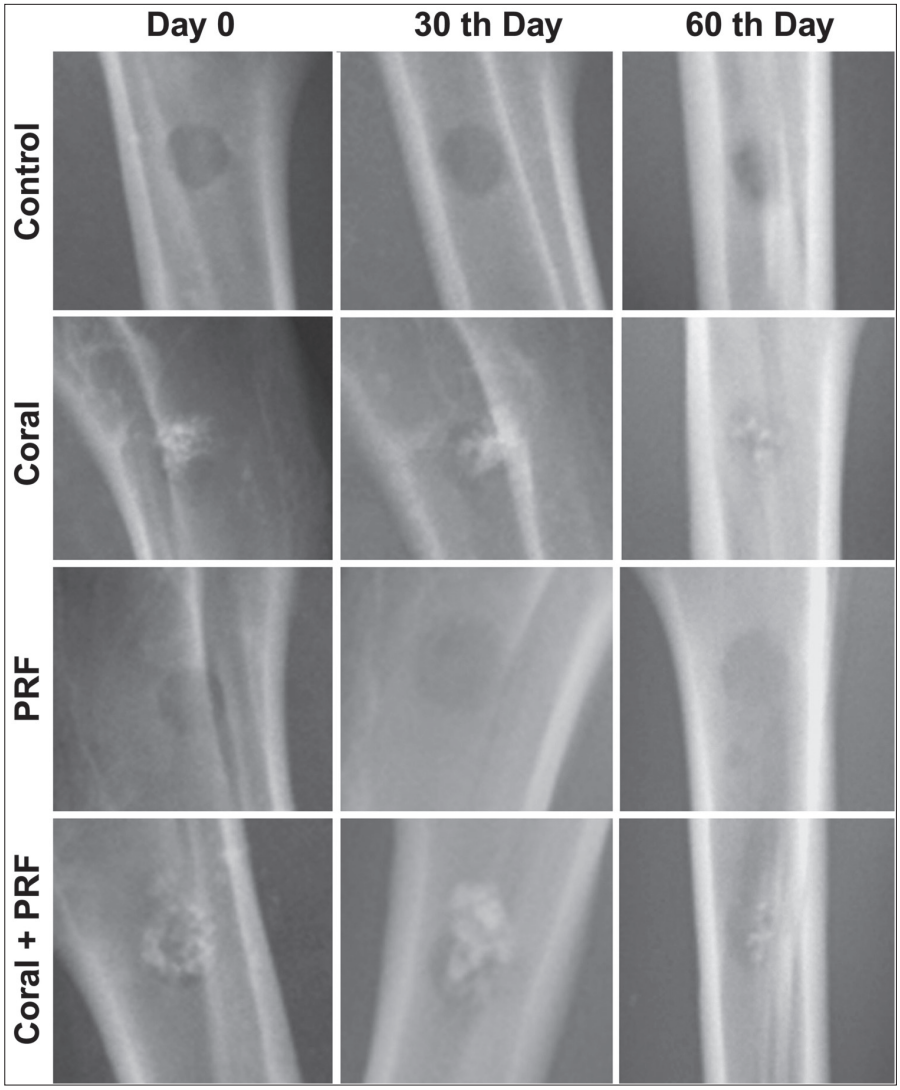

Fig 2. Radiological appearances of the bone defects. PRF: platelet rich fibrin

Table 1. *Radiological evaluation of the defects on 30th and 60th days according to the modified Lane and Sandhu [17] scoring system*

Groups (n=12)	Days	
	30	60
Control	0.50 ± 0.22^{aA}	1.50 ± 0.22^{aB}
Coral	1.83 ± 0.17^{bA}	3.17 ± 0.17^{bB}
PRF	2.67 ± 0.21^{bA}	3.83 ± 0.17^{bB}
Coral+PRF	2.50 ± 0.22^{bC}	3.50 ± 0.22^{bD}

The values are given as mean±SEM; Different superscripts (a,b) in same column represents is a significant difference (P<0.01); Different superscripts (A,B) in same row represents is a significant difference (P<0.01); Different superscripts (C,D) in same row represents is a significant difference (P<0.05); SEM: standard error of the mean

group, the defect was partially filled with bone trabeculae. Havers channel formation and lacuna formation were noted. There was significant bone marrow formation. On the 60th day, bone healing was found to be better in the coral, PRF and coral plus PRF groups than in the control group (P<0.01). The best bone healing was observed in the PRF group (P<0.01).

DISCUSSION

Concentrated platelets are used for bone healing. It is reported that PRF is equipped with many growth factors, and it is effective in bone healing. The growth factors are secreted from α granules 10 min after platelet activation. These factors are transmitted to the wound area in 1 hour, and the healing process begins [19-21]. Platelet-derived growth factor is one of these factors, and this factor accelerates cell proliferation, neovascularization and restructuring in the wound area [8-12,22-24]. It is reported that PRFs have significant advantages for haemostasis and wound healing. It is believed that interleukins in the PRF help wound healing by suppressing inflammation [13,14]. In this study, PRF was obtained according to Choukroun's technique [25,26]. Low speed centrifugation was performed, and we aimed to obtain PRF similar to the natural fibrin network.

To increase bone formation and promote the healing of bone defects, osteoinductive graft materials have been used alone or with osteoconductive graft materials [2,6,15,27,28].

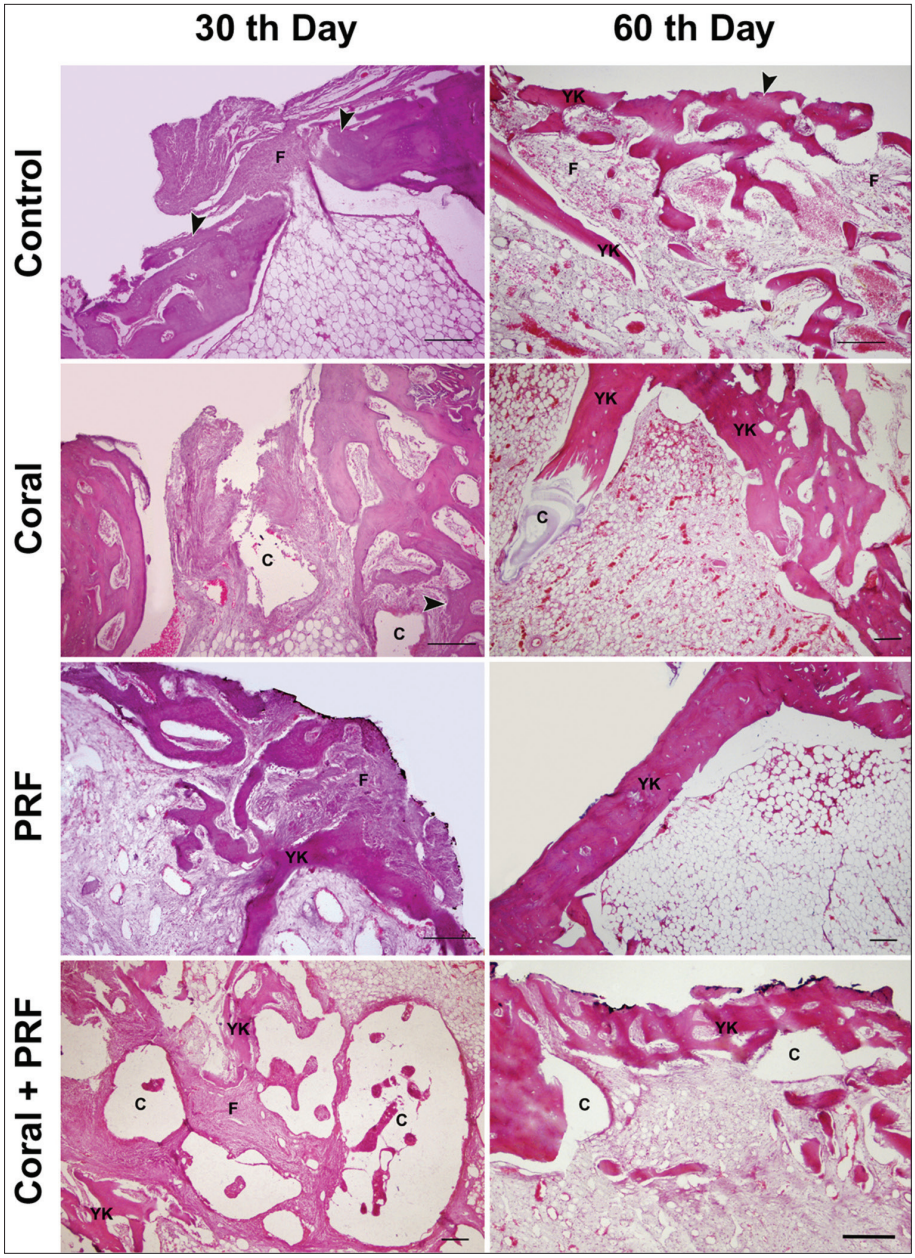

Fig 3. Appearance of the haematoxylin-eosin-stained samples. PRF: platelet rich fibrin, F: fibrous tissue, *Arrow heads:* cartilage tissue, C: coral remnant, YK: new bone, H-E, Bar: 200 µ

Table 2. *Histological evaluation of the defects on 30th and 60th days according to the modified histological scoring system [18]*

Groups (n=12)	Days	
	30	60
Control	1.50±0.22^{A}	3.50±0.22^{Ba}
Coral	2.33±0.21^{A}	5.00±0.37^{Bb}
PRF	2.67±0.33^{A}	6.17±0.17^{Bc}
Coral+PRF	2.33±0.21^{A}	5.17±0.31^{Bb}

The values are given as mean±SEM; Different superscripts $^{(A,B)}$ in same row represents is a significant difference (P<0.01); Different superscripts $^{(a,b,c)}$ in same column represents is a significant difference (P<0.01); SEM: standard error of the mean

In the present study, a tibial defect model was used to evaluate the effects of coral (osteoconductive graft material) and PRF (osteoinductive graft material) on bone healing. We also aimed to create a resource for researchers regarding the acceleration of bone healing.

Healing in bone defects depends largely on the size of the defect. If a bone defect does not heal spontaneously during the experiment, it is a critically sized bone defect [29]. In this study, the 3.5 mm tibial defects in the rabbits were considered to be critically sized bone defects for a 2-month test period.

The coral skeleton has an open, interconnected porous structure, and this structure makes it an important scaffold for bone healing. The pore size of corals is very important. When the coral pores are very small, occlusion by cells will occur. Coral pore size should be at least 150 µm for satisfactory osteoblast and vascular invasion [1,5].

The properties of the bone graft determine the graft resorption rate. The particle size and porosity of the surface are important for graft resorption. Oversized grafts are resorbed more slowly, while porous grafts are resorbed more quickly [1]. The coral used in this study belongs to the *Madreporaria* genus. Its structure is similar to cancellous bone, and it has an aragonite crystal structure. The coral had a 400-800 µm wide interconnected porous structure. This structure had many advantages for vascularization and cell invasion.

It has been reported that in postoperative radiologic examinations, a grafted coral structure was clearly observed, and it was resorbed over time [1,5]. In this study, the coral and its porous structure were clear in radiologic examinations. At the 30th and 60th days postoperatively, coral grafts were partially resorbed, and the density was decreased depending on new bone formation. However, normal bone contrast was not seen. In the coral ($P<0.01$), PRF ($P<0.01$) and coral plus PRF groups ($P<0.05$), callus formation increased over time, and the defects were filled by newly formed bone tissue. Radiological results showed that the graft materials have a stimulating effect on bone healing ($P<0.01$).

Histological examination is necessary to determine bone healing at the cellular level. It is reported that ossification starts in 1-2 weeks, and defects are filled and reorganized in 6-8 weeks [4,16,28]. In this study, the histopathological investigation periods for bone healing were 30 and 60 days.

The histological consequences of this study are in agreement with the findings of other investigators [7,29]. These results showed that bone healing increased during the study period in the coral, PRF and coral plus PRF groups. At the same time, it was shown that bone grafting is required for increasing new bone formation. On the 60th day, in the grafted groups, new bone formation was significantly better than in the control group ($P<0.01$). But it was observed that coral was slightly reduced bone healing in coral plus PRF group compared to the PRF group on the 60th day ($P<0.01$). In addition, on the 60th day, the best bone healing was observed in the PRF group ($P<0.01$).

Bone grafts should be resorbed after the formation of new bone. Additionally, the grafts, which had high osteoinductive properties, should increase new bone formation in a short time and be replaced by new bone. During this study, in agreement with the reports of other investigators [5,28], the resorption of coral grafts continued, but complete resorption was not observed in any cases at the end of the 2 month period. At the same time, foreign body cells or inflammatory reactions were not observed in the coral group.

In conclusion, this study showed that coral, PRF, and coral plus PRF are significantly effective in bone healing. This may be of importance for raising the possibility that PRF may have potential in the treatment of bone defects.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

The Effects of Different Organic Acid Treatments on Some Microflora and Pathogen *Listeria monocytogenes* of White Brine Cheese [1]

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Abstract

The aim of this study was to determine the supplementation of 1% tartaric, malic, ascorbic, fumaric, lactic, acetic and succinic acids in brine on the pH, physical and sensory properties of cheese during storage. In addition, the effect of these organic acids on growth of lactic acid bacteria, yeast/mold and possible inhibitive effect on *Listeria monocytogenes* in brine were investigated. For this purpose, these organic acids were added to 12% brine and stored at +4°C. Physical and sensory properties of cheese were defined and scored by panelists during 1st, 7th, 14th, 21st, 28th, 35th, 42th days. Cheeses ripened in tartaric, malic, fumaric, and lactic acid supplemented brine were scored as the highest points by panelists. *Lactobacillus, Lactococcus* counts significantly decreased in brine samples added with fumaric, lactic and malic acid during cheese ripening process. The highest antimicrobial activity of the organic acids was found against *L. monocytogenes*. It was also found that there was a significant decrease in *L. monocytogenes* counts in malic acid and tartaric acid groups as 2.91 log10 CFU/g and 2.95 log10 CFU/g, respectively. As a result; the supplementation of 1% tartaric acid and malic acid to the classically produced cheese brine were found effective on controlling *L. monocytogenes* which is a significant threat for public health. In addition, it was concluded that tartaric acid can be used commercially in the production of cheese due to its low inhibition effect on lactic acid bacteria, and non-destructive effect on the physical structure of the cheese.

Keywords: *Antimicrobial activity, L. monocytogenes, Organic acid, White cheese*

Beyaz Peynir Salamurasına Farklı Organik Asitlerin İlavesinin Mikroflora ve Patojen *Listeria monocytogenes* Üzerine Etkileri

Öz

Bu çalışmanın amacını laboratuvar ortamında klasik olarak üretilmiş peynirin salamura suyuna %1 oranında katılan tartarik, malik, askorbik, fumarik, laktik, asetik ve suksinik asitin depolama sürecinde peynirin fiziksel ve duyusal özellikleri ile pH değeri üzerine etkileri oluşturmuştur. Ayrıca, salamura suyuna eklenen organik asitlerin laktik asit bakterileri, küf-maya sayısı ve *Listeria monocytogenes* üzerine olası etkileri araştırılmıştır. Bu amaçla organik asitler %12'lik salamura suyuna eklenmiş ve +4°C'de depolanmıştır. Peynirin fiziksel ve duyusal özelikleri 1, 7, 14, 21, 28, 35 ve 42. günlerde belirlenmiş ve panelistler tarafından puanlanmıştır. Peynirin olgunlaşma sürecinde tartarik, malik, fumarik ve laktik asit eklenmiş salamura örnekleri panelistler tarafından en yüksek puanı almışlardır. Peynirin olgunlaşma sürecinde fumarik, laktik ve malik asit eklenmiş salamura örneklerinde *Lactobacillus, Lactococcus* sayıları istatistiksel olarak önemli derecede azalmıştır. Organik asitlerin en yüksek oranda antimikrobiyel etkinliği *L. monocytogenes* karşı tespit edilmiştir. Malik asit ve tartarik asit gruplarında *L. monocytogenes* sayılarında ortalama sırasıyla 2.91 log10 kob/g, 2.95 log10 kob/g oranında önemli derecede azalma tespit edilmiştir. Sonuç olarak; klasik yöntemle üretilen peynirin salamura suyuna katılan %1 oranında tartarik asit ve malik asit halk sağlığı için önemli bir tehdit olan *L. monocytogenes*'in kontrolünde etkili bulunmuştur. Ayrıca peynirde yapısal kusurlara neden olmaması ve laktik asit bakterilerinin üzerine düşük inhibisyon etkisinden dolayı tartarik asitin peynir üretiminde ticari olarak kullanılabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Antimikrobiyel aktivite, L. monocytogenes, Organik asit, Beyaz peynir*

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INTRODUCTION

Food safety is the suitability and protectivity of food consumption, and protected against food-borne health problems. All of the hazards affect the food safety due to biological, chemical, physical contaminants and some erroneous practices in the production line [1-3]. In terms of food safety, one of the hazardous product is cheese. There are more than 130 varieties of cheese production are made in Turkey [4]. Cheese is a ready-to-eat food substance, and during production process microorganisms can easily contaminate and growth. Food pathogens such as Lipolytic *Pseudomonas* spp., *Penicillium* spp. and *Listeria* spp. cause deterioration of quality and threat the consumer health [5-7].

Listeria monocytogenes is an important bacteria in food-borne pathogens because of the psychrotrophic properties. Listeriosis causes important problems in the food industry depend on its bad aspects, besides it gives rise to abortion and meningitis in immunosuppressed adults [8,9]. The incidence of Listeriozis cases in human has increased since 2000 according to the latest data obtained from the European Union's eight countries [10]. In the analysis of data obtained from international food-borne outbreaks between 1988 and 2007 years, 337 of the 4093 outbreaks were reported to be related with dairy products, of which 6.6% were from *L. monocytogenes* [11]. Raw milk can be contaminated with *L. monocytogenes* via the using of unclean equipment during milking, mastitis infections, transport and storage problems. If the hygienic quality of the plant is not sufficient, milk or cheese samples may be contaminated with the bacteria during production process [12].

Fungal agents are the one of the important source of cheese contamination. Molds are microorganisms that have a psychotrophic character which is easily contaminated to the cheese, and can be isolated from many cheese varieties [13]. Besides, yeasts/molds cause change in colour of the cheese surface, shortening of the shelf life and rancidity due to the lipolytic enzymes leading to big economic loses [14].

Different applications are being made to ensure food safety in cheese production. Antimicrobial applications such as pasteurization, different salt concentrations, low water activity, packaging methods, high-low pH and chemical food additives are used for controlling the food safety [15,16]. Recently, consumers have increasingly inclined to natural additives that control microbial synthesis. For this purpose, the organic acids, bacteriocin and essential fatty acids (obtained from animals, plants and microorganisms) used as natural additives such as antioxidants, antimicrobials, sweeteners and colorant have been used increasingly in food production [17]. Organic acids such as lactic acid, acetic acid and citric acid have been used as preservatives in the food sector for a long time. They have antibacterial effects due to the ability of the binding to the cell membrane of the bacteria and reduce the intracellular pH values. Some of the organic acids may also occur chelate with metal ions, and reduce cell membrane permeability by causing cell disruption due to degradation of the substrate transport mechanism of the cell [18].

The present study was undertaken to evaluate the effects of different organic acids which were, added to brine, on pH, physical and sensory properties of cheese during storage. Moreover it was aimed to evaluate the supplementation of these organic acids to brine on the count of lactic acid bacteria, yeast/mold, and also inhibition potential of *L. monocytogenes* during storage days.

MATERIAL and METHODS

Raw milk samples (120 L for stage 1 and 2, separately) were analyzed by using Bentley IBCm and Kombi FTS 600 (USA) analysers. Then, classical cheese was produced from these pasteurized milk samples under the laboratory conditions.

The study was performed in two stages.

Stage 1: Organic acids (1%) were added to the brine (12%) of the clasically produced cheeses. Cheese samples were divided into eight groups as follows: one control and tartaric acid, malic acid, ascorbic acid, fumaric acid, lactic acid, acetic acid and succinic acid groups. All of the samples were put into three kg of sterile tin cans. Sensory and physical characteristics of the cheese samples were detected during the 1st, 7th, 14th, 21st, 28th, 35th, 42th storage days (+4°C). These parameteres were evaluated blindly according to the appearance, smell, taste and intensity within the range from 1 to 5 (1: worse, 2: bad, 3: moderate, 4: good, 5: better) by 10 panelists who taken the course of the cheese technology.

Stage 2: At the end of the storage period, 12% brine samples which include 1% tartaric acid, malic acid, fumaric acid or lactic acid were found suitable for stage 2 because of the sensory-physical structures were preserved and scored above 12 points by 10 panelists. In order to reveal the antimicrobial effect of suitable brine samples with organic acid, *Lactobacillus* spp., *Lactococcus* spp., mold-yeast counts were analyzed at +4°C during the storage conditions on 1st, 7th, 14th, 21st, 28th, 35th and 42th days. Besides, the effects of organic acids were investigated in cheese samples that were inoculated with *L. monocytogenes* (ATCC13932), obtained a final cell density of approximately 10^6 CFU/mL, in the fermentation stage.

Microbial Analysis

***L. monocytogenes inoculation in cheese-milk*:** The raw milk was analysed for the presence of *L. monocytogenes* by using ISO11290-1, 2:2017 method [19]. After the confirmation,

L. monocytogenes ATCC13932 was inoculated to the milk to obtain a final concentration of 6 log10 CFU/g. The white cheese was produced by standard tecnhnique with *L. monocytogenes* inoculated to raw milk. Cheese samples were divided into the five equal parts, and 1% tartaric, fumaric, lactic, malic acid were added to the brine except control group (brine without any supplement). All of the *L. monocytogenes* inoculated cheese groups were stored at +4°C, and test samples were taken at 1st, 7th, 14th, 21st, 28th, 35th, 42th days.

Microbial analysis of cheese samples: Control and brine cheese samples which include 1% organic acid were taken 10 g portions for counting lactic acid bacteria, mold-yeast and *L. monocytogenes*. These 10 g portions were homogenized in stomacher (Seward stomacher, model 400 circulator) in 90 mL of 0.1% buffered peptone water for 2 min. Homogenated serial dilutions (10^{-1}-10^{-8}) were prepared. Dilutions were cultured on MRS Agar (Merck 1,10660) for *Lactobacillus* spp. isolation by using spreading plate technique. Plates were anaerobically incubated (Merck Anaerogen Kit) at 42°C for 48 h and light yellow colored colonies that grown on MRS agar were evaluated as *Lactobacillus* spp. colonies at the end of the incubation period [20].

Lactococcus spp. were cultured on M17 (Merck1.15108) Agar according to the spreading plate technique from the dilutions. Then, plates were (Merck Anaerogen Kit) incubated at 42°C for 24 h, anaerobically. Light yellow colored colonies that grown on M17 agar were evaluated as *Lactococcus* spp. colonies at the end of the incubation period [21]. Yeast- mold counts were cultured from the dilution of YGC agar (Merck 116000) according to spreading plate technique. After the application, plates were incubated at 25°C for 5 days and grown colonies were counted at the end of this period [22].

Listeria monocytogenes was cultured on Palcam Agar and Oxford Agar (Oxoid, CM 856) according to spreading plate technique from the dilutions and then incubated at 37°C for 48 h. At the end of this period, colonies which brown, black and green colours and centrally sunken were counted and evaluated [23].

Statistical Analysis

Analysis of data was made with IBM Statistical Package for Social Sciences (SPSS) software version 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Comparison of 5 independent groups in terms of quantitative variables was performed by Duncan test. Confidence interval was 95% and differences associated with a P value less than 0.05 were considered as statistically significant.

RESULTS

Quality parameters of raw milk (120 L) were determined as dry substance (11.19), fat (3.25%), lactose (4.37%), protein (2.71%), freezing point (-0.519°C), pH (6.8), somatic cell (123.000), total number of bacteria (5.08 log10 CFU/g) in present study. Physical, sensory and pH analysis results of the brine cheese solutions (12% (w/v) salt), which are included 1% (v/v) tartaric, malic, ascorbic, fumaric, lactic, acetic and succinic acids during the storage, shown in *Table 1*. The mean average pH values of cheese brines were also determined as 5.54, 5.25, 5.60, 5.21, 5.42, 5.38, 5.22 and 5.89 for control, tartaric, malic, ascorbic, fumaric, lactic, acetic and succinic acids groups during the storage days, respectively. The highest sensory scores were taken from the tartaric, fumaric, lactic and malic acids' brine solution. One percent of tartaric, fumaric, lactic and malic acid included brine cheese samples were chosen for the microbial analysis (stage 2) because of the highest sensory and good physical scores.

The difference between *Lactococcus* spp. count was not found significant (P>0.05) in tartaric acid added groups during the storage days. When compare the *Lactococcus* spp. counts in fumaric, lactic and malic acid included groups with the control group, average 0.5 log10 CFU/g decrease was found in mentioned groups. Fumaric and malic acids showed higher antimicrobial activities against *Lactococcus* spp. than the lactic acid (P<0.05) which shown in *Table 2*.

Table 1. *Sensory, physical and chemical analysis results (average of three different measurement results)*

Organic Acid Groups	pH Changes of the Organic Acid Groups During the Ripening Period							The Average Scores of the Sensory and Physical Properties Given by the Panelists
	1. day	7. day	14. day	21. day	28. day	35. day	42. day	
Control	6	5.11	5.72	5.59	5.49	5.48	5.43	16.9
Tartaric acid	4.24	5.28	5.64	5.52	5.42	5.38	5.27	14
Malic acid	5.74	5.23	5.83	5.72	5.65	5.59	5.49	13.7
Ascorbic acid	3.75	5.24	5.65	5.55	5.43	5.45	5.43	7.6
Fumaric acid	4.84	4.78	5.79	5.72	5.68	5.64	5.49	15.3
Lactic acid	4.71	4.94	5.73	5.63	5.62	5.57	5.51	16.5
Acetic acid	4.25	4.84	5.69	5.6	5.42	5.41	5.39	10.4
Succinic acid	6.84	5.89	5.89	5.79	5.67	5.61	5.58	9.9

Table 2. *Microorganism counts in the treatment samples during the storage*

Organic Acid Groups	*Changes of Laktococcus* spp., *Lactobacillus* spp. and Mold-yeast Counts of the Organic Acid Groups During the Ripening Period								The Average Statistical Results (D)	
	0	1	7	14	21	28	35	42		
Control	7.54±0.19^{a}	7.85±0.10^{a}	6.76±0.09^{bc}	6.74±0.07^{bc}	7.04±0.08^{b}	6.52±0.08^{c}	5.53±0.034^{d}	5.64±0.36^{d}	6.70±0.19^{a}	
Tartaric acid	7.54±0.19^{b}	8±0.15^{a}	6.77±0.06^{d}	7.20±0.33^{c}	6.59±0.14^{d}	6.48±0.18^{d}	5.85±0.11^{e}	4.71±0.14^{f}	6.64±0.09^{a}	
Fumaric acid	7.54±0.19^{a}	7.12±0.09^{b}	6.63±010^{c}	5.97±0.06^{d}	6.13±0.13^{d}	5.98±0.09^{d}	5.30±0.07^{e}	4.67±0.32^{f}	6.16±0.91^{c}	A
Lactic acid	7.54±0.19^{a}	729±0.04^{a}	7.24±0.27^{a}	6.78±0.2^{b}	6.25±0.14^{c}	6.36±0.16^{c}	4.88±0.10^{d}	4.22±0.09^{e}	6.32±0.14^{b}	
Malic acid	7.54±0.19^{a}	7.30±0.19^{b}	7.11±0.07^{b}	6.06±0.07^{c}	6.01±0.11^{c}	5.94±0.08^{c}	5.09±0.14^{d}	4.20±0.17^{e}	6.16±0.09^{c}	
Control	6.58±0.03^{a}	6.34±0.08^{b}	6.33±0.2^{b}	6.20±0.06^{b}	5.59±0.25^{c}	5.23±0.08^{d}	4.93±0.04^{e}	3.61±0.07^{f}	5.60±0.95^{a}	
Tartaric acid	6.58±0.03^{a}	6.74±0.23^{a}	6.41±0.21^{a}	5.96±0.08^{b}	5.54±0.47^{c}	5.37±0.28^{c}	4.69±0.14^{d}	2.92±0.11^{e}	5.53±0.21^{a}	
Fumaric acid	6.58±0.35^{a}	6±0.18^{b}	5.58±0.19^{c}	5.14±0.07^{d}	5.14±0.12^{d}	4.95±0.09^{d}	4.66±0.13^{e}	3.08±0.14^{f}	5.14±0.99^{b}	B
Lactic acid	6.58±0.03^{a}	6.20±0.06^{b}	6.23±0.2^{b}	5.34±0.12^{c}	5.17±0.14^{cd}	5.02±0.08^{de}	4.75±0.09^{e}	2.77±0.35^{f}	5.26±0.15^{b}	
Malic acid	6.58±0.03^{a}	6.24±0.11^{b}	5.98±0.04^{b}	5.37±0.15^{c}	5.33±0.34^{cd}	5.07±0.13^{d}	4.53±0.11^{e}	3.99±0.00^{f}	5.26±0.08^{c}	
Control	5.08±0.42^{a}	4.81±0.13^{ab}	4.34±0.35^{bc}	4.79±0.32^{ab}	4.74±0.05^{ab}	4.24±017^{c}	4.35±0.07^{bc}	4.45±0.16^{bc}	4.60±0.35^{a}	
Tartaric acid	5.08±0.42^{a}	4.60±0.32^{ab}	4.55±0.07^{ab}	4.36±0.46^{ab}	4.35±0.06^{ab}	3.23±0.18^{c}	3.89±0.57^{bc}	4.17±0.16^{ab}	4.28±0.59^{c}	
Fumaric acid	5.08±0.42^{a}	5.05±0.06^{ab}	4.53±0.83^{ab}	5.01±0.14^{ab}	4.55±0.12^{ab}	4.39±0.12^{b}	4.38±0.11^{b}	4.39±0.15^{b}	4.67±0.42^{a}	C
Lactic acid	5.08±0.42^{a}	5.08±0.09^{a}	4.54±0.15^{b}	4.45±0.41^{b}	4.45±0.20^{b}	4.15±0.12^{b}	4.24±0.06^{b}	4.36±0.08^{b}	4.54±0.39^{ab}	
Malic acid	5.08±0.42^{a}	5.07±0.13^{a}	4.40±0.06^{bc}	4.77±0.26^{ab}	4.30±0.69^{bc}	3.64±0.30^{d}	3.91±0.06^{cd}	4.07±0.14^{cd}	4.40±0.57^{bc}	

$^{a.\ b.\ c.\ d.\ e.\ f}$ P<0.05 is important in the same line; A: Laktococcus spp. B: Lactobacillus spp. C: Mold-yeast count D: Statistical data represent the significant importances according to the organic acid treatments

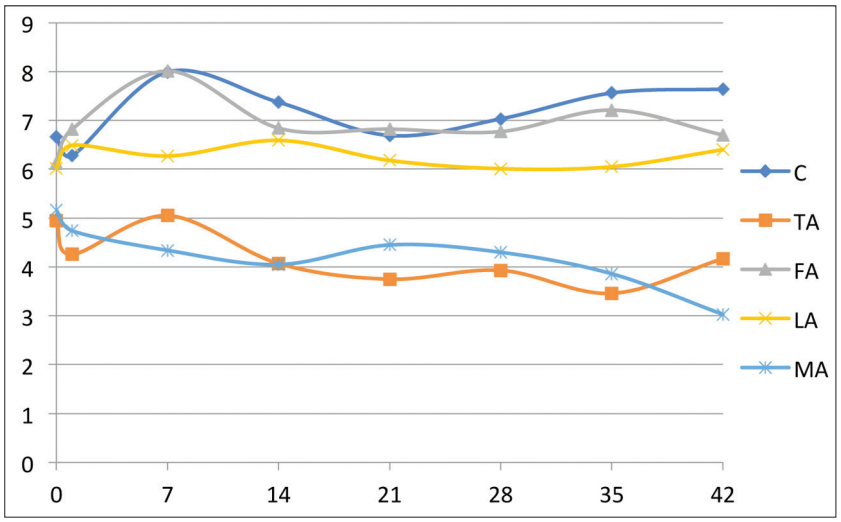

Fig 1. Antimicrobial effects of organic acids on *L. monocytogenes* during storage (C: Control. TA: Tartaric acid. FA: Fumaric acid. LA: Lactic acid. MA: Malic acid)

Lactococcus spp. counts decreased from 7.29 log10 CFU/g to 4.22 log10 CFU/g in lactic acid, from 7.12 log10 CFU/g to 4.67 log10 CFU/g in fumaric acid and from 7.30 log10 CFU/g to 4.20 log10 CFU/g in malic acid added samples at the end of the storage day (42th day), respectively.

The effects of organic acids on *Lactobacillus* spp. count were determined and there was not found significant difference (P>0.05) between the tartaric acid and control groups which shown in *Table 2*. Besides, averagely 0.4 log10 CFU/g decreasing were found in *Lactobacillus* spp. counts in fumaric acid, lactic acid and malic acid including groups. *Lactobacillus* spp. counts decreased from 6.20 log10 CFU/g to 2.77 log10 CFU/g in lactic acid, from 6.24 log10 CFU/g to 3.99 log10 CFU/g in malic acid and from 6.00 log10 CFU/g to 3.08 in fumaric acid included groups at the end of the storage (42th day).

The influence of organic acids on mold-yeast count were determined, and there also was not detected any significant difference between the fumaric acid and control groups (P>0.05). Averagely 0.5 log10 CFU/g decreasing were found in mold-yeast counts in tartaric acid, lactic acid and malic acid included groups which shown in *Table 2*. Mold-yeast counts decreased from 4.60 log10 CFU/g to 4.17 log10 CFU/g in tartaric acid, from 5.07 log10 CFU/g to

4.07 log10 CFU/g in malic acid and from 5.08 log10 CFU/g to 4.36 log10 CFU/g in lactic acid included group at the end of the storage (42nd day).

Listeria monocytogenes counts were found significant between the control and the organic acid brine samples (fumaric, tartaric, lactic and malic acid) during the storage days ($P<0.05$) which presented in *Fig 1*. When compared the *L. monocytogenes* counts between control and organic acid added brine sample groups during the storage days, decreased *L. monocytogenes* counts was detected as 0.22 log10 CFU/g, 0.90 log10 CFU/g, 2.91 log10 CFU/g, 2.95 log10 CFU/g in fumaric acid, lactic acid, malic acid and tartaric acid included brine samples, respectively.

DISCUSSION

Organic acids have been used as preservatives in the food sector for a long time because of the antibacterial properties. These organic acides, diffuse across the bacterial cell membranes, dissociate in the cell cytoplasm, reduce the intracellular pH and lead to cessation of growth or cell death [24]. Some organic acids can be produced by using lactic acid bacteria in fermented foods. Among those acetic acid has synergistic effect with lactic acid on the prevention of fungal growth [25]. Although acetic acid is described as more potent because of its higher pKa value, and level of dissociation inside bacteria cell, acetic, ascorbic and succinic acids disrupted the physical structure of the cheese samples in present study. Besides, sensory characteristics of the brine cheese samples which included acetic, ascorbic and succinic acid were evaluated by 10 panalists, and they did not get enough points for the second stage of the study. Furthermore, the lowest score was detected as 7.6 in ascorbic acid group by panalists. Interestingly, brine samples which contained succinic acid had higher pH levels (pH: 6.84) when compared to control group (pH: 6). There was not found any literature about the addition of organic acids into the brine cheese samples. The decomposition of the physical and sensory structure of the brine samples could be due to quicker decrease in the pH levels of the brine (3.75) in the first experiment day.

The count and the ratio of lactic acid bacteria is an important factor in the fermented food because it forms quality of fermentation, and effets the flavor and aroma of cheese samples. At the beginning of the fermentation, cheese samples, which included organic acids, the *Lactococcus* spp. and *Lactobacillus* spp. counts were determined as 7.54 log10 CFU/g and 6.58 log10 CFU/g, respectively. While the *Lactococcus* spp. counts were similar between the control and the organic acid brine sample groups, an important decrease was observed in the *Lactobacillus* spp. counts during the storage days. It was not found in the literature about counts or ratio of bacteria population in brine cheese samples processed with organic acids. It was suggested that antimicrobial effects of organic acids may be more efficient on *Lactobacillus* spp. than *Lactococcus* spp. because of having different membrane permeability or resistance [26].

Another important factor of cheese production and preservation is mold-yeast count. Shokri et al.[27] evaluated antifungal activity of organic acid treatments on *Trichophyton mentagrophytes* var. mentagrophytes, *Candida albicans*, *Aspergillus fumigatus* and *Malassezia furfur*. The results of the study showed that citric acid has more fungistatic and fungicidal activities than tartaric acid. Besides, the antifungal activity of the acid mixture was similar with citric acid but higher than tartaric acid alone. In contrary, tartaric acid had higher antifungal activity than the other groups ($P<0.05$) in our study. The main target of organic acids and its relatives are cell wall and membrane proteins. The hyphae wall of filamentous fungi contains less protein than the cell wall of yeast. For this purpose, the researcher explained this may be related to the different structures of the fungal cell walls [27].

Recently, there have been performed some scientific researches about the disinfectant or inhibition potentials of organic acids in various areas. One of those is the use of organic acids against food pathogens. Yıldırım et al.[28] showed that lactic acid bacteria, probiotic bacteria and their metabolites can inhibit *L. monocytogenes* in Turkish white cheese at maturation period. In bacterial fermentation due to the production of organic acids, such as lactic, acetic or propionic acids which play a role in the biofermentation of fermented food. Organic acids also include a large spectrum of compounds and many of them are known to be effective antifungal metabolites [29]. In another study, Swaranandam et al.[30] also researched the effectiveness of some organic acids (such as malic acid) in nisin-incorporated soy protein film against *L. monocytogenes*, *E. coli* O157:H7, *Salmonella gaminara*. Incorporated soy protein film was found the fewest survivors of *L. monocytogenes*, *S. gaminara*, and *E. coli* O157:H7 (5.5, 3.0 and 6.8 log10 CFU/mL, respectively). Pintado et al.[31] also defined the effects of nisin, natamycin and malic acids which were incorporated with whey protein films, on inhibitory activity against *L. monocytogenes*, *Penicillium commune* and *P. chrysogenum*. Another study was performed about using of organic acids for decreasing of *L. monocytogenes* counts in biofilms. It was determined that counts of *L. monocytogenes* decreased due to lactic acid washing solution on PVC and stainless steel surfaces. In addition, *S.*Typhimurium, *L. monocytogenes* and *E. coli* O157:H7 were also reduced to the below detection limit (1.48 log10) by using lactic acid (2%) and steam applications [32]. Brown et al.[33] (2018) also investigated the inhibitory effect of acetic acid, citric acid and lactic acid supplementation to the brine of cheese samples aganist *L. monocytogenes* (6 log CFU/m) during the storage period similar with the present study. This result revealed rapid inhibition against *L. monocytogenes* in acetic acid brine samples, and it required non-practical volumes. In

current study, inhibition time was found too long in citric acid added samples compared to lactic acid against *L. monocytogenes* in normal volumes. However, there are very limited numbers of study about the using of organic acids in a dairy product, *L. monocytogenes*. Counts in brine cheese sample groups were affected from organic acids, significantly ($P<0.05$) in the present study *(Fig. 1)*. Initial *L. monocytogenes* counts averagely decreased from 6.67 log10 CFU/g to 0.22 log10 CFU/g, 0.90 log10 CFU/g, 2.91 log10 CFU/g and 2.95 log10 CFU/g in fumaric, lactic, malic and tartaric acid included brine cheese compared with control group, respectively. Besides, tartaric which is the one of the important acid to suppress *L. monocytogenes* counts, and to decrease the *L. monocytogenes* counts from 6.67 log10 CFU/g to 4.95 log10 CFU/g at the 0th day and to 4.17 log10 CFU/g on the 42nd day. Our study also demonstrated the suppressing effects of organic acids on *L. monocytogenes* counts in brine cheese samples, and current results are in aggrement with previous studies. Although, succinic, acetic and formic acids caused structural defects in the brine cheese samples, tartaric, malic, lactic and fumaric acid did not cause physical and sensory deficits. It is also thought that the addition of malic and tartaric acid in brine cheese samples could be used effectively in the control of *L. monocytogenes*.

In conclusion, this research demonstrated useful effects of some organic acids against moulds, yeasts and *L. monocytogenes* contaminations. Morever, supplementation of organic acids in brine solutions has a novel method to enhance the microbial safety and quality of white cheese.

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Research Article

The Effects of Severe Hypoxia on Nitric Oxide Parameters in Hypoxia-tolerant Rodent: *Nannospalax nehringi*

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Abstract

Blind mole rats (BMRs) are solitary rodents which are tolerant to severe hypoxia. The aim of this study is to reveal the changes in nitric oxide (NO) and nitric oxide enzymes (NOS), which are involved in many physiological and pathological processes related with hypoxia, in BMRs under severe hypoxia. For this purpose, 12 subadult (11-15 moths) male *Nannospalax nehringi* were captured in Kars location. Captured BMRs were divided into two groups as Normoxic (NG) and Hypoxic (HG) randomly (n=6). NG were kept in completely dark, normoxic conditions for 52 h. HG were kept inside completely dark glovebox chamber with 7% oxygen flow for 52 h. After experimental protocol, NG were sacrificed under normoxic conditions and HG were sacrificed inside glovebox chamber with 7% oxygen. NO, iNOS, eNOS, nNOS and MDA levels of plasm and homogenized tissue samples were detected spectrophotometrically. All parameters of each sample were found to be high in HG compared to NG. But especially, NO was high in the lung tissues of HG. Additionally eNOS level of the kidney, liver and lung, iNOS levels of the liver and eNOS, and nNOS levels of the brain were found to be markedly high. Consequently, our data on NO and NOS enzyme production in *Nannospalax nehringi* tissues under hypoxia are compatible with the data obtained from other animals, but it contains differences in some points. We believe that these differences are different evolutionary adaptations of BMRs to hypoxia..

Keywords: *Hypoxia, NO, iNOS, eNOS, nNOS, MDA, Nannospalax nehringi*

Hipoksi-toleranslı Rodentte Şiddetli Hipoksinin Nitrik Oksit Parametreleri Üzerine Etkileri: *Nannospalax nehringi*

Öz

Kör fareler (BMR) ölümcül hipoksiye oldukça toleranslı soliter rodentlerdir. Bu çalışmanın amacı, hipoksi ile ilişkili birçok fizyolojik ve patolojik süreçte rol alan nitrik oksit (NO) ve nitrik oksit enzimlerinin (NOS), şiddetli hipoksi altındaki BMR'lerdeki değişimlerini ortaya çıkartmaktır. Buna yönelik olarak Kars arazisinden 12 subadult (11-15 ay) erkek *Nannospalax nehringi* yakalandı. Yakalanan BMR'ler Normoksi (NG) ve Hipoksi grubu (HG) olmak üzere rastgele iki gruba ayrıldı (n=6). NG, 52 saat boyunca tamamen karanlık, normoksik koşullarda tutuldu. HG, 52 saat boyunca tamamen karanlık, içerisine %7'lik oksijen akıtılan glovebox kabin içerisinde tutuldu. Deney sonunda NG, normoksi altında ve HG %7 oksijen içeren glovebox kabin içerisinde öldürüldü. Plazma ve homojenize edilen doku örneklerinden NO, iNOS, eNOS, nNOS ve MDA seviyeleri spektrofotometrik olarak belirlendi. Tüm örneklere ait her parametrenin, NG'ye kıyasla HG'de yüksek olduğu belirlendi. Fakat özellikle, NO'nun, HG akciğer dokularında oldukça yüksek olduğu tespit edildi. Ayrıca böbrek, karaciğer ve akciğer eNOS seviyesinin, karaciğer iNOS seviyesinin ve de beyin eNOS ve nNOS seviyelerinin oldukça yüksek olduğu görüldü. Sonuç olarak, hipoksi altındaki *Nannospalax nehringi*'de NO ve NOS enzim üretimi hakkında elde ettiğimiz veriler, diğer hayvanlardan elde edilen verilerle uyumlu olsa da bazı noktalarda farklılıklar içermektedir. Bu farklılıkların, BMR'lerin hipoksiye karşı sahip olduğu farklı evrimsel adaptasyonlar olduğunu düşünmekteyiz.

Anahtar sözcükler: *Hipoksi, NO, iNOS, eNOS, nNOS, MDA, Nannospalax nehringi*

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INTRODUCTION

Hypoxia is deficiency of normal oxygen tension in tissues and it is a deadly condition for the most animal species. Prolonged exposure to hypoxia results with necrosis, apoptosis and autophagy in cellular level, and so ischemic damages [1]. In cellular level hypoxia also displays various effects on mitochondria, because of mitochondria are major consumers of oxygen [2]. Electron Transport Chain (ETC) components can produce reactive oxygen species (ROS) during ATP production and release them into different compartment of the mitochondria [3]. But under hypoxic situations ETC components is reorganized for adaptation to hypoxic environment and inducing of reductive carboxylation increases ROS production [2]. In multicellular animals, ROS-mediated cellular dysfunctions can be fatal. Therefore, for avoiding from the overproduction of ROS, organisms evolved some protective mechanisms. Upregulation of HIF-1 (Hypoxia Inducible Factor 1) and Nrf2 (Nuclear Factor (Erythroid-derived 2)-Like 2) are examples of cellular hypoxia-protective mechanisms [4,5] and enhanced hematocrit levels or inducing angiogenesis are examples of systemic mechanisms [6,7]. These adaptive mechanisms regulate oxygen economy and so, protect organism against deadly effects of the hypoxia by decreasing ROS production. However, some local mediators such as Nitric Oxide (NO) also play a role in protection from the negative effects of hypoxia.

Nitric Oxide shows local effects and it has an important role in preserving of homeostatic functions under hypoxia, because rapidly inactivated after diffusing into the bloodstream [8]. Releasing of NO as response to low oxygen tension is a compensator mechanism that mediate to enhancing of blood volume in tissues by relaxing of smooth muscles around the vessels. Nevertheless, effects of NO on pulmonary system is different. Pulmonary vascular resistance increases while systemic vascular resistance decreases under hypoxia via NO-mediated and each of these responses is known as protective mechanisms [9]. NO also takes part in different pathological and physiological process like apoptotic balance, proliferation and regulation of the adhesion molecules [10].

Nitric Oxide can be produced enzymatically via Nitric Oxide Synthase (NOS) isoforms or other metabolic process such as cytocrom-c-mediated production [11]. Most known NOS isoforms are Endothelial Nitric Oxide Synthase (eNOS), Inducible Nitric Oxide Synthase (iNOS) and Neuronal Nitric Oxide Synthase (nNOS). Evolutionally, NOS enzyme isoforms found in mammals are thought to have formed after a second duplication following the early tetrapod evolution [12]. Although all isoforms are expressed in various tissues, nNOS is commonly found in neural tissues, eNOS found in vascular endothelial tissues and the third isoform, iNOS, found in a larger tissue spectrum [12,13].

The clades of evolution tree have different looking organisms that adapted to countless lifestyles. In hypoxic habitats, it is possible to find species, which have evolutionary advantages against the deadly effects of hypoxia. There are many hypoxia-tolerant species in Pisces, Amphibia, Reptilia and Aves and also in Mammalia [14,15]. *Nannospalax nehringi* also known as blind mole rats (BMRs) are one of the hypoxia-tolerant animals which belong to Spalacidae family in rodentia [16,17]. BMRs live in highly hypoxic underground tunnels and they differ from other rodents in terms of managing of oxygen economy. They have evolved many physiological, morphological and anti-cancer strategies in millions year [18-20]. Theirs total lung capacity, alveolar surface area and capillary volume is higher than white rats, therefore, theirs pulmonary diffusion capacities are higher by 43% [21]. BMRs have also high mitochondria, myoglobin, neuroglobin and cytoglobin density [22]. Because of these unique features, BMRs are one of the non-model organisms that are frequently studied.

In the light of this information, BMRs are unique animals for understanding the effects of hypoxia. For this purpose, for the first time, we reported levels of NO, eNOS, iNOS and nNOS under normoxia and severe hypoxia levels, and MDA (Malondialdehyde) levels as a stress indicator in *Nannospalax nehringi*.

MATERIAL and METHODS

Experimental Design

This study was carried out twelve subadult (11-15 moths) male *Nannospalax nehringi* (2n=50) which collected from Kars, Turkey. Age determination of BMRs were performed with molar tooth crests methods [23]. The research ethics committee approval for the capture of the BMRs was provided with proper injunction of Kafkas University Local Ethics Committee for Animal Experiments (KAÜ-HADYEK 2018/070). All animals were housed at the Kafkas University, Veterinary Medicine Faculty, Physiology Research Laboratory in individual cages under constant darkness conditions (24D:0L). Animals were fed with fresh vegetable and fruit as *ad libitum*. For the laboratory orientation, animals were housed at the same conditions for three months. After orientation, animals were divided two groups as Normoxic and Hypoxic Group, randomly. Animals in the Normoxic Group (n=6) were housed at normoxic conditions and the constant darkness (24D:0L) for 52 h. Animals in the Hypoxic Group (n=6) were housed in hypoxic glovebox maintained with 7% O_2 + 93% N_2 mixture gas (20 liter/min) for 52 h. Animals in the Normoxic Group were sacrificed with cervical dislocation method under sevoflurane anesthetize after the experiment protocol. Animals in the Hypoxic Group were killed with cervical dislocation method under sevoflurane anesthetize in hypoxic glovebox after the experiment protocol. Tissue samples were homogenized in phosphate buffer (pH: 7.4) and stored at -80°C immediately. Plasma samples were obtained by centrifuged blood (4000 rpm at +4°C) and stored at -80°C.

Biochemical Analysis

Nitric Oxide levels were determined according to the method described by Miranda et al.[24] in that nitrate is reduced to nitrite by $VaCl_3$, and then in acidic environment nitrite was reacted with sulphanilamide to produce colored diazonium compound, which was read at 540 nm. eNOS and iNOS activities are determined by commercial ELISA kit (LSBio, USA). nNOS activity is also determined with commercial ELISA kit (Biocompare USA).

Malondialdehyde as an end product of lipid peroxidation concentrations were measured by the method of Yoshioka et al.[25] based on the reaction between thiobarbituric acid and MDA. The end products were read at 535 nm.

Protein content of each sample were measured using bovine serum albumin as standard according to the method of Lowry et al.[26]. Results were calculated as µmol/L g protein in NO and MDA, IU/L g protein in iNOS, eNOS and nNOS.

Statistical Analysis

SPSS 20.0.0 software was used for statistical evaluation of data, which were expressed as median ± standard deviation. Importance level of difference among the groups was determined by variance analysis test (ANOVA) and Tukey multiple comparison test. P<0.05 was considered as significant.

RESULTS

The NO levels of the brain, kidney, liver, lung and plasma are shown in *Fig. 1*. It was founded that, NO levels of all tissues in Hypoxic Group was increased when compared with Normoxic Group (P<0.001). Also, in the brain, kidney and lung tissues, this increase was found to be two times more than the Normoxic Group.

The results of the brain, kidney, liver, lung and plasma eNOS and iNOS levels, and nNOS levels of brain tissues are summarized in *Fig. 2, 3* and *4* respectively. As a result of statistical analyzes, significant differences was found between all tissues of animals in Hypoxic and Normoxic Group (P<0.001). Liver, lung, and plasma eNOS levels were found to increase approximately four-fold under hypoxia, while brain and liver levels increased ten-fold. Additionally, a three-fold increase was observed in liver iNOS levels, while the levels of iNOS in all tissues increased significantly under hypoxia. Also it was determined that, hypoxia causes approximately four-fold increase in nNOS levels of brain tissues.

The MDA levels of the brain, kidney, liver, lung and plasma are shown in *Fig. 5*. Statistically significant increase was determined in MDA levels of all tissues under hypoxia when compared with Normoxic Group (P<0.001).

DISCUSSION

As a result of the mutual harmony among the organisms, our world has an average oxygen concentration between 20-21% at sea level [27]. Therefore, the oxygen pressure that many organisms can tolerate is in quite limited

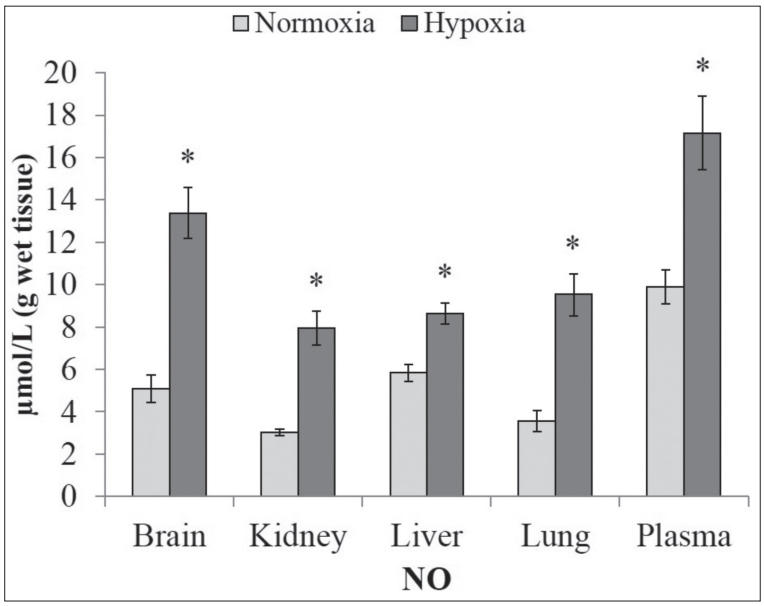

Fig 1. NO levels under normoxic and 7% hypoxic conditions in *Nannospalax nehringi* (* P<0.001)

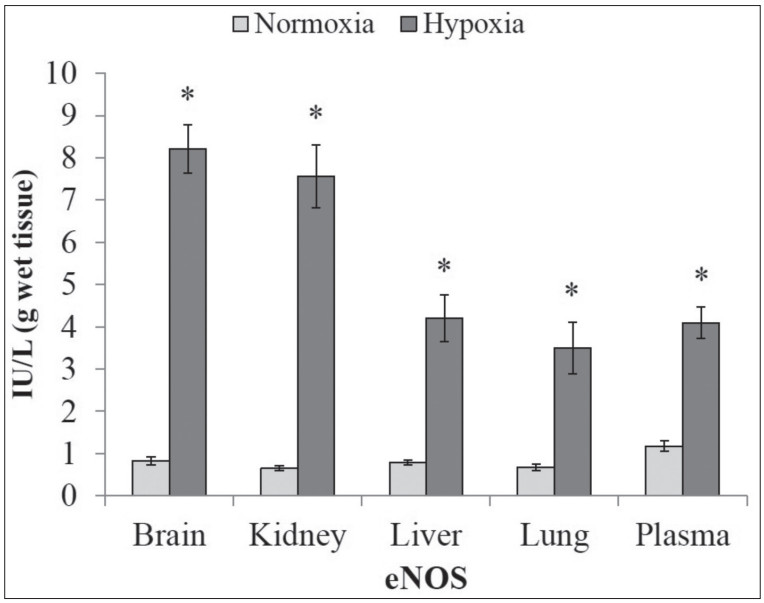

Fig 2. eNOS levels under normoxic and 7% hypoxic conditions in *Nannospalax nehringi* (* P<0.001)

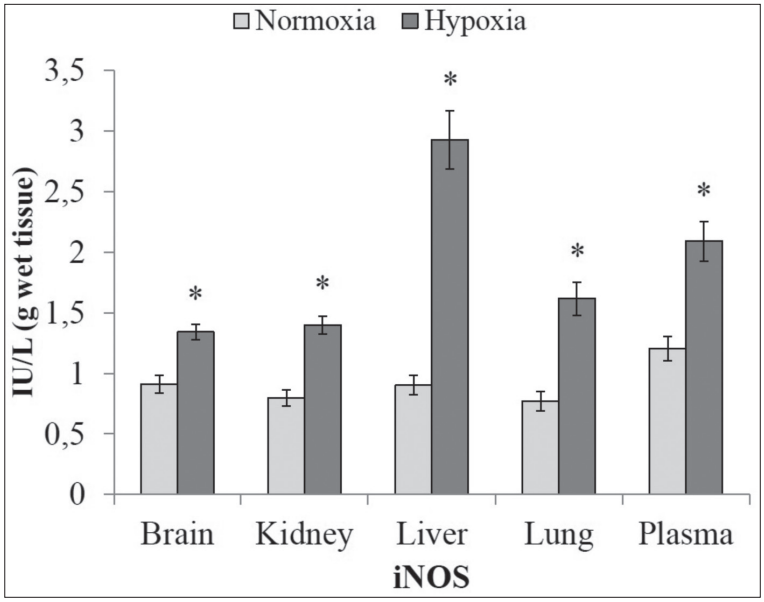

Fig 3. iNOS levels under normoxic and 7% hypoxic conditions in *Nannospalax nehringi* (* P<0.001)

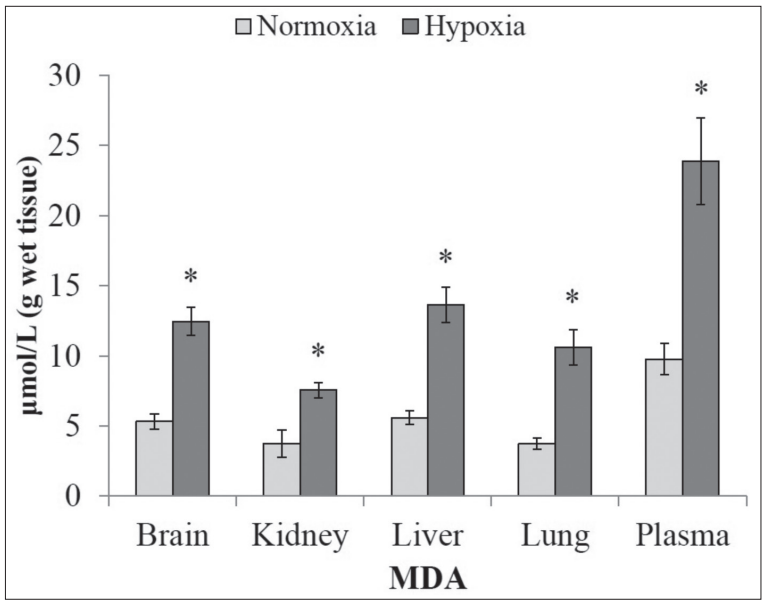

Fig 5. MDA levels under normoxic and 7% hypoxic conditions in *Nannospalax nehringi* (* P<0.001)

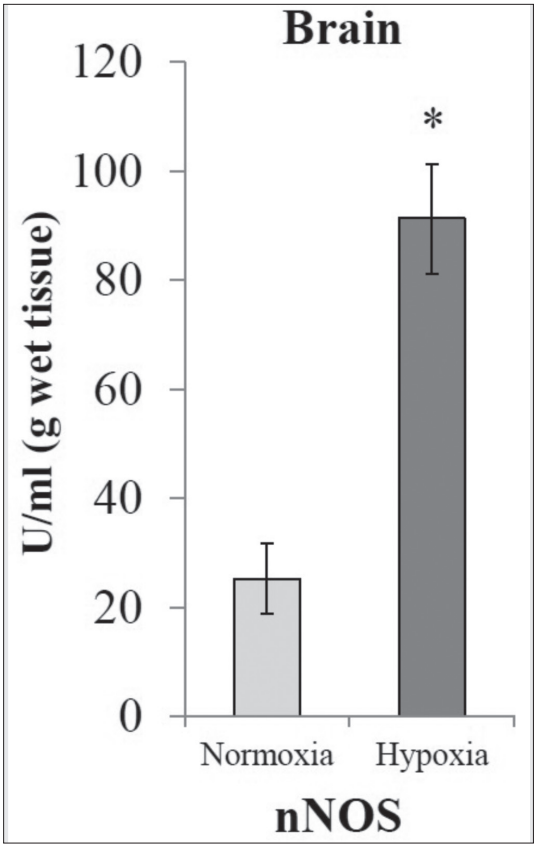

Fig 4. Brain nNOS levels under normoxic and 7% hypoxic conditions in *Nannospalax nehringi* (* P<0.001)

range. Organisms try to maintain their homeostasis by processing the molecules that they can get energy, with various chemical reactions. Many organisms need to oxygen for these processes and they can not survive without oxygen. Habitats of BMRs are highly hypoxic and their characteristics are change with soil structure and seasonally [20]. In present study, no losses of reflexes or behavior changes were observed in the animals during the 52 h hypoxia administration. But hypoxia is a mortal condition for the most organisms. Long-term hypoxia causes various types of cell death [28,29] or, at best, cellular damages by producing to ROS [30]. The most affected biomolecules from the free radicals are lipid-structured molecules. Therefore, evaluating of MDA which the end produce of lipid peroxidation is one of the methods that using for following the ROS producing [31,32]. We observed that hypoxia causes an increase in the MDA levels of all BMRs tissues.

Nitric Oxide have an important role in adapting to hypoxia because they cause smooth muscles relaxing, thereby they mediate to enhancing of blood volume in the tissue via vasodilatation [33,34]. It is seen that there are different NO levels among different human populations, depending on their geographical characteristics. Erzurum et al.[35] showed that, Tibetan highlanders who live at the high altitudes and durable to the hypobaric hypoxia, that have higher NO levels than other people who live at the sea levels. Interestingly, NO also plays a central role in protective effect of intermittent-hypoxia against different conditions including cardiovascular diseases and ischemia [36]. Thus, it can be say that, NO may one of the key for adapting to hypoxia in the evolutionary process. In our study, we observed that, NO levels in brain, kidney, liver and plasma samples of BMRs increase up to 2.6, 1.47 and 1.7 times respectively. However, it is known that NO has different effects on pulmonary system [37].

Using of NOS antagonists increases pulmonary and systemic vascular resistance under normoxic conditions, and these data refer to protective role of NO against the hypoxia-mediated damages [9]. This phenomenon also have tested in different animal species. It was observed that hypoxic ventilation has no any effect on the NO levels of buffer-perfused rabbit's lung [38]. On the contrary, a decrease in both NO levels of isolated pig lungs [39] and, aortic and pulmonary arterial nitrite levels in cardiopulmonary bypassed pigs [40] exposed to hypoxia have been shown. In the present study, we detected approximately 2.6 fold increase in NO products levels of BMRs lung tissues. We think that this may be another adaptation that BMRs have. However, cause of these different results is may be originate from differentiation of experimental methods.

Nitric Oxide Synthases inhibition results with low blood flow and critical oxygen pressure levels in cortical and medullary area of kidney [41]. Also, eNOS specifically plays an important role in the controlling of vascular tension which effects glomerular filtration rate in the kidney [42]. Similarly, our data show that, kidney of BMRs have high NOSs expression under the hypoxia. Especially eNOS have 11.6 fold increase in kidney. It indicate that, eNOS is an important component for running of physiological activities under hypoxia in the kidney of BMRs. Lung is another critical organ for the animals. Balasubramaniam et al.[43] showed that hypoxia impairs alveolarization in the eNOS deficient mouse, and also decreased expression of the eNOS is related with impaired integrity of injured lung [44]. Also, it was showed that, levels of eNOS protein increase in lung of rats but not iNOS after hypoxic exposure [45]. Nevertheless, nNOS is not considered to have an important role in the lungs [45]. However, we observed a significantly increase both eNOS and iNOS levels in lung tissues of BMRs. Previous studies have demonstrated that pulmonary diffusion capacities of BMRs are higher than rats [21]. High eNOS and iNOS levels under hypoxia may be another component of these adaptations. Also, it is known that, iNOS production increases in ischemic human liver [46]. Similarly, we found hypoxia causes significant increase of iNOS and eNOS production in the liver tissue of BMRs. We believe that this is a compensative process which are protect the vital organ liver.

Nitric Oxide production is also closely associated with HIF-1α and HIF-1 activity can be prevent or activate by the NO sources because of their compounding pharmacological actions [47]. HIF-1α is stabilized under hypoxia and controls the iNOS and eNOS transcription, but in brain, nNOS activity is necessary for the HIF-1α stability [33]. Brain is the major organ that sensitive to O_2 pressure. Therefore, effects of NOS enzymes on the brain are more complicate and nNOS plays a critical role in hypoxia protection in the central nervous system. It is thought that nNOS is a negative regulator for inhibiting of NO overexpression, because of nNOS is evaluated as NOS derivative which associated with presence of O_2 [48]. Hypoxia-ischemia models in mice brain causes markedly increase in both protein and mRNA levels of nNOS [49]. van den Tweel et al.[50] observed that hypoxia-ischemia induced brain injury in neonatal rats and cause an increase in nNOS while eNOS decreases after hypoxia-ischemia but iNOS is not effect. Additionally, inhibition of nNOS and iNOS decreases adverse effects of hypoxia-mediated brain ischemia [51]. Therefore some researchers argue that nNOS inhibition afford neuroprotection in ischemia models [52,53]. In our study, we have found that hypoxia increases eNOS expression 9.8 fold, iNOS expression 1.47 fold and nNOS expression 3.6 fold in the brain tissues. Unlike the most animals, BMRs live in hypoxic underground tunnels and severe hypoxia is a part of BMRs life. For this reason, we believe that high increase of the brain nNOS and iNOS levels may be another protective element that *Nannospalax nehringi* have against the hypoxia.

Consequently, our data indicate that, although production of NO and NOS enzymes in tissues of *Nannospalax nehringi* are compatible with data obtained from other animals, it contains differences in some points. We think that these differences may be another characteristic features that BMRs have. Therefore, further research should aim to test our data with different animal research methods for reveal the properties of NO in BMRs.

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Research Article

Molecular Identification of *Listeria monocytogenes* and *Escherichia coli* O157: H7 Isolated from Fresh Kashar Cheese and Milk Creme [1]

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Abstract

In this study, it is aimed to analyze the risk factors related to *Listeria monocytogenes* and *Esherichia coli* O157:H7 monitoring in fresh kashar cheese and milk creme in the province of Istanbul. It has 100 pieces of fresh kashar cheese and 100 pieces of milk creme material. Samples were confirmed by PCR methods and genetic basis with conventional microbiological sowing methods and analysis methods. Another case for the study is to obtain significant epidemiological environment for the field strains in the country in terms of *L. monocytogenes* and *E. coli* OH157: H7, where high polymorphic protein structures and rapid genetic variation exist. In the study, 4 (2%) of *E. coli* OH157: H7 and 6 (3%) of *L. monocytogenes* were found among 200 milk slices and fresh kashar cheese samples. Those microorganisms can easily reproduce in natural environment such as soil and silage and with primer/secondary contamination, they appear to be health threatening food related pathogens. It is suggested to have studies for optimal food safety and this can be provided in accordance with appropriate legislation.

Keywords: *PCR, Food safety, L. monocytogenes, E. coli*

Taze Kaşar Peyniri ve Süt Kaymağından İzole Edilen *Listeria monocytogenes* ve *Esherichia coli* O157:H7'nin Moleküler Tanımlaması

Öz

Bu çalışma ile İstanbul ilinde satışa sunulan, taze kaşar peyniri ve süt kaymağı örneklerinde *Listeria monocytogenes* ve *Esherichia coli* O157:H7 varlığı ile halk sağlığı açısından oluşturabileceği risklerin değerlendirilmesi amaçlanmıştır. Araştırma kapsamında rastgele örneklem yöntemi ile toplanmış olan 100 adet taze kaşar peyniri ve 100 adet süt kaymağı materyal olarak kullanılmıştır. Örnekler, konvansiyonel mikrobiyolojik ekim teknikleri kullanılmasının yanısıra PCR yöntemi ile moleküler ve genetik bazda doğrulanmıştır. Çalışmanın farklı bir amacı da, yüksek polimorfik protein yapılarına sahip ve hızlı genetik varyasyon yeteneği bulunan *L. monocytogenes* ve *E. coli* OH157:H7 açısından ülkemizdeki saha suşları hakkında önemli epidemiyolojik verilerin elde edilmesidir. Çalışma sonucunda, analiz edilen toplamda 200 adet olan süt kaymağı ve taze kaşar peyniri örneklerinden 4 adedi (%2) *E. coli* OH157:H7 ve 6 adedi (%3) ise *L. monocytogenes* açısından pozitif olarak tespit edilmiştir. Söz konusu mikroorganizmalar, toprak ve silaj gibi doğal çevre ortamlarında rahatça üreyebilmesiyle ve primer/sekonder kontaminasyonlar nedeniyle halk sağlığını ciddi şekilde tehdit eden gıda kaynaklı patojenler arasında yer almaktadır. Optimal gıda güvenliğinin sağlanması ve gıdanın mevzuata uygun şekilde tüketiciye ulaşması yönünde çalışmalar yapılması önerilmektedir.

Anahtar sözcükler: *PCR, Gıda güvenliği, L. monocytogenes, E. coli*

INTRODUCTION

Foodborne pathogens are considered to be a significant risk factor for public health in developed and developing countries [1] due to their ability to spread throughout the world For almost all of the foodborne infections, the surface of the staff and the surfaces in the facilities, the tools and equipment used in the food production and processing and the sources of contamination from the end consumers, the points where the food prepared in

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bulk and/or the food prepared in the institutions such as hotels, schools, workplaces and hospitals are consumed is defined as a serious risk factor [2]. Inadequate hygiene conditions, and/or microbiologically charged hands can be the weakest chain of food safety chains and seriously threaten public health [3].

Cheese is the mostly consumed variety of food in the world. At the same time cheese is a milk product, which is easily degradable by reducing the rate of humidity, moisture, converting it to a product that can be kept undisturbed for a long time (up to 10 years from 4-5 days depending on the type of cheese and storage conditions) [4]. Chemical and microbiological quality of raw poultry to be used in cheese making is of great importance for consumer health. Otherwise, some of the saprophytes and pathogens in the raw milk can pass to cheese, which can threaten consumer health. In addition, it should be kept in mind that, in the case of toxigenic pathogens such as *Listeria monocytogenes* and *Escherichia coli* in the raw milk, the toxins produced by the agents are not denatured at such temperatures and the important risk factors for consumer health continue even when the pasteurisation procedure is applied [5]. In addition, kashar cheese amongst the cheese varieties constitute the primary risk group for consumers due to short-term maturation procedures [6].

L. monocytogenes is a foodborne pathogen causing gastro-enteritis, septicemia, central nervous system infections, maternal-fetal infections and abortions in humans. Listeriosis caused by this microorganism has the highest mortality rate (reaching 40%) even though it has a lower incidence than other foodborne pathogens, leading to the fact that microorganism is among the foodborne pathogens most seriously threatening public health in medical literature [1]. *E. coli* is an important display of fecal contamination and hygienic applications in milk, dairy products. *E. coli O157:H7* pathogenity is related to Shiga toxins, and intimin. It is known that Shiga toxins are associated with symptoms such as Hemorrhagic Colitis (HC), Hemolytic Uremic Syndrome (HUS) and bloody diarrhea [7,8].

The fact that the agent can be easily grown in natural environments such as soil and silage and isolating it from raw and processed foods, such as milk and its products, meat and its products, vegetables and seafood, seriously raises the risk to consumer's health [9]. Six large listeriosis outbreaks have been reported in the United States and Canada between 1979-1999. According to the results of the investigations, it was determined that the hospitalized cases had been contaminated from green leaf salad, carrot, potato, pasteurized milk, pork products, raw milk, hot dog sandwich, chocolate and various cheeses [10].

The aim of this study is to investigate the presence of *L. monocytogenes* and *E. coli O157: H7* in fresh kashar cheese and milk creme, which have extremely high potential risks for consumer health in milk and products. In addition, in the study, it is aimed to type isolates obtained from samples by molecular methods. Another aim of the study is to demonstrate the advantages/disadvantages of methods used, which are, confirming the isolates on the molecular/genetic basis by PCR procedures in addition to the use of conventional microbiological seeding techniques. However, it is also aimed to obtain important epidemiological data on the field strains in the country in terms of *L. monocytogenes* and *E. coli O157: H7*, which have high polymorphic protein structures and rapid genetic variation ability.

MATERIAL and METHODS

In the scope of the research, 100 fresh kashar cheese and 100 milk creme collected by random sampling method were examined. In the kashar cheese samples, specimens subjected to short-term maturation such as primary risk group were collected. From the point of milk cremes, samples which are offered for sale in the open are preferred. In order to provide sample homogenization, an equal number of sales points were visited for each category at Asian and European sides of Istanbul. The collected samples were delivered to the laboratories of the Faculty of Veterinary Medicine of Istanbul University by observing the antisepsis rules and preserving the cold chain and the samples were started to be analyzed in the laboratory on the same day. Detailed breakdown data of the samples collected during the study are shown in *Table 1*.

Table 1. *Detail breakdown table of fresh kashar and milk samples collected*

Province	Region	Sample Name	Sample Number	Sales Point Type	Explanation
Istanbul	Europe	Fresh kashar cheese	50	Sales points selling on the market or dairy products (cheeses being the point of selling breakfast products)	Products that have been subjected to short-term maturation, preferably openly offered for sale
Istanbul	Asia	Fresh kashar cheese	50	Fix/weekly market	
Istanbul	Europe	Milk creme	50	Sales points selling on the market or dairy products (cheeses being the point of selling breakfast products)	Preferably products that are offered for sale in open form
Istanbul	Asia	Milk creme	50	Fix/weekly market	
Total	200 samples				

Sample Isolation and Identification Procedure

***Listeria monocytogenes*:** 25 g samples were transferred into 225 mL BLEB (Buffered Listeria Enrichment Broth Base) (Merck, Germany), incubated at 30°C for 4 hours and then supplemented with selective agents and 25 mg/L natamycin in media at 48°C for 48 h. At the 24th h of incubation, Oxford agar (Merck, Germany) and Palcam agar (Merck, Germany) were passaged and incubated for 48 h at 35°C. At the end of the 48th h of incubation, passage was made to the Chromogenic Listeria Agar Base (Merck, Germany), one of the *L. monocytogenes/ivanovii* differential selective agar. *Listeria* spp. cultures were purified by passage of susceptible Trypticase anagen (TSA) (Merck, Germany) agar containing yeast extract [11]. Identification of suspicious isolates was performed by Gram staining, catalase, motility, dextrose, maltose, rhamnose, mannitol, xylose fermentation, esculin hydrolysis, nitrate reduction. In addition, CAMP test with *S. aureus* was performed and it was determined whether the isolates had CAMP factor [12].

***Escherichia coli/E. coli O157: H7*:** Asepsy conditions were followed by dilution and homogenization procedures first in sterile containers and then transferred to the laboratory. Afterwards, Tryptone Bile X-glucuronide (TBX) (Merck, Germany) by the spread method into Petri dishes. This Petri dishes were incubated at 44°C for 24 h and typical colonies were counted at the end of the incubation period. For *E. coli O157*: *H7*, homogenization was followed by Modified Tryptic Soy Broth (MTSB) (Merck, Germany) and incubation at 37°C for 24 h. Afterwards, 10 mcL of Sorbitol Mac Conkey Agar (SMAC) (Merck, Germany) was passed from each medium and incubated at 37°C for 24 h. At the end of the incubation period, "straw" color columns in the SMAC Agar were trasnferred to *E. coli* O157:H7 medium (EOH). After an incubation period of 18-24 h, typical pink colonies on EOH were as "suspected". Then, suspected colonies were transferred to cefixime-tellurite (Merck, Germany) (CT-SMAC) to determine CT resistance and the purity of the suspected colonies and the colonies were incubatedat 42°C for 18-24 h. CT resistant colonies were tested for the utility of sorbitol and methyumbelliferyl-β-glucuronide in modified Haemorrhagic Coli (Merck, Germany) broth. Suspected colonies were analyzed to determine of fermentation lactose and sucrose in Triple Sugar Iron agar (Merck, Germany) slants, indol production, methyl red and Woges Proscauer reactions, citrate utilisation (the IMVIC tests) and typical colony morphology on Levine Eosine Methylene Blue agar (L-EMB Merck, Germany) Presence of the O157 antigen was investigated by the latex agglutination test using the *E. coli* O157 test kit (Oxoid). Antisera contained in a commercially available O:H serotyping kit Escherichia coli antisera (SEIKEN, Denka Seiken Co., Ltd., Tokyo, Japan) was utilised for O:H serotyping following the manufacturer's manual [12].

PCR

The presence of *L. monocytogenes* and *E. coli O157: H7* in all samples collected as planned by the PCR procedure will be sought. For this purpose, the PCR procedure defined for each microbiological parameter was applied to the isolates evaluated as suspect or positive after the sowing procedure with conventional methods for microbiological parameters *(Table 2)* [12].

Electrophoresis

The PCR products were 2% (wt/vol) electrophoresed in agarose containing ethidium bromide, and the source of specific bands is searched with the help of UV trans-illuminator. Positive control groups containing DNA-free negative control group and phenol extracted specific active DNA for each parameter were also used during the procedure.

RESULTS

The collected samples were analyzed for *L. monocytogenes* and *E. coli O157:H7* using both conventional microbiological sowing methods and PCR. According to the results obtained in the study four samples of fresh kashar cheese subjected to short time maturation were found to be positive for *E. coli O157:H7* and six samples for *L. monocytogenes* out of two hundred milk samples analyzed. The results of our study are shown at *Table 3*.

Products obtained after PCR are shown in *Fig. 1* and *Fig. 2*.

DISCUSSION

According to the results obtained in the study, 4 (4%) of 100 milk creme analyzed were contaminated with *E. coli O157: H7*, 3 (3%) were contaminated with *L. monocytogenes* and 100 fresh short-term maturated 3 (3%) of the samples of kashar cheese were positive for *L. monocytogenes*.

Table 2. *Primer sets and properties to be designed according to different serotypes used in our work*

Primer No	Sequence (5'–3')	Target Gene/Amp (bp)	Target Microorganisms
1	GCTGATTTAAGAGATAGAGGAACA	*actA* 827	*L. monocytogenes*
2	TTTATGTGGTTATTTGCTGTC	*actA* 827	*L. monocytogenes*
3	GATAGACTTTTCGACCCAACAAAG	*shigaliketoxin*/208	*E. coli O157:H7*
4	TTGCTCAATAATCAGACGAAGATG	*shigaliketoxin*/208	*E. coli O157:H7*

Specifically designed primers were replicated and used in this study (GenBank accession no. NC-003210 for L. monocytogenes and strain: Sakai, substrain: RIMD 0509952, serovar: O157:H7 E. coli O157:H7)

Table 3. *Detail breakdown in terms of L. monocytogenes and E. coli O157:H7 analyzed in fresh kashar cheese and milk creme samples*

Microorganism Information	Sample Name	Example Province/Region	The Total Number of Samples Analyzed	Positive Sample Number
E. coli 0157:H7	Fresh kashar cheese	İstanbul/Europe	50	0
E. coli 0157:H7	Fresh kashar cheese	İstanbul/Asia	50	0
E. coli 0157:H7	Milk creme	İstanbul/Europe	50	3 (6%)
E. coli 0157:H7	Milk creme	İstanbul/Asia	50	1 (2%)
L. monocytogenes	Fresh kashar cheese	İstanbul/Europe	50	2 (4%)
L. monocytogenes	Fresh kashar cheese	İstanbul/Asia	50	1 (2%)
L. monocytogenes	Milk creme	İstanbul/Europe	50	2 (4%)
L. monocytogenes	Milk creme	İstanbul/Asia	50	1 (2%)

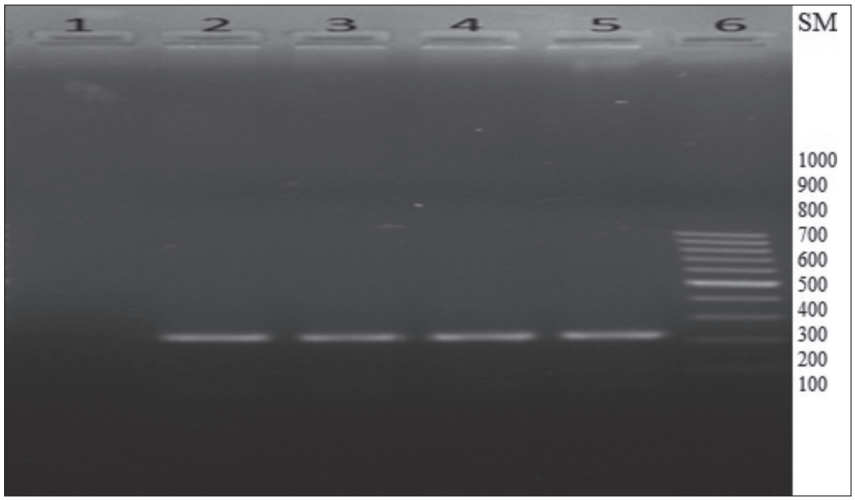
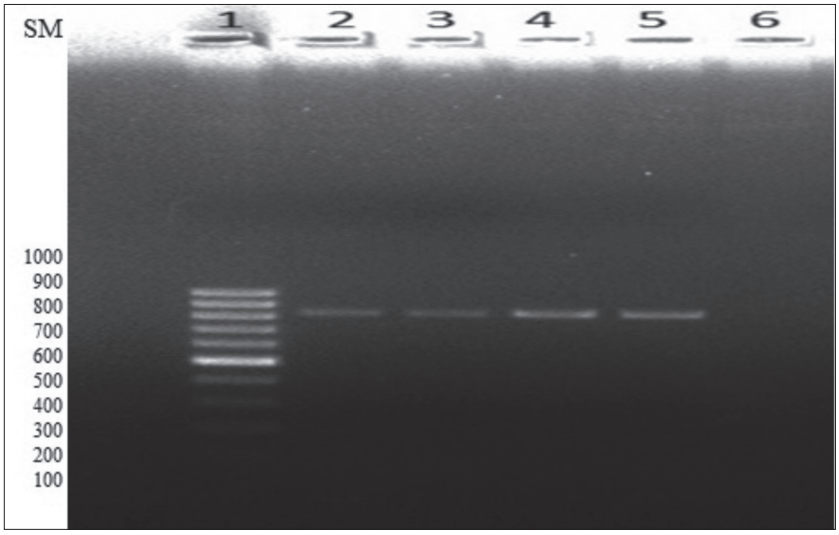


Fig 1. Agarose gel electrophoresis of amplified PCR products of *E. coli O157: H7* strains detected (1 - NC, 2/5 - *O157:H7* shigaliketoxin 208 bp, 6 - sm)

Fig 2. Agarose gel electrophoresis of PCR products of amplified *L. monocytogenes* strains (1 - sm, , 2/5 - *L. monocytogenes* - actA, 827 bp, shigaliketoxin, 6 - NC)



Although there are few cases of listeriosis in Brazil, it has been reported that pathogenic bacteria are isolated repeatedly from the dairy farms. For example, in a survey on 437 products in Brazil, 3 (0.7%) were detected in retail products and 1 was in dairy farm products (0.2%) *L. monocytogenes* [13]. Tümbay et al.[14] in a study conducted over feta cheese in Turkey, collected from different points of sale, 323 cheeses of 5.8% contaminated with Listeria spp and they found that it was 3.4% were *L. monocytogenes*. Sağun et al.[15], found 3.93% of *L. monocytogenes* in herbage cheese samples in their study on raw milk and herb cheeses from Van and the region. Çetinkaya et al.[4] detected *L. monocytogenes* in one of 51 samples of Shavak type white cheese. Loncarevic et al.[10] found a *L. monocytogenes* level of 6% in a study on 333 imported cheese in Sweden. Kevenk et al.[9] found *L. monocytogenes* in 5 milk samples

(5%) in 100 milk samples and 9 milk samples out of 110 (8.2%) in a study of 210 total dairy products. Aksoy et al.[16] found *L. monocytogenes* in 16 (5.3%) dairy products in 300 food samples . Şanlıbaba et al.[17] raported that *Listeria* spp. the prevalence were found in homemade cheese (9.09%), raw milk (8.19%) and white cheese (3.64%), respectively. *L. monocytogenes* strains were isolated from raw milk and homemade cheeses.

In a study by Ombarak et al.[18] in Egypt, it was found that 109 (55 raw milk, 41 local cheese, Karish, 13 local cheese Ras) samples were contaminated with *E. coli* out of 187 regional milk products examined. *E. coli* bacteria have been reported to be potentially harmful to consumers if they carry virulence genes and 69 of the 187 strains tested (36.9%) were found to have one or more virulence genes. Can and Elmalı [3] studied 71 traditional cheese products and 60 mincing samples and found that 17 (13%) of *E. coli O157* and 16 (16.2%) of 131 samples had *E. Coli O157:H7*. Sağlam and Şeker [19] they have isolated 3 (3%) *E. coli O157* from 100 samples in their study using conventional culture methods and serological confirmation tests on slip samples sold in Afyonkarahisar at local public markets. Ioanna et al.[8] found that *E. coli O157:H7* survived throughout the production process in the local (*Cacioricotta*) cheeses with a 90-day maturation period, increased concentration on the first day of maturation, remained stable until 35 days, and decreased until the end of the hardening period. They pointed out that the 90 day maturation period for the contaminating cheese was not sufficient to completely destroy the pathogenic bacterium. They also argued that adding starter cultures during cheese construction was an important factor in reducing contamination with *E. coli O157:H7*. In another study done, Hashemi et al.[6] observed strong antibacterial activity against slipped *E. coli O157:H7* and other common pathogenic bacterial strains fermented with *Lactobacillus plantarum* strain.

Findings from our study are similar to those of the above mentioned researchers. Three microbial analyzes of *L. monocytogenes* (3%) of 100 samples of short-term maturation cheese, which were analyzed according to the findings obtained in our study, were finally determined and the positivity of the factors in the mentioned samples was confirmed by PCR procedures. As a result of this study, presence of *E. coli*, a hygiene indicator and *L. monocytogenes*, a listeriosis agent, in kashar cheese and milk creme, which can cause food poisoning requires more serious inspection for food safety applications. It is an important risk factor for public health that milk and dairy products are not given sufficient attention to hygiene conditions during production.

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Research Article

Effect of Multi-enzyme Produced By a Single Fungus on Growth Performance and Some Carcass Parameters of Broiler Chicks Fed on Maize-Soya Based Diets

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Abstract

The present study was conducted to test whether a multi-enzyme produced by a fungus would keep performance of broiler chicks receiving a diet with almost 3% less nutrients (energy, protein, amino acids, calcium and phosphor) under two different housing density. In both experiments, positive control received standard broiler diet, negative control received a diet with 3% less nutrients than standard broiler diet and enzyme group received the negative control diet+supplemental enzyme. In the first trial with 1000, day-old chicks housed 10 birds/m^2, only 1% improvement in feed conversion efficiency and body growth was achieved with enzyme supplementation in contrast to the negative control at the end of 35 days feeding period. In the second trial with 1200, day-old-chicks accommodated 16 birds/m^2. The results showed that enzyme improved broiler performance almost 3.5% in contrast to the performance obtained from the negative control. In conclusion, supplementing broiler diets based on maize/soya with a multi-enzyme produced by a single fungus provides a great potential to gain the gap created by formulating almost 3% nutrients reduction in broilers housed with 16 birds/m^2 density. However, providing high comfort and better rearing condition as the birds at 10 birds/m^2 density, positive effects of the enzyme was limited.

Keywords: *Enzyme, Broiler, Body growth, Feed conversion efficiency, Rearing density*

Bir Mantar Tarafından Üretilen Multi-enzimin Mısır-Soya Bazlı Rasyonla Beslenen Broyler Civcivlerinin Büyüme Performansı ve Karkas Parametrelerine Etkisi

Öz

Mevcut çalışma, 35 günlük bir besi döneminde iki farklı kümes yoğunluğunda, yaklaşık %3 daha az besin (enerji, protein, aminoasit, kalsiyum ve fosfor) içerikli yem tüketen broyler civcivlerde bir mantar tarafından üretilen multi-enzimin performansı koruyup koruyamayacağını test etmek için yapılmıştır. Her iki çalışmada, pozitif kontrol standart broyler yemi, negatif kontrol standart broyler yeminden %3 daha az besin maddesi içeren yem ve enzim grubu negatif kontrol yemi+takviye enzim tüketmiştir. İlk denemede günlük yaşta 1000 civciv 10 civciv/m^2 şeklinde yerleştirilmiş, deneme sonunda enzim takviyesiyle negatif kontrole zıt şekilde yalnızca yemden yararlanma oranı ve vücut gelişiminde %1'lik iyileşme sağlanmıştır. İkinci denemede günlük yaşta 1600 civciv 16 civciv/m^2 şeklinde yerleştirilmiştir. 35 gün sonunda elde edilen sonuçlar enzim takviyesinin negatif kontrolden elde edilen sonuçlara zıt şekilde broyler performansını yaklaşık %3.5 iyileştirdiğini göstermiştir. Sonuç olarak, mısır/soya bazlı broyler rasyonlarına bir mantar tarafından üretilen multi-enzim ilavesi 16 civciv/m^2 yoğunlukta yerleştirilmiş broylerlerde yem formulasyonundan doğan %3 besin maddesi boşluğunu doldurmak üzere büyük potansiyel sağlamıştır. Ancak, yüksek konfor ve 10 civciv/m^2 yoğunluğu gibi daha iyi yetiştirme şartlarında, enzimin olumlu etkisi sınırlandırılmıştır.

Anahtar sözcükler: *Broyler, Büyüme performansı, Yemden yararlanma etkinliği, Enzim, Yerleşim sıklığı*

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INTRODUCTION

The use of exogenous enzymes to enhance nutrient availability of animal feeds has been reported as back as 1925 [1]. The commercial application of feed enzymes as a feed additive however has a history of less than 30 years. Over the last two decades, many studies have been conducted to determine effects of exogenous enzymes and their potential use in poultry nutrition [2-17]. The main focus of those studies with regards to monogastric nutrition was for the use of non-starch polysaccharide (NSP) -degrading enzymes such as β-glucanase and xylanase in barley, rye and wheat, in an attempt to alleviate the problems associated with increased digesta viscosity caused by the use of such viscous grains in animal diets.

In fact, formulating poultry diets based on their indigestible components has been a common concept [18]. Maize is considered a homogenous commodity that is highly digestible for broilers [19], although some reports have found considerable variation between samples [20] and others that maize starch digestibility may be as low as 85% at the terminal ileum [21]. This may be due to the fact that some forms of starch cannot be degraded by endogenous carbohydrases [19] because of their different chemical structure and physical properties [22]. Such starch is called as resistant starch and presents the opportunity for the use of exogenous feed enzymes in poultry diets [23].

As the second main ingredient of poultry feeds, soya is considered a good source of protein and amino acids and is probably the most popular vegetarian protein source used in broiler feeds. There is, however considerable variability for digestibility between soybean meal samples. Therefore, corn-soya based poultry diets are suggested to be supplemented with exogenous feed enzymes not as a pro-nutrient, but rather as a means of improving homogeneity and digestibility.

In practice, exogenous enzymes in poultry diets are used in three ways. Firstly, it could be used as a feed additive with a potential nutritional (protein, amino acids, energy etc.) value contributing the diet on top, secondly, reducing the nutrient (protein, amino acids, energy etc.) content of diet up to a potential amount which would be gained by supplemental enzyme, and thirdly, on top without calculating any nutritional contribution. It has practically been claimed that most of NSP-enzymes used in broiler diets provide an almost 2-3% improvement of feed digestibility on average, meaning a significant feed cost reduction without impairing bird performance.

The present study was conducted to evaluate the effects of dietary supplemental multi-enzyme produced by a single fungus *(Talaromyces versatilis)* in maize-soya diets with reduced nutrient contents (energy, protein, amino acids, calcium and phosphor) according to its potential enhancement (almost 3%) on the growth performance, mortality, carcass weight, yield and abdominal fat weight of broiler chicks fed on maize-soya based diets under our experimental condition in two consecutive trials differing in animal density during the rearing period from day 0 to 35.

MATERIAL and METHODS

The present study comprising two trials differing in animal density were carried out in the Broiler Unit of Experimental Farm of the Department of Animal Science, Faculty of Agriculture, University of Çukurova-Turkey. All the protocols used in this experiment are approved by the Animal Experiments Local Ethics Committee of Çukurova University, Adana-Turkey.

Day old broiler chicks (ROSS 308) were used in both trials. The first experiment was conducted using one thousand chicks (500 male and 500 female) accommodated in 20 pens with a density of 10 birds/m^2 for 35 days. While the second experiment was run with a density of 16 birds/m^2 using 1200 (600 males and 600 females) chicks up to 35 days of age. A conventional feeding programme was applied during the both experiments, in which standard starter (days 0 to 10), grower (days 11-21) and finisher (days 22 to 35) diets were used. All diets were based on corn and soya. Ingredient and nutritional composition of the diets used in Experiments 1 and 2 are given in *Table 1* and *Table 2,* respectively.

As the possible effect(s) of supplemental enzyme (50 g/ton feed) on enhancing nutrient value of the diet is known to be varied according ingredient content of the diet, potential reduction in nutrients contents of the experimental diet was determined using Predictor of Rovabio-Advance [25], in which the ingredient composition and consumption level of each diet of each trial were loaded then the Predictor calculated what the reduction level as same as the levels gained by supplemental enzyme should be for energy, protein, digestible amino acids, calcium and available phosphorus for each diet used in Trials 1 and 2 *(Table 3)*.

At the beginning of the trials all the chicks were weighed, sexed and grouped with a similar mean weigh and same number of each sex, then accommodated in 20 pens in a completely randomized design with 3 treatments with 6 or 7 replicates *(Table 4)* each. Each pen (replicate) was equipped with a tube feeder and water drinkers on litter; wood shaving litter 7-8 cm height. Chicks were fed 35 days *ad libitum* under a commercial rearing condition (23:1 light: dark photoperiod, thermoneutral temperature). In the first trial total 50 chicks (25 females+25 males) were accommodated in each pen sized 5 m^2 (2x2.5m; having 10 birds/m^2), while in the second trial total 60 chicks (30 females+30 males) were housed in each pen sized 3.75 m^2 (1.875 x 2.0m; having 16 birds/m^2 as applied by the industry).

Table 1. *Ingredient and nutritional compositions of the diets used in Experiment 1*

Ingredients (%)	Standard (for control diets)			Reduced Nutrient Contents (for negative control diets)		
	Starter (0-10d)	Grower (11-21d)	Finisher (22-35d)	Starter (0-10d)	Grower (11-21d)	Finisher (22-35d)
Yellow corn	48.49	51.33	46.30	52.06	53.26	49.76
Fullfat soya	23.41	23.00	26.00	14.66	22.36	26.00
Soybean meal (46% CP)	16.29	10.00	-	21.80	9.84	-
Soybean meal (44% CP)	-	-	5.83	-	-	4.20
Rice (feed grade)	-	-	5.00	-	-	5.00
Maize gluten meal (60% CP)	2.85	3.22	-	2.63	2.60	-
Meat bone meal (35% CP)	2.00	2.00	2.50	2.00	2.00	2.50
Poultry offal meal	2.00	2.00	2.50	2.00	2.00	2.50
Sunflower meal (34% CP)	2.00	3.00	4.00	2.00	3.00	4.00
DDGS (Corn)	-	2.00	3.00	-	2.00	3.00
Soya Oil	-	0.99	3.07	-	0.50	1.26
Marble powder	0.80	0.76	0.59	0.70	0.75	0.59
DCP (18% P)	0.60	0.30	-	0.62	0.31	-
DL-Methionine	0.33	0.27	0.27	0.33	0.27	0.25
Sodium bicarbonate	0.33	0.27	0.18	0.33	0.27	0.18
L-Lysine	0.31	0.32	0.22	0.30	0.30	0.23
L-Threonine	0.12	0.09	0.06	0.11	0.08	0.05
Common salt	0.17	0.15	0.18	0.16	0.16	0.18
Trace Mineral Premix*	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin Premix**	0.10	0.10	0.10	0.10	0.10	0.10
Choline-60	0.05	0.05	0.05	0.05	0.05	0.05
Coccidiostat (Sacox)	0.05	0.05	0.05	0.05	0.05	0.05
Total	100.00	100.00	100.00	100.00	100.00	100.00
Nutrients (%)						
ME-Poultry (MJ/kg)***	12.55	12.97	13.39	12.17	12.59	13.01
Dry matter	88.37	88.35	88.05	88.06	88.20	87.98
Crude protein	24.24	22.46	20.37	23.68	21.90	19.85
Crude fibre	3.71	3.74	4.05	3.63	3.75	4.05
Crude fat	7.83	8.42	10.42	5.60	7.85	8.69
Crude ash	5.79	5.25	4.88	5.69	5.22	4.84
Ca	1.00	0.90	0.85	1.00	0.90	0.85
Available P	0.49	0.44	0.43	0.49	0.44	0.42
Na	0.18	0.17	0.16	0.18	0.17	0.16

** Each kg of vitamin premix contains 13.500.000 IU Vit. A, 4.000.000 IU Vit. D_3, 100.000 mg Vit. E, 5.000 mg Vit. K_3, 3.000 mg Vit. B_1, 8.000 mg Vit. B_2, 60.000 mg Niacin, 18.000 mg Ca-D-Pantotenate, 5.000 mg Vit. B_6, 30 mg Vit. B_{12}, 2.000 mg Folic Acid, 200 mg D-Biotin and 100.000 mg Vit. C*
*** Each kg of trace mineral premix contains 100.000 mg Manganese, 80.000 mg Iron, 80.000 mg Zinc, 8.000 mg Copper, 200 mg Cobalt, 1000 mg Iodine, 150 mg selenium (sodium selenite), 500.000 choline chloride*
**** ME value of the diet is calculated by individual ME value of each feedstuff [24] multiple by its ratio in the diet*

During the experiment, body weight, feed intake, feed conversion efficiency and mortality were recorded on days 7, 14, 21, 28, 35 on subgroup (replicate) bases. At the end of the trial on day 35 all the birds were weighed, 10 birds were sampled for each subgroup, then total 200 birds were transferred to a commercial slaughter house to slaughter for carcass analyses. Live weight, slaughter weight, hot and cold carcass weight, carcass yield (100 x [cold carcass weight/slaughter weigh]) abdominal fat (consists of a mass of adipose tissue located in the abdominal cavity adjacent to the pelvic bones; [26]) weight were measured. Feeds used in the trial were analysed for dry matter, ether extract, crude ash, crude protein and crude fibre according to AOAC [27] procedure.

Table 2. *Ingredient and nutritional compositions of the diets used in Experiment 2*

Ingredients (%)	Standard (for control diets)			Reduced Nutrient Contents (for negative control diets)		
	Starter (0-10d)	Grower (11-21d)	Finisher (22-37)	Starter (0-10d)	Grower (11-21d)	Finisher (22-37d)
Yellow corn	41.32	46.70	43.64	45.88	50.50	48.30
Fullfat soya	24.00	18.00	25.00	16.18	16.01	25.00
Soybean meal-44	15.23	15.04	6.40	20.22	16.09	4.98
Wheat shorts	0.00	0.00	6.00	0.00	0.00	6.00
Corn gluten meal-60	6.82	3.93	0.00	3.52	2.41	0.00
Corn flour	3.00	3.00	3.00	3.00	3.00	3.00
Meat & bone meal-35	3.00	2.50	2.73	3.00	2.50	2.58
DDGS (Golden)	2.00	3.00	3.00	2.50	3.00	3.00
Sunflower meal-34	2.00	3.00	5.00	3.00	3.00	4.00
Marble powder	0.70	0.69	0.60	0.69	0.69	0.67
Soybean oil	0.00	2.34	3.24	0.00	1.00	1.08
DCP-18	0.37	0.28	0.00	0.41	0.30	0.00
L-Lysine	0.32	0.31	0.24	0.32	0.29	0.24
DL-Methionine	0.29	0.29	0.29	0.32	0.30	0.29
Sodium bicarbonate	0.26	0.22	0.16	0.26	0.22	0.17
Common Salt	0.17	0.18	0.20	0.17	0.18	0.20
L_Threonine	0.11	0.12	0.10	0.13	0.11	0.09
Trace Mineral Premix*	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin Premix**	0.10	0.10	0.10	0.10	0.10	0.10
Toxin Bindere (Unike Plus)	0.10	0.10	0.10	0.10	0.10	0.10
Choline-60	0.06	0.05	0.05	0.05	0.05	0.05
Coccidiostat (Sacox)	0.05	0.05	0.05	0.05	0.05	0.05
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Nutrient (%)						
ME-Poultry (MJ/kg)***	12.55	12.97	13.39	12.17	12.59	13.01
Dry matter	88.56	88.23	88.53	88.02	87.88	88.12
Crude protein	25.71	22.51	19.96	24.00	21.60	19.22
Crude fiber	3.87	3.88	4.01	4.07	3.93	3.85
Crude fat	7.05	8.31	10.53	5.69	6.70	8.49
Crude ash	6.04	5.52	5.16	6.04	5.50	5.07
Ca	1.00	0.90	0.83	1.00	0.90	0.83
Available P	0.49	0.44	0.41	0.49	0.44	0.40
Na	0.18	0.17	0.16	0.18	0.17	0.16

** Each kg of vitamin premix contains 13.500.000 IU Vit. A, 4.000.000 IU Vit. D_3, 100.000 mg Vit. E, 5.000 mg Vit. K_3, 3.000 mg Vit. B_1, 8.000 mg Vit. B_2, 60.000 mg Niacin, 18.000 mg Ca-D-Pantotenate, 5.000 mg Vit. B_6, 30 mg Vit. B_{12}, 2.000 mg Folic Acid, 200 mg D-Biotin and 100.000 mg Vit. C, Phytase (500 U/kg)*
*** Each kg of trace mineral premix contains 100.000 mg Manganese, 80.000 mg Iron, 80.000 mg Zinc, 8.000 mg Copper, 200 mg Cobalt, 1000 mg Iodine, 150 mg selenium (sodium selenite), 500.000 choline chloride*
**** ME value of the diet is calculated by individual ME value of each feedstuff [24] multiple by its ratio in the diet*

The data obtained in the study were analysed using GLM procedure of SAS; the Statistical Analysis System [28] and Duncan's New Multiple Range Test in SAS were used to identify significant differences among treatments means.

RESULTS

The results obtained in the experiments 1 and 2 are presented according to the statistical analyses, given in the relevant tables and discussed in the frame of present literature.

Table 3. *Calculation of potential reduction in nutrient contents of the experimental diets containing 50 grams enzyme over its define matrix value per kg using Predictor for Experiments 1 and 2*

Nutrients	Experiment 1		Experiment 2	
	Nutritional Up-Lift Potential of 50 g Enzyme	Matrix Value of Each kg Enzyme	Nutritional Up-Lift Potential of 50 g Enzyme	Matrix Value of Each kg Dietary Enzyme
ME-Poultry (kcal/kg)	92.8	1.855.499	93.3	1.866.555
Crude Protein	0.530	10.567	0.550	11.045
Dig. Lysine	0.029	579	0.032	641
Dig. Methionine	0.005	94	0.007	136
Dig. Cystine	0.012	241	0.013	266
Dig. Meth. + Cyst.	0.017	335	0.020	401
Ca	0.025	588	0.024	478
Available P	0.029	500	0.028	563

Table 4. *Treatment groups and replicates number used in the Experiments 1 and 2*

Treatment Groups	No of Replicates (subgroups)	Treatment Groups	Enzyme (50 g/ton)
1	6	Positive Control-standard diet	NO
2	7	Negative control (reduced nutrient contents) diet	NO
3	7	Negative control + Enzyme supplemented diet*	YES

Table 5. *Effect of multi-enzyme produced by a single fungus on feed intake (g/bird) of broilers in Experiment 1*

Age (day)	Treatment Groups			SED	P=
	Positive Control	Negative Control	Negative Control + Enzyme		
7	143.6^{b}	147.3^{ab}	148.9^{a}	0.862	0.072
14	544.5^{b}	558.6^{ab}	566.3^{a}	3.086	0.034
21	1269	1275	1302	6.993	0.147
28	2357	2328	2365	11.14	0.362
35	3544	3560	3560	15.22	0.878

SED: *Standard error of difference between means;* ^{a,b} *means in the same row with different letters are significantly different (P<0.05)*

The results obtained in the experiment 1 with respect to feed intake, body weight gain, feed conversion efficiency, slaughter weight and carcass parameters and mortality are presented in *Tables 5, 6, 7, 8* and *9* respectively.

During the first two weeks of the experiment 1, the birds of the groups receiving reduced nutrient contents (negative control and/or negative control + enzyme) consumed feed higher amount than the birds of positive control *(Table 5)*. However, from the week 3 and later, all birds attained similar feed intake without any significant difference (P>0.05). The results with respect to feed intake during the first two weeks suggest that the birds receiving diets with reduced nutrient contents tried to compensate nutrient intake by increasing feed consumption for the period in which broilers grow fast and almost increased their body weight 12 folds.

At the beginning of the experiment 1, all the groups had similar body weight. Throughout the experiment, all the groups had similar growth performance. At the end of the experiment 1, the group receiving positive control diet achieved the best performance. The groups receiving supplemental enzyme with negative control diet had a performance, which was 1% better than the group receiving negative control diet but 0.5% lower than the group receiving positive control diet *(Table 6)*.

Similar to body weight gain, feed conversion efficiency was also not affected by enzyme significantly during the first four weeks of the experiment 1 (P>0.05). However, the cumulative data obtained at the end of the experiment showed that the group receiving positive control diet attained the best performance. Their feed conversion rate was found to be numerically better than that of the group receiving negative control diet with enzyme, but significantly better than that of the group receiving only negative control diet. 3% reduction in nutrient content of

Table 6. *Effect of multi-enzyme produced by a single fungus on body weight gain (g/bird) of broilers in Experiment 1*

Age (day)	Treatment Groups			SED	P=
	Positive Control	Negative Control	Negative Control + Enzyme		
Initial Body Weight (g/bird)	44.4	44.4	44.8	0.099	0.189
7	128	130	129	0.927	0.630
14	459	470	473	3.118	0.210
21	1014	1004	1008	4.045	0.609
28	1753	1702	1727	9.433	0.165
35	2479	2442	2467	10.69	0.371

SED: *Standard error of difference between means*

Table 7. *Effect of multi-enzyme produced by a single fungus on feed conversion efficiency (g intake : g gain) of broilers in Experiment 1*

Age (day)	Treatment Groups			SED	P=
	Positive Control	Negative Control	Negative Control + Enzyme		
7	1.12	1.13	1.15	0.007	0.352
14	1.19	1.18	1.19	0.007	0.772
21	1.25	1.26	1.29	0.007	0.127
28	1.34	1.36	1.36	0.005	0.239
35	1.43^{b}	1.45^{a}	1.44^{ab}	0.004	0.081

SED: *Standard error of difference between means;* ^{a,b} *means in the same row with different letters are significantly different (P<0.05)*

Table 8. *Effect of multi-enzyme produced by a single fungus on slaughter weight and carcass parameters (g/bird) of broilers in Experiment 1*

Parameters	Treatment Groups			SED	P=
	Positive Control	Negative Control	Negative Control + Enzyme		
Slaughter weight (at 35 d)	2504	2471	2498	29.75	0.893
Hot carcass weight	1912	1882	1910	21.70	0.814
Cold carcass weight	1814	1790	1814	21.56	0.864
Carcass yield (%)	72.47	72.42	72.65	0.055	0.183
Abdominal fat weight	28.40^{b}	34.47^{a}	33.04^{a}	0.475	**0.001**

SED: *Standard error of difference between means;* ^{a,b} *means in the same row with different letters are significantly different (P<0.05)*

the diet deteriorated feed conversion efficiency at about 1.4%, but enzyme supplementation could compensate the half (0.7%) of this deterioration *(Table 7)*.

The results with respect to carcass analyses showed that there were no differences obtained for slaughter weight, hot carcass weight, cold carcass weight and carcass yield of the treatment groups (P>0.05). However, the groups receiving negative control diet with or without enzyme attained significantly heavier abdominal fat than the group receiving positive control diet *(Table 8)*.

The results obtained from the first experiment suggest that almost 3% reduction in nutrient contents of the diet reduce broiler performance numerically (P>0.05) but increased abdominal fat statistically (P<0.05) under our condition of Experiment 1. The increase in abdominal fat may be attributed to increased feed/energy intake of the birds to meet their protein requirement. The increase in abdominal fat should also be associated with the deterioration in feed conversion efficiency. The results with respect feed intake, body weight gain and feed conversion efficiency suggest that positive effects of supplemental enzyme to the diet with reduced nutrients contents were not significant and also lower than the expected.

The results obtained in the experiment 2 with respect to feed intake, body weight gain, feed conversion efficiency, slaughter weight and carcass parameters are presented in *Tables 9, 10, 11* and *12*, respectively.

The results with respect to feed intake showed that nutrient reduction almost 3% affected feed intake significantly (P<0.001). During the experiment 2, the birds receiving reduced nutrient contents consumed feed higher (2-3%) amount than the birds receiving positive control or

Table 9. *Effect of Effect of multi-enzyme produced by a single fungus on feed intake (g/bird) of broilers in Experiment 2*

Age (day)	Treatment Groups			SED	P=
	Positive Control	Negative Control	Negative Control + Enzyme		
7	140[b]	144[a]	137[c]	0.648	0.0007
14	585[b]	630[a]	594[b]	2.715	<.0001
21	1248[a]	1244[a]	1163[b]	7.068	0.0001
28	2218[ab]	2249[a]	2158[b]	11.90	0.0164
35	3494[ab]	3552[a]	3462[b]	12.24	0.0217

SED: *Standard error of difference between means;* [a,b] *means in the same row with different letters are significantly different (P<0.05)*

Table 10. *Effect of multi-enzyme produced by a single fungus on body weight gain (g/bird) of broilers in Experiment 2*

Age (day)	Treatment Groups			SED	P=
	Positive Control	Negative Control	Negative Control + Enzyme		
Initial Body Weight (g/bird)	44.4	45.7	44.6	0.473	0.518
7	110	110	106	0.825	0.139
14	400	399	398	1.876	0.914
21	885[a]	863[ab]	854[b]	5.192	0.082
28	1568	1568	1542	8.573	0.382
35	2371[a]	2330[b]	2359[ab]	7.340	0.088

SED: *Standard error of difference between means;* [a,b] *means in the same row with different letters are significantly different (P<0.05)*

Table 11. *Effect of multi-enzyme produced by a single fungus on feed conversion efficiency (g intake : g gain) of broilers in Experiment 2*

Age (day)	Treatment Groups			SED	P=
	Positive Control	Negative Control	Negative Control + Enzyme		
7	1.28	1.32	1.29	0.009	0.2377
14	1.46[c]	1.58[a]	1.49[b]	0.014	**<.0001**
21	1.41[b]	1.44[a]	1.36[c]	0.003	**<.0001**
28	1.41[b]	1.44[a]	1.40[b]	0.003	**0.0005**
35	1.47[b]	1.52[a]	1.47[b]	0.002	**<.0001**

SED: *Standard error of difference between means;* [a,b,c] *means in the same row with different letters are significantly different (P<0.05)*

negative control + enzyme diets *(Table 9)*. The difference between feed intake of the negative control and negative control+enzyme groups was significant (P<0.05). This result suggest that birds receiving negative control diets tried to sustain nutrient intake by increasing feed intake throughout the study. Supplemental enzyme seems to be worked as expected by liberating some extra nutrients, which helps birds to sustain nutrient flow through intestines.

At the beginning of the experiment 2, all the groups had similar body weight. Throughout the experiment, all the groups had similar growth performance but at the end of the experiment, the group receiving positive control diet achieved the best performance. The group receiving supplemental enzyme had a performance, which was 1% better than the group receiving negative control diet but 0.5% lower than the group receiving positive control diet *(Table 10)*.

From the second week of the experiment 2, feed conversion efficiency was also affected by enzyme supplementation significantly similar to body weight gain (P<0.001). The overall data obtained at the second, third, fourth and last weeks of the experiment 2 showed that the best feed efficiency was obtained by the group receiving positive control or negative control+enzyme diets. Their feed conversion rate was found to be significantly (P<0.05) better than that of the group receiving negative control diet. Enzyme supplementation was improved feed conversion efficiency by about 3.5% *(Table 11)*.

The results with respect to carcass analyses showed that there were no significant differences obtained for slaughter weight, hot carcass weight, cold carcass weight and carcass yield of the treatment groups. However, the groups receiving negative control diet with or without enzyme attained significantly heavier abdominal fat

Table 12. *Effect of multi-enzyme produced by a single fungus on slaughter weight and carcass parameters (g/bird) of broilers in Experiment 2*

Parameters	Treatment Groups			SED	P=
	Positive Control	Negative Control	Negative Control + Enzyme		
Slaughter weight (at 37d)	2622	2582	2619	25.01	0.769
Hot carcass weight	1954	1910	1946	21.05	0.670
Cold carcass weight	192	1882	1916	19.68	0.680
Carcass yield (%)	73.25	72.92	73.17	0.241	0.840
Abdominal fat weight	30.73[a]	32.89[ab]	35.93[a]	0.784	**0.036**

SED: *Standard error of difference between means; [a,b] means in the same row with different letters are significantly different (P<0.05)*

than the group receiving positive control diet *(Table 12)*.

The results of the second experiment suggest that almost 3% reduction in nutrient contents of the diet reduce broiler performance (P<0.05) under the condition of Experiment 2. The results also suggest that positive effects of supplemental enzyme to the diet with reduced nutrients contents were significant for feed intake and feed conversion efficiency as expected.

DISCUSSION

The results of the present study showed that exogenous multi-enzyme produced by a single fungus used in maize-soya based diets improve nutritional value of the diets up to 3%. The improvement found to be varies according to animal density. In the first experiment during which the animals reared at low density (10 birds/m^2), the improvement with the supplemental enzyme was about only 1%, but when the birds were accommodated at high density (16 birds/m^2 industrial level) the improvement with the supplemental enzyme in performance was over 3%. As the source of main ingredients, maize and soya, of the diets and the other experimental conditions used in the both trials were the same, the difference between the improvement obtained from the both experiments could be attributed to the stocking density.

In fact, studies on stocking densities in broiler production have produced variable conclusions. Some studies show large benefits in reducing stocking density, while others show little or no differences. BILGILI and HESS [29] conducted a study examining densities of 0.8, 0.9 or 1.0 square foot per bird. Body weight, feed conversion, mortality, carcass scratches and breast meat yield were improved significantly when birds were given more space. A limited improvement with enzyme addition under better welfare condition should be expected, as modern broilers perform better when given more space. However, the ultimate goal in broiler production is to maximize pounds of chicken produced per square meter while preventing production losses due to overcrowding. Under this condition, enzyme supplementation seems to offer easy and effective way to maximize the product by promoting feed conversion efficiency. The improvements in feed conversion efficiency could be attributed to better digestion and higher absorption of nutrients liberated by the enzyme. It is well known that the most abundant carbohydrates in maize and soya are cellulose, arabinoxylans, pectins, oligosaccharides and starch [30]. Some of these compounds are relatively resistant to digestion such as cellulose, arabinoxylans and pectins. Many different primary enzymes are required to degrade these carbohydrates such as cellulase and cellobiohydrolyse to degrade cellulose, xylanase and arabinofuranosidase to degrade arabinoxylans, α-galactosidase to degrade the oligosaccharides and amylase to degrade starch.

The enzyme, produced by *Talaromyces versatilis*, used in the present study, has been introduced by ADISSEO [25]. It does have many different types of xylanases (Endo -1.4 β-xylanase β-xylosidase), β -glucanases (Endo - 1.3 1.4 β-glucanase Laminarinase), pectinases (polygalacturonase, Pectin esterase, Endo-1.5 α-arabinanase, α-galactosidase, rhamnogalacturonase), proteases (aspartic protease, metallo protease) and celluloses (Endo-1.4 β-glucanase, cellobiohydrolase, β-glucosidase) are associated with arabinofuranosidases, essential debranching enzymes (α-arabinofuranosidase, α-glucuronidase, ferulic acid esterase) besides some others (Endo-1.4 β-mannanase, β-manosidase).

It is well known that arabinoxylan is one of the main non-starch polisaccarides components in cell wall of most cereals and can partially be solubilised by processing [31]. It is composed of a xylose backbone chain carrying arabinose branches [32]. Arabinoxylans are primarily hydrolyzed by endo-1,4-b-xylanase activity, cleaving the (1,4)-linkages of the xylan backbone [20]. Due to their narrow specificity towards a given bond, distinct enzymes are required for the degradation of arabinoxylans. While xylanases hydrolyze the xylose backbone, their activity is frequently hampered by the presence of arabinose branches which prevent access to the central xylose chain. FOURIE [23] reported remarkable improvements with multi-enzym (xylanase, glucanase, cellulase, xyloglucanase, endo-xylanase, galactomannanase, mannanase and pectinase) in energy and protein digestibility of broiler received maize-soya based diets. He suggest that enzyme addition to balanced vegetarian maize-soya diets may result in significant financial gains. Similar results have also been

reported in more recent studies; using a combination of amylase, xylanase and protease was reported to be effective in improving the growth profiles of broilers fed maize-soybean-rapeseed-cotton mixed diets [33]. The multy-enzyme used in the present study does also have arabinofuranosidases, which is known as debranching enzymes, are able to cleave arabinose from the backbone and enhance the efficacy of xylanases. Removing the branches greatly helps the xylanases gain access to the xylose backbone [34].

The results obtained in the present study suggest that exogenous multi-enzyme containing many different types of xylanases, ß-glucanases, pectinases, celluloses and arabinofuranosidases is able to improve nutritional value of maize-soya up to 3%, as it can compensate the reduction made 3% nutrients (energy, protein, amino acids, calcium and phosphor) by better feed conversion efficiency.

As a result, in the first trial, providing no feeding competition with high comfort and better rearing condition as the birds were accommodated with low density (10 birds/m^2), positive effects of the enzyme was shadowed. Supplemental multi-enzyme seems be work partly and it gains almost 1% nutrients through better feed conversion efficiency and growth performance. In the second trial, providing feeding competition with low comfort and moderate rearing condition similar to the industry, as the birds were accommodated with high density (16 birds/m^2), positive effects of the enzyme was obvious. Supplemental multi-enzyme seems to work markedly and it gains weight almost 3.5% nutrients by better feed conversion efficiency and growth performance.

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Research Article

Effect of Cysteamine and 13-Cis-Retinoic Acid on Bovine *In Vitro* Embryo Production

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Abstract

One of the main factors decreasing the success of an *in vitro* embryo production is oxidative stress-mediated reactive oxygen species resulting in cellular damages. The present study was carried out to evaluate the effects of addition of different doses of cysteamine and 13-cis-retinoic acid during *in vitro* maturation on oocyte maturation and blastocyst formation. Five-hundred oocyte complexes were randomly collected from slaughterhouse and assigned to five groups; oocytes matured *in vitro* maturation (IVM) medium without any supplementation (control), IVM medium supplemented with 50 µM or 100 µM cysteamine, IVM supplemented with 5 nM 13-cis-retinoic acid and IVM supplemented with 10 nM 13-cis-retinoic acid. Matured oocytes were fertilized and cultured. Oocytes and embryos were evaluated for nuclear maturationand blastocyst formation, respectively. The highest numbers of oocytes in the cumulus expansion, cleavage, murola and blastocyst stages were seen in cows treated with 13-cis retinoic (5 nm) (92%), cysteamine (100 µL) (74%), cysteamine (50 and 100 µL) (48%), cysteamine (100 µL) and 13-cis retinoic (24%) and finally cysteamine (100 µL) (22%), respectively.Nuclear maturation of oocytes decreased significantly in the media supplemented with 10 nM 13-cis-retinoic acid ($P<0.05$). The results of the present study demonstrated that IVM supplementation with cysteamine and a low concentration of 13-cis-retinoic acid improved the efficacy of an *in vitro* production of bovine embryo.

Keywords: *Cysteamine, 13-cis-retinoic acid, Bovine, In vitro embryo production*

Sığır *İn Vitro* Embriyo Üretiminde Sistamin ve 13-Cis-Retinoik Asitin Etkisi

Öz

In vitro embriyo üretiminin başarısını azaltan en önemli faktörlerden biri oksidatif stres aracılı reaktif oksijen türleri ve bunun sonucu oluşan hücresel hasarlardır. Bu çalışma, *in vitro* olgunlaşma süresince farklı dozlarda sistamin ve 13-cis-retinoik asit ilavesinin oosit olgunlaşması ve blastosit oluşumu üzerine etkililerini araştırmak amacıyla yapılmıştır. Beş yüz oosit kompleksi kesimhaneden rastgele toplandı ve beş gruba ayrıldı; ilave katkısız *in vitro* olgunlaştırma medyumu (IVM)'nda olgunlaşan oositler (kontrol), 50 µM veya 100 µM sistamin katkılı IVM'de olgunlaşan oositler, 5 nM 13-cis-retinoik asit katkılı IVM'de olgunlaşan oositler ve 10 nM 13-cis-retinoik asit katkılı IVM'de olgunlaşan oositler. Olgunlaşan oositler fertilize ve kültüre edildi. Oositler ve embriyolar sırasıyla nükleer olgunlaşma ve blastosit oluşumu bakımından değerlendirildi. Kümülüs genişlemesi, çentiklenme, morula ve blastosit evrelerindeki en yüksek oosit sayıları sırasıyla 13-cis-retinoik (5 Nm) (%92), sistamin (100 µL) (%74), sistamin (50 ve 100 µL) (%48), sistamin (100 µL), 13-cis-retinoik (%24) ve son olarak sistamin (100 µL) (%22) uygulananlarda gözlemlendi. Oositlerin nükleer olgunlaşması 10 nM 13-cis-retinoik asit katkılı medyumda anlamlı oranda düştü ($P<0.05$). Bu çalışmanın sonuçları IVM içerisine sistamin ve düşük konsantrasyonda 13-cis-retinoik asit katkısının sığır embriyosunun *in vitro* üretimini iyileştirdiğini göstermiştir.

Anahtar sözcükler: *Sistamin, 13-cis-retinoik asit, Sığır, İn vitro embriyo üretimi*

INTRODUCTION

An *in vitro* production (IVP) of bovine embryos has become a popular technology which may solve part of reproductive issues in modern dairy farms. Achieving appropriate protocols and media which produce highly qualified embryos may improve the efficiency of IVP which is still as low as 30-40% [1]. The procedures of the IVP of embryo are consist of an *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) of embryos. IVP is a sequential process and failure in each step may influence the final result [2-4]. One of the most important factors with detrimental effects on the quality of gametes and embryos under culture conditions is oxidative stress

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(OS) [2-4]. Oxidative stress induces mitochondrial, DNA, RNA, and protein damages [2-4], inhibition of sperm-oocyte fusion [2-4] and finally embryo cell apoptosis [2-4]. Free radicals such as reactive oxygen species (ROS) interact with oocytes and embryos and decrease blastulation rates in embryo development [2-4]. Therefore, it is important to decrease the levels of ROS formation during an IVP.

Progress toward the simplification and modification of a medium used for IVP, especially for the reduction of ROS, may increase the efficacy of embryo production in commercial aspects. *In vivo* mammalian cells possess efficient antioxidant systems such as catalase or superoxide dismutase as well as thiol compounds that act as metabolic buffers which scavenge active oxygen species. These systems are more critical for important processes such as the maturation, formation, and growth of gametes and embryos. In recent years, various materials with antioxidative properties have been examined to reduce the risk of ROS formation in IVP procedure [5].

Vitamin A is a fat-soluble unsaturated isoprenoid and is well known to regulate development, cellular growth, differentiation, and maintenance of tissue function. Vitamin A may influence bovine ovarian follicular development, steroidogenesis, oocyte maturation, embryo and conceptus development and uterine environments [6-8]. The effects of vitamin A and its metabolites including 9-cis-retinal forms photoactive isorhodopsin, 11-cis retinal forms which is present in the retina, and 13-cis-retinoic acid which is a metabolite of retinol found in many tissues of bovine reproduction is not only exerted as an antioxidant, but also acts as a local modulation for the development and differentiation of cells [6-8]. Xing and Sairam [9] showed that retinoid has direct effects on the modulation of the gonadotropin receptor promoter [9]. Another way by which vitamin A is postulated to affect reproduction is the induction of midkine (neurite outgrowth-promoting factor 2) by retinoic acid (RA) in cumulus-granulosa cells [10]. Moreover, some studies have suggested that RA may regulate nitric oxide synthesis in granulosa cells [11]. In the past two decades, a number of studies have examined different concentrations of cis-retinoic acid on the efficacy of bovine IVP media. Results showed that the addition of cis-retinoic acid into the IVM medium increased bovine blastocyst development and hatching rate [12]. Duque et al.[8] showed that the cytoplasmic competence of bovine oocytes improved in the presence of cis-retinoic acid during prematuration. Moreover, Livingston et al.[13] demonstrated that the addition of retinol to the IVM medium may improve the embryonic development and blastocyst rate of bovine oocytes.

Another way to scavenge active oxygen species is the synthesis of glutathione (GSH) which protects the cell from OS damages. The production of GSH depends on the existence of cysteine [14], and the synthesis of cysteine depends on the availability of cysteamine as a thiol compound that reduces cystine to cysteine [15]. The effect of cysteamine supplementation to IVM media on nuclear maturation rates, cleavage rates, and blastocyst development is controversial. Balasubramanian et al.[16] indicated that the supplementation of cysteamine during bovine IVM had no effect on the cleavage rate but enhanced embryo development. Furthermore, Oyamada et al.[17] concluded that the addition of cysteamine to IVM had no effect on the nuclear maturation of oocytes but improved the fertilization rate and developmental competence of bovine embryos.

Regarding uncertain roles of cysteamine and 13-cis-retinoic acid on bovine *in vitro* embryo production, the present research was planned to test the effects of different concentrations of cysteamine and 13-cis-retinoic acid on nuclear maturation rate, cumulus expansion, cleavage rate, and morula and blastocyst formation in an *in vitro* bovine embryo production.

MATERIAL and METHODS

Ethical Consideration

The study was approved by the Ethical Council of Research of the Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran. Iran (910950746). Verification of this research project and the licenses related to sampling process were approved by the Prof. Mohammad Amin Eslampour and Dr. Mehran Farhoodo Moghadam.

Chemical Reagents

All chemicals used in the present study were purchased from Sigma Chemical Company (Sigma-Aldrich, St. Louis, MO, USA).

Collection of Oocytes

Bovine ovaries were obtained from slaughtered cows (Isfahan, Iran) and placed in a thermoflask containing 0.9% NaCl supplemented with 100 IU/mL penicillin and 100 µg/mL of streptomycin at 30-35°C and transported to the laboratory within 1 h. Ovaries were washed twice in distilled water and once in freshly prepared saline. Cumulus-oocyte complexes (COCs) were aspirated from the antral follicles of 2-8 mm using an 18-gauge needle attached to a 10 mL sterile disposable syringe, pooled in 50 mL conical tubes, and allowed to gravitate for 10 min. COCs were assessed morphologically and only the oocytes with three or more layers of compact and non-atretic cumulus with a homogeneous cytoplasm were selected.

Experimental Design

From June to August 2017, a total of 500 oocytes were divided in five maturation media, including Group (1):

without any supplemented material (control); Group (2): IVM supplemented with 50 µM cysteamine; Group (3): IVM supplemented with 100 µM cysteamine; Group (4): IVM supplemented with 5nM 13-cis-retinoic acid; and Group (5): IVM supplemented with 10 nM 13-cis-retinoic acid. The 13-cis-retinoic was solved in ethanol, aliquoted, and stored at -20°C in darkness. In each group, 50 oocytes were assessed for nuclear maturation and 50 oocytes were placed in the IVP protocol.

In Vitro Maturation (IVM)

All selected COCs were washed in triplicate in washing medium (90% H-Tissue culture medium-199 (TCM 199), 10% Fetal calf serum (FCS), 2.5 mg/mL $NaHCO_3$, 5 µL/mL GlutaMAX, 1 µg/mL penicillin/streptomycin). COCs were incubated in maturation media (using 5-10 COCs per 50-100 µL of maturation medium) in tissue culture dishes under mineral oil and matured for 22-24 h at 38.5°C in an atmosphere of 5% CO_2 in highly humidified air. The base of IVM medium was 25 mM HEPES-buffered TCM 199 supplemented with 2 mM sodium pyruvate, 1 mM l-glutamine, gentamicin (50 µg/mL), 10% steer serum, porcine FSH (1 µg/mL), and 17 beta-estradiol (1 µg/mL).

In Vitro Fertilization (IVF) and In Vitro Culture (IVC)

Sperm separation was performed using a swim-up procedure. The semen from a single bull which had been previously tested for IVF efficiency in the laboratory was used. A total of 1000 µL semen was used for swim up. The frozen-thawed semen was carefully added to button of tube of 1.5 mL Tyrode's albumin lactate pyruvate (TALP) and incubated for 1 h. After that, 500-700 µL of the upper layer of the supernatant containing motile spermatozoa was removed. The spermatozoa were centrifuged at 200 × g for 10 min provided pellet formation and supernatant had been removed then pellet was resuspended with 0.6-0.8 mL of HEPES-TALP medium. Spermatozoon concentration was determined with a haemocytometer, and IVF was accomplished by incubating oocytes and sperm cells together for 18-20 h at 39°C in 5% CO_2 and high humidity. Heparin was used as a capacitor agent in IVF medium. The concentration of spermatozoa was of 2×10^6 cells/mL in 200 Lof medium containing 25 COCs per well.

After 18-20 h, the presumptive zygotes were removed from the IVF medium, cumulus cells were denuded by repeated gentle pipetting, washed several times in a pre-warmed culture medium, and then transferred into IVC droplets made of modified synthetic oviductal fluid (mSOF) added with 1 mM glucose and 3 mg/mL of bovine serum albumin (BSA). The embryo culture was maintained at 39°C, 5% CO_2, 5% O_2, and 90% N_2 under mineral oil and examined under a stereomicroscope after 24 h for fertilization rate. On Day 2-3 of IVC, cleavage rate was evaluated, unfertilized oocytes were removed, and cleavaged embryos were transferred into fresh IVC medium. On Day 5-7, embryos were evaluated for morula and blastocyst formation.

For the morphological evaluation of oocytes, the cumulus cells were first stripped in a medium containing hyaluronidase (0.01% w/v). Then, denuded oocytes were fixed in 60% methanol and stained with 1 mg/mL of Hoechst 33342 (Sigma-Aldrich) to assess their nuclear maturation status. Finally, nuclear maturation rate was assessed by evaluating oocytes that reached the M II stage.

Statistical Analysis

Statistics were subjected to Microsoft office Excel (version 15; Microsoft Corp., Redmond, WA, USA). Statistical analysis was performed by means of the SPSS 24.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences among different groups were analyzed by One Way analysis of variance (One Way ANOVA). Moreover, differences among means were analyzed by Duncan's test. A probability of P value <0.05 was considered to be statistically significant.

RESULTS

Totally, two-hundred and fifty oocytes in different groups were evaluated for nuclear maturation (MII). Oocytes staining method with Hoechst (nuclear maturation) is shown in *Fig. 1*. *Fig. 2* represents the nuclear maturation rate (MII) in bovine oocytes matured in the control and other supplemented IVM groups. The proportion of oocytes that reached the metaphase II stage was similar in control, cysteamine, and RA (5 nm)-treated group. However, statistically significant difference was seen for the proportion of oocytes reached to Metaphase II stage between 10 nM RA and other studied groups (P<0.05).

A total of 250 oocytes were placed in five groups and

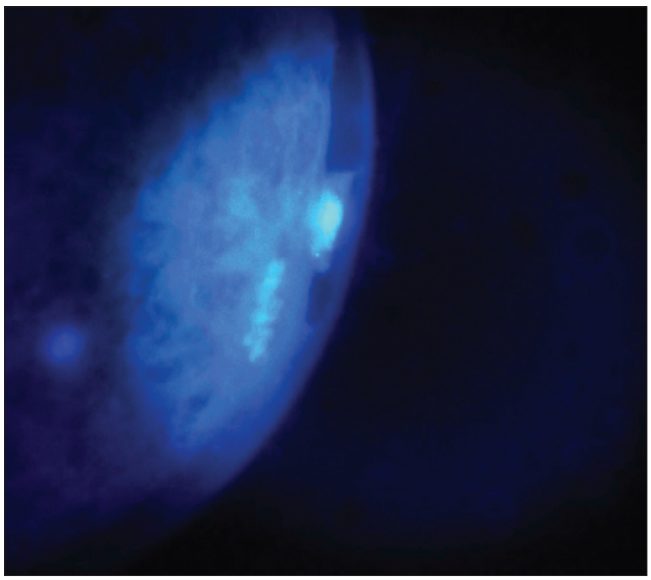

Fig 1. Oocytes appearance stained with Hoechst (nuclear maturation) staining method

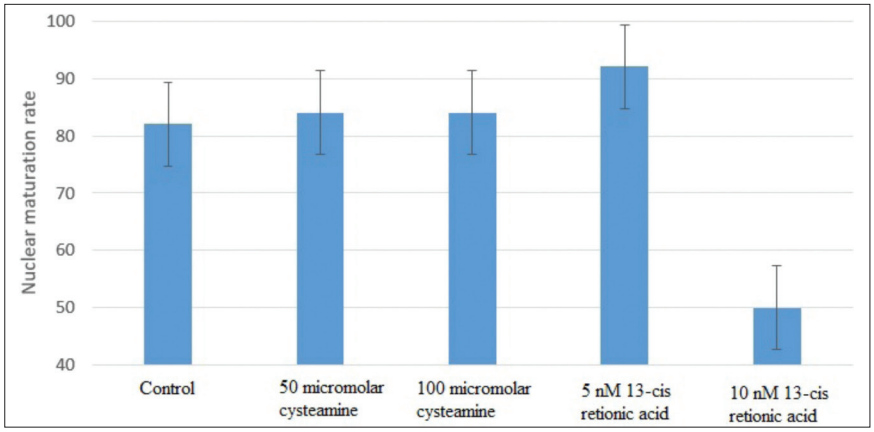

Fig 2. Nuclear maturation rate (MII) in bovine oocytes matured in the control and other supplemented IVM groups. Different superscripts show significant differences (P<0.05)

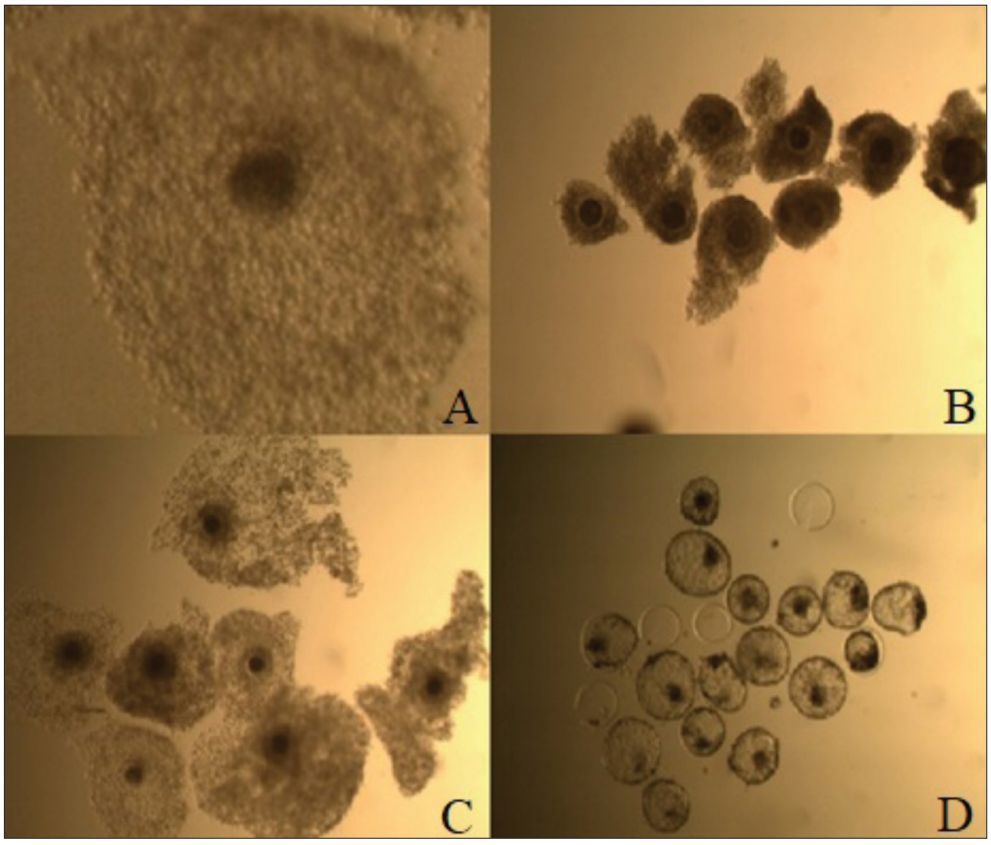

Fig 3. Cumulus expansion of COCs (A), immature oocytes (B), mature oocysts (C) and blastocyst (D)

evaluated for cumulus expansion, cleavage rate, morula, and blastocyst formation. *Fig. 3* represents the cumulus expansion of COCs (A), immature oocytes (B), mature oocysts (C) and blastocyst (D). *Fig. 4* represents the cleavage process 3 days after fertilization.

Table 1 represents the cumulus expansion, cleavage rate, morula and blastocyst formation in bovine oocytes matured in the control and other supplemented IVM groups. A total of 500 oocytes were studied. Each group contained 100 oocytes and a total number of 50 oocytes were studied for nuclear maturation and other 50 oocytes were entered to cumulus expansion, cleavage, murola and blastocyst stages. Cows treated with 13-cis retinoic (5 nm) had the highest numbers of oocytes in the nuclear maturation (92% (46/50). We found that the highest numbers of oocytes in the cumulus expansion, cleavage, murola and blastocyst stages were seen in cows treated with 13-cis retinoic (5 nm) (92%), cysteamine (100 µL) (74%), cysteamine (50 and 100 µL) (48%), cysteamine (100 µL) and 13-cis retinoic (24%) and finally cysteamine (100 µL) (22%), respectively. There were significant differences in cumulus expansion and cleavage rate between cows treated with 13-cis-retinoic acid (10 nm) and other studied groups. After cleavage, the percentage of embryos that reached morula and blastocyst stages were higher in oocytes matured in the medium supplemented with cysteamine and 13-cis-retinoic acid (5 nm) (P<0.05) than those matured in media supplemented with 13-cis-retinoic medium (10 nm).

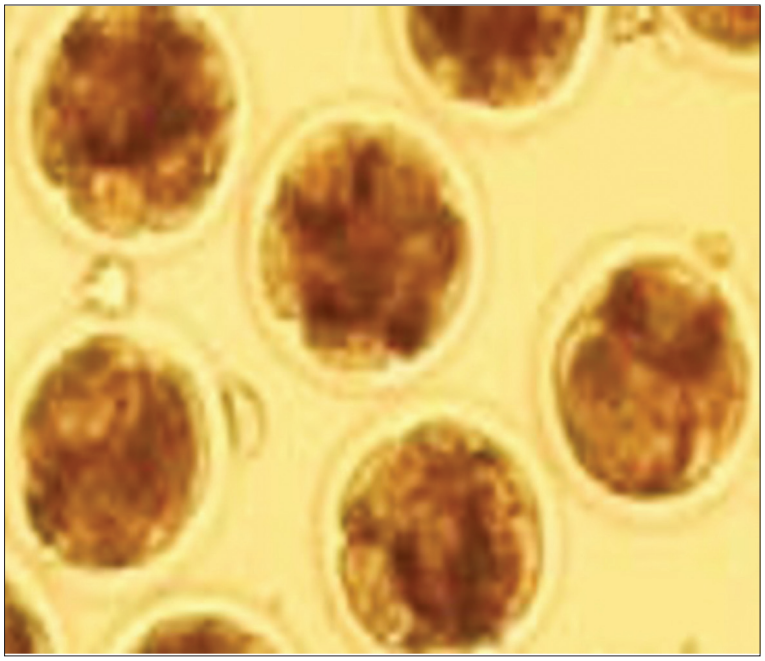

Fig 4. Cleavage process 3 days after fertilization

of bovine oocytes improved in the presence of 9-cis-retinal acid during pre-maturation. Moreover, Livingston et al.[13] demonstrated that the addition of retinol to the IVM medium may improve the embryonic development and blastocyst rate of bovine oocytes. Nasiri et al.[18] reported that the rate of oocytes that reached to the metaphase II stage of maturation significantly increased with 2 and 4 μM t-RA compared to the control and sham groups ($P<0.05$). Additionally, number of fertilized oocytes was significantly higher in 4 μM RA compared to the control ($P<0.05$) which was mainly similar to our findings. Rodríguez et al.[19] reported that retinoid prescription caused significant increase in the blastocyst development in bovine embryonic cells. Akçay et al.[1] reported similar findings about the maturation and fertilization of bovine oocytes under the effects of serum, steroid and gonadotropins. Similar findings about the effective activities of cis-retinal acids on proportion of oocytes that reached the meta-

Table 1. *Cumulus expansion, cleavage rate, morula and blastocyst formation in bovine oocytes matured in the control and other supplemented IVM groups*

Groups	Nuclear Maturation	Stages (%)*			
		Cumulus Expansion	Cleavage	Murola	Blastocyst
Control	41 (82)	35 (70)	23 (46)	8 (16)	6 (12)
Cysteamine (50 μL)	42 (84)	34 (68)	24 (48)	11 (22)	10 (20)
Cysteamine (100 μL)	42 (84)	37 (74)	24 (48)	12 (24)	11 (22)
13-cis retinoic (5 nm)	46 (92)	34 (68)	18 (36)	12 (24)	10 (20)
13-cis retinoic (10 nm)	25 (50)	20 (40)	12 (24)	6 (12)	4 (8)

** Numbers of oocytes in each group is 50*

DISCUSSION

The preservation, maturation, formation, and growth of gametes and embryos in an *in vitro* condition are inferior to those of *in vivo*, indicating that an *in vitro* condition induced various cellular and metabolic stress situations and, therefore, these cells need more attention and energy to adapt to the environment. In the past three decades, *attempts* have been made to achieve appropriate *in vitro* media for decreasing these stresses and increasing capabilities for creation of embryos with acceptable fertility.

The effects of retinol as an antioxidant in IVP on nuclear maturation rates, cleavage rates, and blastocyst development were examined by some researchers [1-4,8,13,18]. This study evaluated different concentration of 9-cis-retinal acid on the efficacy of bovine IVP media is examined in a study and results revealed that the addition of 9-cis-retinal acid into the IVM medium increased bovine blastocyst development and hatching rate [12] which were similar to our findings. Duque et al.[8] showed that the cytoplasmic competence

phase II stage and also *in vitro* embryo production were reported from Spain [20], India [21], Iran [22] and United States [23]. Öztürkler et al.[2] reported that the percentages of oocyte maturation, cleavage, 8-cell and morula stage embryos in L-ergothioneine supplemented group were significantly higher compared with the other groups. They showed that the percentages of oocyte maturation in L-ergothioneine group and L-ascorbic acid group were 2.67 and 1.22 times higher than control group, respectively. Similar reproductive effects have been reported for nitric oxide concentrations, estradiol-17β progesterone in cows by Pancarci et al.[3] and Pancarci et al.[4].

However, the possible mechanisms of the positive effects of RA on oocytes are hypotheses, but RA may promote cytoplasmic maturation of oocytes via its modulatory effects on the gene expression of gonadotropin receptors, midkine, cyclooxygenease-2 and nitric oxide syntheses in cumulus-granulosa cells [8,24]. Additionally, RA induced cortical granules prior to maturation. Also, the cortical granules distribution after RA exposure formed a uniform

monolayer beneath the oolemma with lesser clustering once RA pre-matured oocytes were allowed to mature in the absence of RA [8,24]. Moreover, treatment of cumulus-enclosed oocytes with cis RA during meiotic arrest was observed to improve cortical granule migration, increase subsequent blastocyst development and increase total cell number [8,24]. Gomez et al.[25] suggested that retinoid administration may improve mRNA quality based on the observation that 9-cis RA increased poly-(A) mRNA content in meiotically arrested oocytes. Poly-(A) mRNA content of oocytes treated with 9-cis RA or ethanol vehicle was greater in matured oocytes than in oocytes prematured in the presence of 9-cis RA and then matured. These results suggest a role for RA in the improvement of developmental competence of oocytes. However, the exact timing (and possibly also the concentration) of RA exposure is critical since it alters the normal RA migration and distribution.

The effect of cysteamine supplementation to IVM media on nuclear maturation rates, cleavage rates, and blastocyst development is controversial [16,17]. Balasubramanian et al.[16] evaluated the effect of cysteamine (100 µM) of the IVM medium on chilled and non-chilled embryos. Cysteamine supplementation during IVM had no significant effect on oocyte maturation or fertilization but increased the proportions of oocytes developing to the blastocyst stage ($P<0.05$). Furthermore, Oyamada et al.[17] reported that the addition of 100 µM cysteamine to the IVM medium of bovine oocytes significantly increased ($P<0.05$) the intracellular GSH concentration in the oocytes, improving capacity of fertilization and development competence following vitrification, but had no positive effect on nuclear maturation. Dematos et al.[26] were the first that examine the effects of 100 µM cysteamine in IVM on GSH synthesis, subsequent development of embryos and the ability of freezing of bovine embryos. They showed that the addition of cysteamine in IVM increases the GSH of *in vitro*-matured oocytes to protect the embryo until the blastocyst stage, thus increasing the efficiency of *in vitro* blastocyst production from immature oocytes [26]. The effect of cysteamine added during the IVM (0, 50, 100, and 200 µM) of sheep oocytes on GSH synthesis and embryo development was examined by Zullo et al.[3] which represented similar findings with those of cow. Dematos et al.[27] evaluated the effect of cysteamine in maturation and culture medium on the developmental rate and embryo quality of bovine oocytes. They showed that the percentage of embryos developed to the blastocyst stage was significantly higher ($P<0.05$) when 100 µM of cysteamine was added during IVM, and this was further improved when 100 and 50 µM of cysteamine were present during IVM and IVC, respectively ($P<0.05$). Cysteamine supplementation during IVM improves embryo development and better-quality embryos can be obtained if cysteamine is also added during the first stages of embryo development [27]. Lojkic et al.[28] cultured bovine embryos in different concentrations (0, 50, 100, and 200 µM) of cysteamine. The results of their study revealed that the supplementation of IVC media with 100 µM cysteamine increased the blastocyst yield ($P<0.05$) without affecting the hatching rate. Furthermore, the addition of 100 µM cysteamine to an IVC media improved embryo quality, which may lead to the improvement of the IVC system for bovine embryos [28]. Significant effects of cysteamine on an *in vitro* maturation and embryo development were also reported from Japan [29], Turkey [30], Italy [31], Belgium [15] and Netherlands [32].

The results of our study showed that cysteamine supplementation and a low concentration of 13-cis-retinoic acid in IVM had no positive effect on nuclear maturation in comparison to the control group but improved the embryo development of bovine oocyte. In the present study, although nuclear maturation rate and the first step of embryo development were similar among cysteamine, low concentration of 13-cis-retinoic acid, and control groups, the cumulative effect of supplementation was detected in the blastulation formation of embryos. These results suggest that, when cysteamine stimulates GSH synthesis during IVM, increased sources of GSH help embryos develop faster and reach the blastocysts stage. In addition, 13-cis-retinoic acid in the first step of IVP may improve the maturation, development, and differentiation of embryonic cells [33,34].

In conclusion, we have demonstrated that enriching the IVM medium with cysteamine and a low concentration of 13-cis-retinoic acid improves IVEP efficiency in cattle. It is likely that the presence of cysteamine and a low concentration of 13-cis-retinoic acid should promote early embryo development by facilitating the completion of oocyte cytoplasmic maturation, ensuring a normal pro-nuclear development and hence improving cleavage rate and the overall embryo yield. In future, these results may be of great help for obtaining a culture medium most suitable for oocyte nuclear and cytoplasmic development.

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Research Article

Study of *FecX*^{G} Polymorphism in Beetal Goat and Its Phylogenetic Relationship

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Abstract

The objective of the current study was to detect the incidence of *FecX*^{G} mutation in 141bp fragment of exon 2 of BMP-15 gene in Beetal goat and to determine its phylogenetic relationship with other goat breeds. In this study 60 Beetal goats were randomly selected and divided equally into two groups on the basis of their body weights. The PCR-RFLP and DNA sequencing techniques were employed to explore polymorphism in exon 2 of BMP-15 gene. Upon digestion with Hinf1, the purified product showed heterozygous carriers with two bands (111bp, and 54bp) in all animals. The analysis of polymorphism for *FecX*^{G} in Beetal goat indicates that the genetic factor responsible for difference in growth rates is not related to the reported mutated alleles of BMP-15 gene. Therefore, it is suggested that polymorphisms in the other regions of BMP-15 gene should be explored which might be responsible for the difference of growth rates in Beetal goat. Phylogenetic tree of mRNA of BMP-15 gene was constructed and the results revealed Teddy as outgroup. Moreover, among all the breeds used in this analysis Teddy is also phenotypically different from other due to its smaller size and greater prolificacy. However, all the remaining breeds were present in a single large clade, with Black Bengal and Markhoz goat in a further single sub-clade and showing maximum divergence from the common ancestor. The Beetal was next to Black Bengal and Markhoz and along with their common ancestor they were present in a single clade.

Keywords: *FecX*^{G} *polymorphism, BMP-15 gene, Beetal goat, PCR-RFLP, Phylogenetic relationship*

Beetal Keçisinde *FecX*^{G} Polimorfizmi ve Filogenetik İlişkisinin İncelenmesi

Öz

Bu çalışmanın amacı Beetal keçisinde BMP-15 geninin ekzon 2'sinde 141 bp fragmanında *FecX*^{G} mutasyonunun insidansını ve diğer keçilerle olan filogenetik ilişkisini belirlemektir. Çalışmada 60 Beetal keçisi rastgele seçildi ve vücut ağırlıklarına göre eşit iki gruba ayrıldı. BMP-15 geninin ekzon 2'sindeki polimorfizmi araştırmak amacıyla PCR-RFLP ve DNA sekanslama teknikleri kullanıldı. Tüm hayvanlarda, Hinf1 ile kesilme sonrasında elde edilen saf ürün iki bantlı (111bp ve 54bp) heterozigot taşıyıcılar olarak gözlemlendi. Beetal keçilerinde *FecX*^{G} polimorfizm analizi, büyüme oranlarındaki farkın oluşmasından sorumlu olan genetik faktörlerin BMP-15 geninin mutasyonlu alleli ile ilgili olmadığını gösterdi. Bu nedenle, BMP-15 geninin diğer bölgelerindeki polimorfizmin, Beetal keçilerinde büyüme oranlarındaki farklılığının oluşmasından sorumlu olabileceği düşüncesi ile araştırılması önerilmiştir. BMP-15 geninin mRNA'sının filogenetik ağacı oluşturuldu ve elde edilen sonuçlar Teddy'nin dışgrup olduğunu ortaya koymuştur. Ayrıca, bu çalışmada kullanılan tüm ırklar arasında, Teddy daha küçük boyutu ve daha fazla üretkenliği nedeniyle diğer ırklardan fenotipik olarak farklıydı. Ancak, geri kalan tüm ırklar tek bir büyük kuşakta bulunurken Siyah Bengal ve Markhoz keçisi başka bir alt kuşakta yer aldı ve ortak atadan azami sapma gösterdiler. Beetal, Siyah Bengal ve Markhozdan sonra ortak ataları ile birlikte tek kuşakta yer almaktadır.

Anahtar sözcükler: *FecX*^{G} *poliformizm, BMP-15 geni, Beetal keçisi, PCR-RFLP, Filogenetik akrabalık*

INTRODUCTION

Goats are vital indigenous assets of Pakistan and have an ample share in the annual production of milk and meat besides providing enough income to rural families [1]. Beetal is one of the most famous goat breed of Punjab province of Pakistan; moreover, it is also present in some parts of Indian (East) Punjab. It is very popular due to its greater body size and higher potential for meat and milk yield especially in rural areas of Punjab province [2]. However, information regarding the genetic potential of Beetal for meat production is scarce which merit some well-

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planned genetic and genomic studies. There is substantial need to explore the candidate genes for growth traits in this goat breed. Bone morphogenetic protein-15 (BMP-15), Growth hormone (GH), and Insulin like growth factor-1 (IGF-1) genes are reported as candidate genes for growth and skeletal development in goat [3-5].

The BMP family of proteins is the largest subgroup of the transforming growth factor-ß (TGF-ß) superfamily [3] and act as a major molecule in tissue and organ development [5,6]. It plays a vital role in the functioning of connective tissue, brain, kidney, and muscle [7]. The BMP-15 is the one of the crucial members of BMP family which is famous for affecting reproductive [4,8,9], and growth traits [10,11] in small ruminants. It is reported to enhance proliferation and differentiation of cells by promoting mitosis and controlling gene expression [12,13]. Some mutations in the BMP-15 gene have been reported to be associated with the ovulation rate in different sheep breeds, especially the *FecX^{B}* mutation in Booroola breed and *FecX^{G}* in Inverdale breed [7,14-16]. The present study is based on the hypothesis that *FecX^{G}* polymorphism in BMP-15 gene might be associated with difference in growth rate viz body weight and body measurements in Beetal goat. Therefore, primarily the study was aimed to identify polymorphism at the *FecX^{G}* loci in BMP-15 gene in the Beetal goat using PCR-Restriction Fragment Length Polymorphism (RFLP) method. Moreover, the secondary objective was the determination of relationship of this precious genetic resource of Pakistan with other goat breeds of the region (South Asia) through phylogenetic analysis.

MATERIAL and METHODS

Ethics Statement

Ethical permission (number DR/151) was obtained from the Ethical review committee for animal research of University of Veterinary and Animal Sciences, Lahore, Pakistan, before start of the study.

Experimental Population

In total, 60 healthy unrelated female Beetal goats of about one year of age were randomly selected from Small Ruminant Training and Research Centre, University of Veterinary and Animal Sciences, Lahore, Pakistan. Animals were equally divided into two groups on the basis of difference in their body weight and body measurements i.e. 30 in higher body weight group and 30 in lower weight group. The body weight of higher weight category animal ranged from 55-60 kg while low weight category animals were having weight of 40-45 kg. All animal were stall fed and additionally one kg concentrate was also offered to each animal on daily basis. The animals were reared in the sub-tropical climatic condition of central Punjab of Pakistan. Phenotypic data for different morphometric traits including heart girth, body height, body length, and body weight were recorded.

DNA Extraction

Blood samples with a volume of 5 mL were taken from jugular vein of each goat by using sterile 5ml syringe having a 22 guage needle fixed to it. The blood sample was immediately poured, after removing the needle, into 50 mL falcon tubes containing 160 µL of 0.5 M EDTA. 100 µL of each blood sample were transferred into Eppendorf tubes. 1000 µL TEB buffer (Tris HCL 10 mM, EDTA 2 mM) was added and samples were vortexed for 5 min and then centrifuged at 1000 rpm for 5 min. A pellet was made at the bottom of eppendorf. After discarding the remaining solution, 20 µL proteinase K enzyme was added along with 60 µL SDS (10%) solution and 100 µL of TNE buffer (Tris HCL 10 mM, NaCL 400 mM, EDTA 2 mM). Samples were vortexed for 5 min and placed in water bath at 58°C for overnight digestion. In fully digested samples, PCL (Phenol 25: Choloroform 24: Iso-amyle 1) was added. Three layers were formed after centrifugation at 1000 rpm for 10 min. Samples were washed with 70% ethanol and again centrifuged at 1000 rpm for 10 min. A solid pellet of DNA was formed and dissolved in 100 µL double distil water. DNA was stored at 4°C or -20°C. The concentration of DNA was measure in Nanodrop spectrophotometer (ThermoFisher) instrument.

PCR Amplification

Polymerase chain reaction (PCR) was carried out in a final reaction volume of 20 µL in C1000 Touch™ Thermal Cycler (BIO-RAD, USA). The primers for the amplification of exon 2 of BMP-15 are given in *Table 1*. The reaction mixture consisted of 150 µM dNTPs, 1.2 mM $MgCl_2$, 2.0 mM 10 × buffer, 30 ng each forward and reverse primer and 1 Unit of Taq DNA polymerase. The PCR reaction cycle protocol was 5 min at 95°C; 30 cycles at 94°C for 45 s, annealing at 60°C for 30 s, extension at 72°C for 45 s. The PCR products were visualized in Gel Doc™ EZ imager (BIO-RAD, USA) following electrophoresis through 2% agarose gel.

Table 1. *Primers and PCR amplification parameters used to amplify BMP-15 gene*

Gene	Sequence	[1]Tm (°C)	Size of Amplicon (bp)	Reference
BMP-15E2F	CACTGTCTTCTTGTTACTGTATTTCAATGAGAC	68.17	141	[17]
BMP-15E2R	GATGCAATACTGCCTGCTTG	62.41		

E2: Exon 2; F: Forward Primer; R: Reverse Primer; [1]Tm Melting temperature (°C)

PCR-RFLP Analysis

The digestion of PCR products was done with restriction enzyme HinfI at 37°C for 12 h. After digestion, the samples were stored at 4°C. The digested product was visualized in Gel Doc™ EZ imager (BIO-RAD, USA) following electrophoresis through 2% agarose gel.

Sequencing

The amplified region was sequenced using an ABI 3130 Automated DNA Sequencer (Applied Biosystems, USA). Sequence data were aligned using CLUSTALW algorithm in MEGA 7 software [18].

Phylogenetic Analysis

The mRNA (1184bp) sequences of BMP15 gene of seven different goat breeds including Beetal goat, Black Bengal goat, Markhoz goat, Tibetan goat, Lezhi black goat, Ganjam goat, and Teddy goat were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/nucleotide/) and used to construct phylogenic tree in Mega7 software [18]. The accession numbers of these goat breeds are given in *Fig. 5*. The phylogenetic tree was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [19]. The tree was made by using Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 7 nucleotide sequences.

RESULTS

A 141bp amplified DNA fragment was subjected to 2% agarose gel electrophoresis. The results showed that amplified fragment size was consistent with the expected size as determined from the gene sequence information *(Fig. 1)*.

The amplicons showed a 141bp product in the present study *(Fig. 1)*. The RFLP analysis revealed two bands. One band was appeared at 111bp and other band was at 54bp. All the tested Beetal goats were found carriers of *FecX*^{G} mutation as the restriction site for Hinf1 was observed in all the samples *(Fig. 2)*. The restriction site of Hinf1 enzyme is showed in *Fig. 3* from the chromatogram produced for Beetal goat in the current study and same mutation is shown in the sequences of Beetal goat in *Fig. 4*.

The 1184bp long mRNA of BMP15 gene of some Chinese, Indian, Iranian, and Pakistani goat breeds including Beetal, Black Bengal, Markhoz, Tibetan, Lezhi black, Ganjam, and Teddy goat were retrieved from NCBI to construct phylogenic tree. The phylogenetic tree showed Teddy goat as outgroup and its sequence was different from other goat breeds when clustered at 1*100 nucleotide substitution level *(Fig. 5)*. However, all the remaining breeds were present in a single large clade, with Black Bengal and Markhoz goat in a further single sub-clade. The Beetal was next to Black Bengal and Markhoz and along with these two breeds and the common ancestor they were present in a single clade.

DISCUSSION

The present study was carried out to find the polymorphism of *FecX*^{G} of BMP-15 gene in Beetal goat by PCR-RFLP method and also by sequencing. From the study, it was found that all the genotype of Beetal goat showed two bands of 111bp and 54bp after digestion with Hinf1 enzyme. It's suggested that although digestion was done at the said locus indicating the presence of mutation but this mutation was observed in the all the tested animals. Out of the total 60 animals, tested in this study, half of them

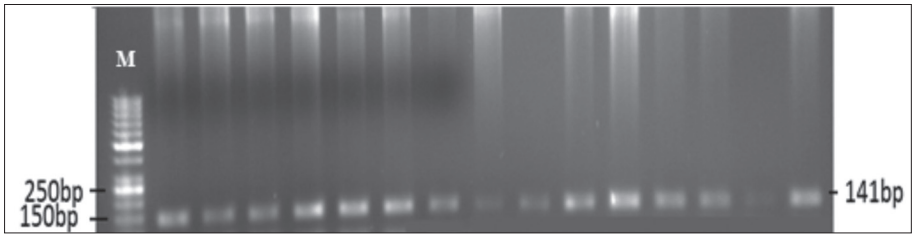
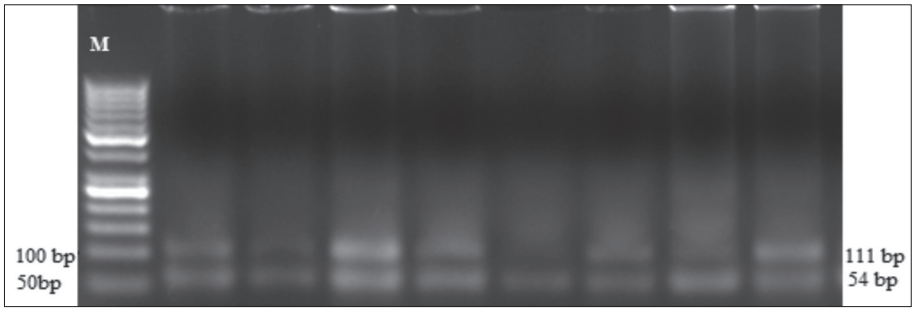


Fig 1. Amplified products of exon2 of BMP-15 gene by PCR. In Lane1 the letter 'M' represent the Ladder

Fig 2. Hinf1-RFLP patterns of BMP-15 gene. This pattern was observed in all tested animal. In Lane1 the letter 'M' represent the Ladder (50bp)



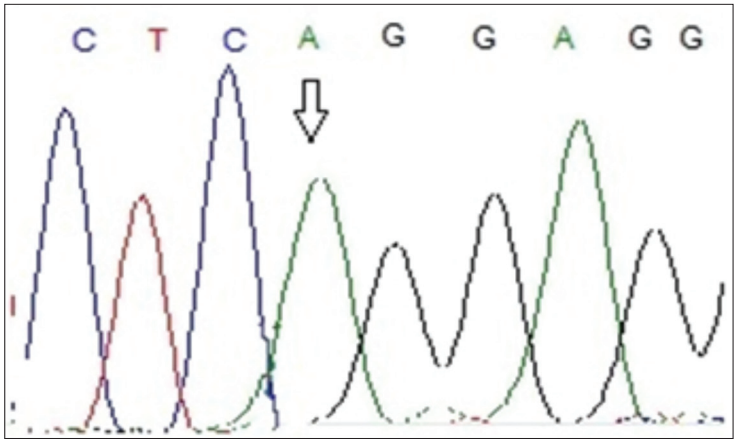


Fig 3. Showing the *FecX*^{G} mutation in the Beetal goat

no evidence of mutation in *FecX*^{G} in Iranian goats by using PCR-RFLP technique and all animals were monomorphic. Similarly, He, Chu [22] also observed absence of mutation in BMP-15 gene in six breeds of Chinese goats. However, contrarily mutations at five different points in exon 2 of BMP-15 gene were found associated with prolificacy and growth in some breeds of sheep [23]. This might be due to difference in the biological effect of the mutations among different species and it had also been suggested recently that difference in BMP-15 mutation may be associated with the differences in ovulation rate and growth rate among various species [24]. In current study, monomorphic nature of *FecX*^{G} suggests that current mutation in BMP-15 gene cannot be regarded as the major polymorphism

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B_H              TTTGTCTCGT CGAATGCAGT GAGGGTCTCA GGAGGGTAAC TCTTTCAGGC
CTTTAGGGAG
B_H              TTTGTCTCGT CGG-TGCAGT GAGGGTCTCA GGAGGGTAAC TCTTTCAGGC
CTTTAGGGAG
B_H              CTGGTATCAT CG-GTGCAGT GAGGGTCTCA GGAGGGTAAC TCTTTCAGGC
CTTTAGGGAG
B_L              CTGCAATC-T CG-GTGCAGT GAGGGTCTCA GGAGGGTAAC TCTTTCAGGC
CTTTAGGGAG
B_L              GTTCAGTACG CGTGCGAACT ACACTACTCA GGAGGGTGCC TCTTTCAGGG
CTTTAGGGAG
B-L              GTTCAGTACG CGTGCGAACT ACACTACTCA GGAGGGTGCC TCTTTCAGGG
CTTTAGGGAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
....|....|
B_H              AGGTTTGGTT TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTAACAAG
AAGACAGTGA
B_H              AGGTTTGGTC TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTAACAAG
AAGACAGTGA
B_H              AGGTTTGGTC TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTAACAAG
AAGACAGTGA
B_L              AGGTTTGGTC TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTAACAAG
AAGACAGTGA
B_L              AGGTTTGGTC TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTACCTCG
AAGACAGTGA
B_L              AGGTTTGGTC TTCTGAACAC TCTGAGTCTC ATTG-AAATA CAGTAACAAG
AAGACAGTGA
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Fig 4. Sequence of Beetal goat showing the mutation of *FecX*^{G} in all the tested Beetal goat (B). B_H are the high weight Beetal goat and B_L are the low weight Beetal goat

were of low body weight and other half was having higher body weight. The results showed that the variation in body weight of animals was not due to *FecX*^{G} mutation because *FecX*^{G} was observed in both heavy and low body weight animals. It is very likely that some other variants in BMP-15 or in some other candidate genes might be involved in affecting/controlling body weight in Beetal goat. In agreement with the findings of present study the Polley [20] found that all known point mutations in BMP-15 and GDF9 genes were monomorphic in the Black Bengal goat. Likewise, Deldar-Tajangookeh, Shahneh [21] observed associated with growth traits of goat. Further investigation should be directed at other loci of BMP-15 gene and also in some other genes by using larger sample size.

Phylogenetic tree of mRNA (1184bp long) of BMP-15 gene indicated the Teddy goat as outgroup from the other breeds *(Fig. 5)* of China, India, Iran, and Pakistan. The difference of the Teddy with other breeds was based on the seven nucleotides sequences, out of total 1184bp used to construct the phylogenetic tree. However, all the remaining six breeds including Black Bengal, Markhoz,

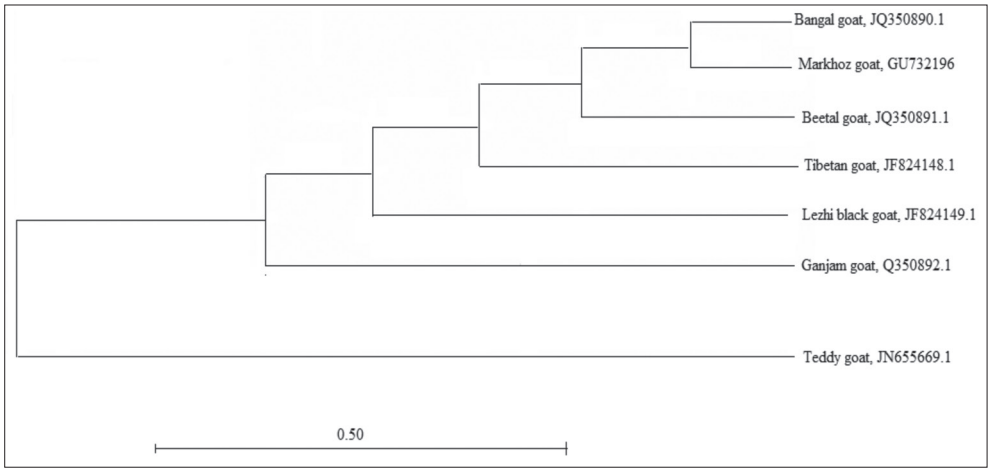

Fig 5. Neighbour-joining phylogenetic tree of mRNA of BMP-15 gene of different goat breeds

Beetal, Lezhi Black, and Ganjam goat breeds were grouped into a single mega clade. However, Black Bengal and Markhoz goat were clustered together in a single sub-clade and both of them showed the maximum divergence from the common ancestor followed by Beetal goat. The phylogenetic tree in this study was made on the basis of mRNA sequence of exonic regions of BMP15 gene which is a famous candidate gene for fecundity in small ruminants; therefore, it is likely that the difference shown among the breeds by the phylogenetic tree is the difference in the sequence of exons of BMP15 genes, in narrow sense, and in their prolificacy in broader sense. In agreement with the litter size in the six breeds present in a single larger clade is about 1.3 to 1.8 [25-27] whereas Teddy is the most prolific breed of Indian sub-continent and famous for producing triplet and quadruplet in addition to twins [28].

However, the Ganjam goat was closer to Teddy goat in the tree compared with other goat breeds. The reason for this might be that both share some similarities in their phenotypic appearance like coat color, medium size body height, shape of head and face, and bear etc. Moreover, in addition to these two breeds the other breeds in this study are mainly black coat color. Additionally both of them are meat-type breeds only whereas the remaining breeds are mainly used for dairy and hair/fiber (mohair) production. It is highly recommended that phylogenetic trees of these goat breeds should be developed in future studies by using larger genetic data sets (whole genome sequence) will better explain their relatedness and relationship with each other.

Based upon present finding, it may be concluded that polymorphism in *FecX*^{G} i.e genetic factor responsible for difference in growth rates is not related to the reported mutated alleles of BMP-15 gene in Beetal goat. Therefore, polymorphisms in the other regions of BMP-15 gene should be explored which might be responsible for the difference of growth rates in Beetal goat. Phylogenetic tree of mRNA of BMP-15 gene of different goat breeds revealed that all breeds goat were present in a single large clade, with Black Bengal and Markhoz goat in a further single sub-clade and showing maximum divergence from the common ancestor. The Beetal was next to Black Bengal and Markhoz and along with their common ancestor they were present in a single clade. Teddy goat act as outgroup and phenotypically different from other due to its smaller size and greater prolificacy.

Author Contribution

AB and IZ designed the study. MI and MS did do the Lab work, whereas AB and IZ did run all the analyses. Finally AB, and IZ drafted the manuscript and all authors read and approved the final draft.

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Research Article

The Investigation of the Effect of Ozone Therapy on Gout in Experimental Rat Models

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Abstract

The effect of ozone treatment in experimental rat gout arthritis was investigated. Twenty adult albino male rats were divided into 4 groups: Group 1 (Control) was followed without any medication, Group 2 (Gout control) was injected with a single dose of 0.2 mL (10 mg) monosodium urate (MSU) intraarticulary to 1st metatarsophalangeal joint to create experimental Gouty arthritis. Group 3 (Gout + Ozone) was injected intraarticulary to 1st metatarsophalangeal joint with a single dose of 0.2 mL (10 mg) MSU to generate experimental gout, followed by ozone therapy to the rats a single dose of 1 mL (10 µg/mL) intraperitoneally daily for 3 weeks. The Group 4 (Ozone control) ozone therapy was given a single dose of 1 mL (10 µg/mL) intraperitoneally daily for 3 weeks without gout formation. The results were recorded and compared statistically between the groups. In conclusion, the low level of inflammation in the third group (Gout + Ozone) was considered statistically significant as compared to the second group. With Ozone treatment the gout joint has been shown to have reduced acute inflammation, it has not been affected by the chronic proliferation process.

Keywords: *Gout, Ozone, Immunomodulators, Anti-inflammatory agents*

Deneysel Olarak Gut Oluşturulmuş Ratlarda Ozon Terapinin Etkisi

Öz

Bu çalışmada deneysel olarak Gut artriti oluşturulmuş rat ekleminde ozon tedavisinin etkisi araştırıldı. Yirmi adet albino cinsi erişkin erkek rat 4 gruba bölünerek: 1. Gruba (Kontrol) herhangi bir ilaç verilmeden takip edildi, 2. Gruba (Gut kontrol) deneysel Gut artriti oluşturulmak üzere tek doz 0.2 mL (10 mg) monosodyum ürat (MSU) 1. metatarsofalengeal ekleme enjekte edildi, 3. Gruba (Gut + Ozon) deneysel Gut oluşturulmak üzere tek doz 0.2 mL (10 mg) monosodyum ürat (MSU) 1. metatarsofalengeal ekleme intraartiküler olarak enjekte edildi ve arkasından ratlara 3 hafta süreyle günde tek doz 1 mL (10 µg/mL) intraperitoneal ozon terapi verildi, 4. Gruba ise (Ozon kontrol) Gut oluşturulmaksızın 3 hafta günde tek doz 1 mL (10 µg/mL) intraperitoneal ozon terapi uygulandı. Sonuçlar patolojik olarak kayıt altına alındı ve gruplar arası istatistik olarak karşılaştırıldı. Sonuç olarak 3. Gruptaki (Gut + Ozon) inflamasyon düzeyindeki düşüklük 2. Gruba göre istatistikse olarak anlamlı kabul edildi. Ozon tedavisinin Gut oluşturulan eklemde akut inflamasyonu azalttığı gösterilse de kronik olan proliferasyon sürecine etkisi görülmedi.

Anahtar sözcükler: *Gut, Ozon, Antiinflammatuar ajan, İmmünomodülatörler*

INTRODUCTION

Gout disease is the most common cause of inflammatory arthritis in adults at the time of the US registry [1]. Gout disease is a chronic disorder associated with self- limiting acute gout attacks (gout flare), caused by accumulation

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of monosodium urate (MSU) deposits in joints and surrounding soft tissue and bursa [1]. Acute attacks of gout disease are one of the most important causes of adverse health-related quality of life [2,3]. It has also been reported that 12 to 15% of individuals aged 40-60 years worldwide have hyperuricemia and 3% are affected with gout arthritis [4]. As the molecular basis of gout disease, oxidative stress has been noted in studies that have shown promise in the pathogenesis of gout.

In the extracellular compartment, the uric acid molecules are present in 98% of the MSU at dense sodium concentration and physiological pH. If the urate concentration exceeds 6.8 mg/dL, the urate molecules will crystallize and precipitate and play a role in the initiation of the inflammatory reaction. In addition to exceeding the value of the urate density, conversion to MSU crystal formation is also implicated in a number of factors such as ambient temperature, hydrogen ion formation, concentration of cations, and the viscosity of the joint fluid [5,6]. This suggests that Gout arthritis is more common in peripheral joints, such as the first metatarsophalangeal joint [7]. MSU crystals are detected and presented to phagocytes by receptors such as Toll-Like Receptor (TLR) 1 and 2 and surface antigens such as CD14 [8-10]. In this context, anti-inflammatory treatments have found wide use in the treatment of gout arthritis, especially acute gout attack (gout flare) and chronic gout prophylaxis. These include interleukin 1 antagonists (anakinra, canakinumab, rilonacept), uricosuric drugs (probenecid, benzbromarone), xanthine oxidase inhibitors (allopurinol, febuxostat), cyclooxygenase 2 (COX2) inhibitors, colchicine and steroids that inhibit the migration of inflammatory cells [11].

Medical ozone treatment consists of 95% Oxygen (O_2) and 5% Ozone (O_3) mixture. The present rates are therapeutic ratios. There is toxic effects of ozone (O_3) ratio above 5% in ozone treatment [12]. On the other hand, ozone therapy has shown that ozone can be safely used at doses between 10 and 80 µg/mL and immunomodulators, anti-inflammatory, bactericidal, antifungal and analgesic effects are at these doses [13]. Medical ozone therapy affects anti-inflammatory activity in two phases. Studies have shown that in the first phase, the levels of proinflammatory mediators such as prostaglandin E_2, phospholipase A_2, cyclooxygenase I-II, callicrein and bradykinin are reduced. In addition, ozone treatment reduces levels of molecules responsible for joint destruction such as metalloproteinase, collagenase, and gelatinase. In the second phase, ozone therapy increases the levels of antioxidant enzymes, molecules and cytokines. Examples include oxidative shock proteins (hemo-oxygenase-1), Interleukin 4 and Interleukin 10, Transforming Growth Factor-β (TGF-β). It has also been shown that ozone therapy induced new vessel formation (neoangiogenesis), increases nitric oxide (NO) endorphin, adrenocorticotropic hormone (ACTH) and cortisol levels [14].

In the face of this wide spectrum of activities, today's ozone therapy is available in many different system diseases such as osteomyelitis, chronic obstructive pulmonary disease, hepatitis, cystitis, rheumatoid arthritis, osteoarthritis, back pain, multiple sclerosis and coronary artery disease [15]. The aim of our study was to examine the effects of nontoxic ozone administration on the gout-induced joint model in therapeutic doses and to create an alternative to current in the future.

MATERIAL and METHODS

Following approval from the Kafkas University Local Experimental Animals Ethics Committee (KAÜ-HADYEK/2017-012) twenty adult albino male rats were used in the study. Rats were randomly divided into 4 groups and group 1 (Control, n=5) was followed up without any drug. 0.2 mL (10 mg) MSU was injected intra-articularly to 1st metatarsophalangeal joint through the right footpad to create experimental gout in the 2nd group (Control group, n=5) and was followed without additional treatment. A single dose of 0.2 mL (10 mg) MSU was injected intraarticularly to 1st metatarsophalangeal joint through the right footpad to create an experimental gout in group 3 (Gout + Ozone, n=5) followed by intraperitoneal 1 mL (10 µg/mL) ozone therapy daily in rats. Group 4 (ozone control, n=5) Ozone therapy was administered intraperitoneally 1 mL (10 µg/mL) daily without gout. Group 3 and group 4 administered once daily dose of 1 mL (10 µg/mL) intraperitoneally for 3 weeks. After 3 weeks of treatment, euthanasia was performed according to the condition of the rats and then the right hind paws were cut, and histopathology examined.

RESULTS

Confirmed radiological imaging of rats with gout *(Fig. 1)*. The pathological results of the groups were compared statistically (P=0.003) *(Table 1)*. In addition, the rate of suppression of inflammation in the ozone group was significantly higher than the control group (P=0.005).On the other hand, no statistically significant inflammation was observed in the 4th group which was given only ozone according to the control group (P=0.03). Control group (1st group) and ozone control group (4th group) and gout control group (2nd group) and ozone control group (3th group) were compared between each other in terms of epithelization *(Fig. 2A,B,C,D)*. There was no statistically significant difference between the groups.

DISCUSSION

Gout is a common disease that causes severe pain and loss of work power during an acute exacerbation. The etiopathogenesis has been well researched with a wide range of treatments ranging from monoclonal antibody therapy to anti-inflammatory therapy in the treatment

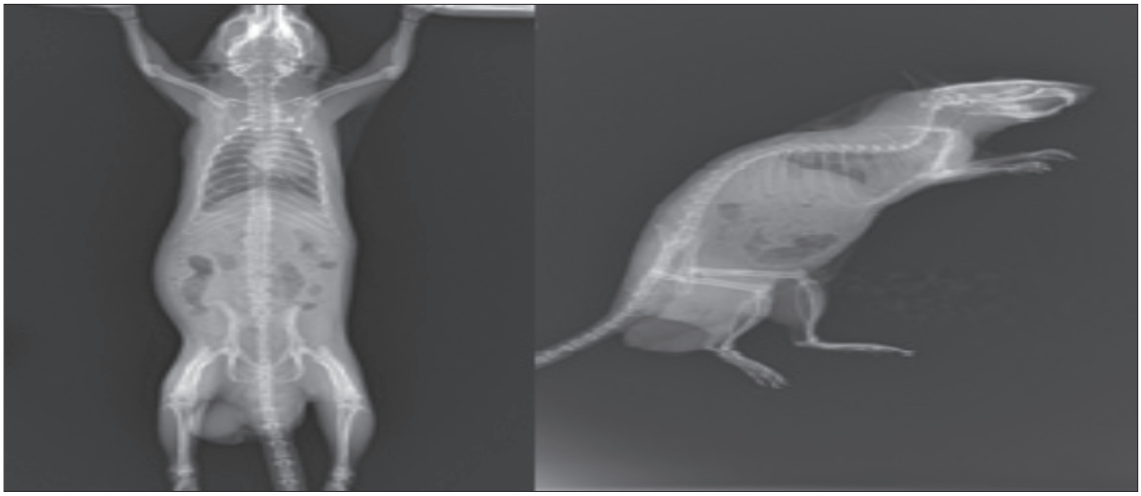

Fig 1. Radiological image of gut-generated rats

Table 1. *Histopathological evaluation of the groups*

Rat Number	Group Name	Inflammation	Proliferation	Reactive Epithelium
1	Control	No	No	No
2	Control	No	No	No
3	Control	No	No	No
4	Control	No	No	No
5	Control	No	No	No
6	Gout control	Distinct	Yes	Yes
7	Gout control	Distinct	Yes	Yes
8	Gout control	Distinct	Yes	Yes
9	Gout control	Distinct	Yes	Yes
10	Gout control	Distinct	Yes	Yes
11	Gout+Ozone	Moderate	Yes	Yes
12	Gout+Ozone	Moderate	Yes	Yes
13	Gout+Ozone	Moderate	Yes	No
14	Gout+Ozone	Slight	Yes	No
15	Gout+Ozone	Slight	Yes	Yes
16	Ozone control	No	No	Yes
17	Ozone control	No	No	No
18	Ozone control	No	No	No
19	Ozone control	No	No	No
20	Ozone control	Slight	No	No

of the disease. These treatments are given in the form of step-by-step treatment and are constantly updated as both side effects and treatment costs are considered [1]. Glucocorticoids and non-steroidal anti-inflammatory drugs are the leading cost-effective treatments for gout. Although its efficacy in long-term therapy has been proven, alternative forms of treatment are still needed in terms of potential side effects (Chronic Renal Failure, Diabetes Mellitus, Cushing Syndrome). In addition, xanthine oxidase inhibitors (allopurinol), selective xanthine oxidase inhibitor (febuxostat), drugs that increase uric acid excretion (losartan, fenofibrate), turgous drugs (rasburicase) that provide resorption of tofus are tested among current medical treatments [16]. In the treatment of gout, two main titles are being considered today; to reduce the uric acid increase and to minimize the destructive effect of increased uric acid.

Ozone treatments instead of conventional therapy has been experimented on for many diseases because of the better understanding of the mechanism of action and lowering of treatment costs. It has been shown that ozone

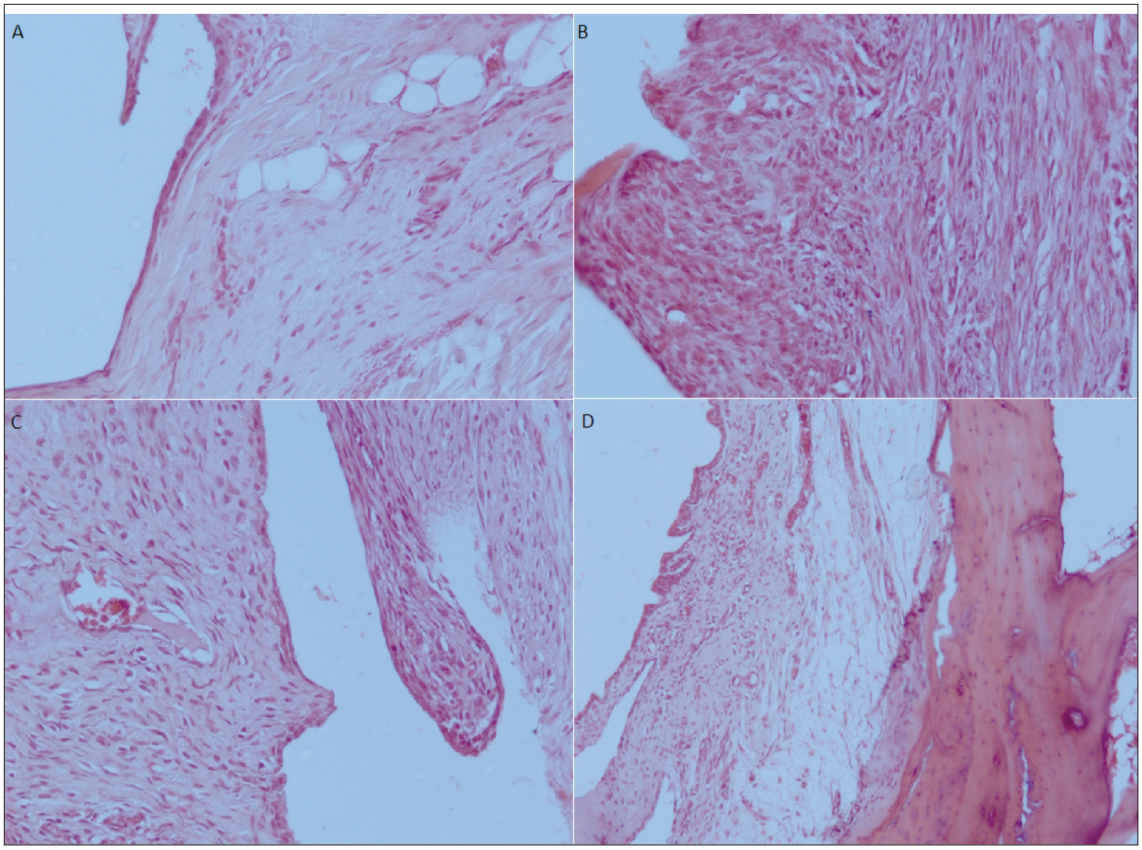

Fig. 2. Microscopic view of histopathological sections. A. Control group showing normal synovial and perisynovial tissue (H&E, 200x); B. Gouty arthritis group with reactive epithelial changes and active chronic inflammatory cells (H&E, 200x); C. Gouty arthritis + Ozone treatment group with mild active chronic inflammatory cells and no reactive changes (H&E, 200x); D. Ozone control group showing minimal reactive epithelial change an no inflammation (H&E, 100x)

treatment reduces serum uric acid levels in patients with osteoarthritis and also reduces VAS (visual analogue scale) and WOMAC (Western Ontario and Mac master index) scores and reduces joint pain [17,18]. Li and Ni [19] evaluated gut arthritis-associated pain-associated VAS scores in their study of ozone at a dose of 20 µg/mL on subjects with gout disease and found that the VAS score fell from 5.35±2.78 to 3.30±2.21. As mentioned above, although there are studies in which the ozone treatment is given in the form of major hemotherapy in hyperuricemic patients and intraarticular in osteoarthritic patients, there is no study in the literature that examines the ozone effect in experimental gout joints in the literature. In ozone-treated patients, as indicated in other publications, it has been shown that the molecules responsible for joint destruction are reduced. In the study we conducted, a significant proliferation increases in the ozone Gout group was observed compared to the control group. Bilge et al.[20] studies on ozone treated rats with osteomyelitis found higher total serum antioxidant capacity and histopathologically found more positive results in terms of inflammation and abscess formation. Similarly, in our study ozone-induced inflammation was significantly suppressed. Although our study did not prevent the formation of proliferation or reactive epithelium when given intraarticularly, it showed suppressed inflammation, and there was no significant pathologic change in the joints in the osteonecrosis test group 4. As a result, intraarticular ozone treatment was considered to be one of the advanced treatment options considering the potency and efficacy in Gout.

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Research Article

Phylogenetic Analysis of Bovine Respiratory Syncytial Virus from Calves with Respiratory Disorders

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Abstract

Bovine respiratory disease (BRD) causes economic losses related to a reduction in weight gain of affected animals, veterinary treatment costs, death, etc. One of the important respiratory tract disease viruses is the bovine respiratory syncytial virus (BRSV). In this study, it is aimed to report the molecular characterization of detected BRSVs. Therefore, nasal samples from three calves in a herd seen severe respiratory disorders were examined for BRSV and other possible viral etiological agents by PCRs and the amplicons were sequenced. In the phylogenetic tree, BRSV circulating in this herd is clustered with the genetic subgroup III BRSVs deposited in GenBank from some other countries. This study on the molecular characterization of BRSV circulating in calves would contribute for future studies on the epidemiology of this infection and the development and/or choice the effective vaccines in Turkey.

Keywords: *BRSV, Calves, Genotype, Subgroup III*

Solunum Sistemi Hastalığı Olan Buzağılarda Saptanan Bovine Respiratory Syncytial Virusun Filogenetik Analizi

Öz

Sığırlarda solunum sistemi hastalıkları, etkilenen hayvanların kilo alımında azalma, veteriner tedavi masrafları, ölüm gibi sebeplerle ekonomik kayıplara neden olmaktadır. Bovine Respiratory Syncytial Virus (BRSV), solunum sistemini etkileyen viral etkenlerin başında gelmektedir. Bu çalışmada BRSV'nin saptanması ve tespit edilen etkenlerin moleküler karakterizasyonlarının ortaya konulması amaçlanmıştır. Bu amaçla, buzağılarda ağır solunum yolu sistemi bulguları gözlenen bir işletmedeki üç buzağıdan alınan burun akıntısı örnekleri BRSV ve diğer viral etkenler yönünden PCR ile test edilmiştir. RT-PCR sonucunda pozitif bulunan örneklerin dizin bilgileri elde edilmiştir. Yapılan filogenetik analiz sonucunda bu sürüde tespit edilen BRSV'nin genetik olarak subgrup III içerisinde yer aldığı ortaya konulmuştur. Buzağılarda saptanan BRSV'nin moleküler karakterizasyonu üzerine yapılan bu çalışma, bu enfeksiyonun epidemiyolojisi ve etkili aşıların geliştirilmesi ve/veya seçimiyle ilgili gelecekteki çalışmalara katkıda bulunacaktır.

Anahtar sözcükler: *BRSV, Buzağı, Genotip, Subgrup III*

INTRODUCTION

Bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus type 3 (BPIV3), bovine herpesvirus type 1 (BHV-1), bovine adenovirus (BAV), bovine coronavirus (BCoV) and bovine viral diarrhea virus (BVDV) are important pathogens associated with the bovine respiratory disease complex (BRDC) [1]. It is known that each of these agents can cause infection either alone or in combination with the bacterial agents and the other viruses [2]. Economic effects of BRSV infection together with the other BRDC agents to the cattle industry should not be underestimated due to the mortality, treatment expenses, and slower growth of affected animals [1,3]. BRSV infection could mostly cause mild disease in adult cattle, the infection could be quite a severe infection in calves and even leads to outbreaks and deaths [4].

Bovine respiratory syncytial virus belongs to the genus

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Orthopneumovirus in the family *Pneumoviridae* and contains 10 genes encoding 11 proteins. Three of them are transmembrane glycoproteins and located on the surface of the viral envelope. These are the large attachment glycoprotein (G), which mediates viral attachment to the host cell; the fusion protein (F), which enables fusion of virus with the host cell, and the small hydrophobic protein (SH). Other proteins are nucleocapsid associated nucleoprotein (N), phosphoprotein (P), the viral RNA-dependent polymerase protein (L), M2-1, and M2-2 and the matrix protein M. Finally, there are 2 non-structural (NS) proteins that accumulate in infected cells, NS1 and NS2 [5].

Based on the reaction patterns with monoclonal antibodies, BRSV has been divided into four antigenic subgroups, designated as A, B, AB and untyped although there is only one serotype of BRSV [6,7]. Further studies on the nucleotide sequencing of the G protein gene and the reaction with neutralizing antibodies, the antigenic subgroups were confirmed, although there is genetic variability in these subgroups [1]. Moreover, the genetic and antigenic heterogeneity of G protein along with the analysis of the genes-encoding N and F protein has been used to characterize BRSV strains [8,9]. Currently, seven genetic subgroups of BRSV strains have been determined by the nucleotide sequencing of attachment G protein [8].

Despite the serological and virological data on the presence of BRSV and contribution on respiratory infections [2,10,11], the circulating genotypes were not previously reported in Turkey. The aims of the study were i) to investigate the etiological agent(s) causing severe respiratory disorders in a herd and ii) to describe the molecular characterization of BRSVs detected in Turkey.

MATERIAL and METHODS

History of Infection and Study Design

At the beginning of September of 2016, a severe outbreak characterized by respiratory distress, fever, cough, sneeze, etc. in calves, aged 7-85 days, was seen in a herd including approximately 3000 Brown Swiss cattle in Eskişehir. This herd was closed and had been restocking only from internal animal source. Animals over 1 year of age are kept under the same roof, although newborns are grouped under a different roof for every 3 months of age. However, they do mingle freely during the grazing. Animals analyzed in this study were vaccinated with a commercial vaccine (Elite 9-HS; Boehringer Ingelheim Vetmedica, Inc., Germany). The morbidity and the mortality (rate up to 50% and 20%, respectively) became higher during the outbreak, notably between November and February, despite the treatment using antibiotics and other drugs to prevent symptoms and also the infection.

Extraction and Screening of Samples By PCR

Nasal secretions were collected from three calves with clinical signs using sterile swabs and immediately after collection viral transport medium (1 mL) was added into swabs. The samples were sent to the laboratory on ice and stored at -80°C, until analyses. Following the submission of nasal swabs to our laboratory for diagnostic purposes, all samples were examined for BRSV and other possible etiological agents such as BCoV, BPIV3, BVDV, and BHV-1 by PCRs. Viral RNA/DNA was purified using Trizol LS reagent (Thermo Scientific, USA) according to manufacturer's instructions. Briefly, synthesis of cDNA was achieved following denaturation of RNA at 70°C for 5 min. The cDNA was synthesized using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (RT) (Thermo Scientific, USA) and random hexamers (Thermo Scientific, USA) by incubating at 25°C for 10 min, and thereafter at 37°C for 1 h. MMLV-RT was therefore inactivated at 70°C for 10 min. PCR/RT-PCRs for the detection of mentioned viruses were performed by using the specific primers according to the protocols reported elsewhere [12-16]. BRSVs in nasal samples were detected by nested RT-PCR using the primer sets B1/B2A: 5'-AATCAACATGCGTGCAGTTAG-3'/5'-TTTGGTCATTCGTTATAGGCAT-3' (711 bp) and B3/B4A: 5'-GTGCAGTTAGTAGAGGTTATCTTAGT-3'/5'-TAGTTCTTTAG ATCAAGTACTTTGCT-3' (481 bp) targeting F gene region [15]. The PCR mixture was incubated for 5 min at 96°C. Then, 40 amplification cycles (1 min at 94°C, 1 min at 55°C and 1 min at 72°C) were performed followed by a final extension step at 72°C for 10 min. The nested PCR was performed under the same reaction conditions with inner primers. To molecularly characterize of BRSVs, primers (G2.5/F2.7: 5'-AGACATTAAAGAGGGCTTGGA-3'/5'-CTGCA CTGCATGTTGATTGA-3') targeting G gene region were used with an expected amplicon size of 1030 bp [9]. The mixture for PCR was preheated at 98°C for 30 sec, subjected to 35 cycles of 30 sec at 98°C, 30 sec at 60°C, 30 sec at 72°C and a final 7 min incubation at 72°C. An isolate previously identified in our department was used as the positive control virus. The PCR amplicons of the samples and the positive control virus were analyzed by 1% agarose gel electrophoresis and visualized under UV light.

Sequence and Phylogenetic Analysis

Purification of the amplicons and sequencing were performed from by a commercial company. Following the sequencing of these amplicons, multiple sequence alignments were prepared by the MUSCLE algorithm as implemented in AliView Software [17,18]. Cognate sequences of reference BRSVs representing different subgroups for the G protein gene were retrieved from Gen Bank through the BLAST engine. Phylogenetic analysis of nucleotide sequences of the G protein gene was performed using MEGA 6.06 software [19]. The phylogenetic tree was constructed using the maximum likelihood method and Tamura-Nei nucleotide substitution model and the statistical significance was estimated by bootstrap analysis (1000 replicates). The nucleotide identity table was

computed using online tools (SIAS, http://imed.med.ucm.es/Tools/sias.html).

RESULTS

All nasal swab samples (n=3) were positive for BRSV by RT-PCR targeting F gene region while they were negative other mentioned viruses tested *(Fig. 1)*. Additionally, herd manager reported that they sent all samples to another routine diagnostic laboratory and the samples were negative for bacterial agents. RT-PCR targeting G protein gene region for the detection of a genetic subgroup of BRSV produced correct size amplicons (1030 bp) only in two nasal samples *(Fig. 2)*. Genome sequences of G protein gene of BRSVs were deposited in GenBank under the following accession numbers: MH133326 and MH133327.

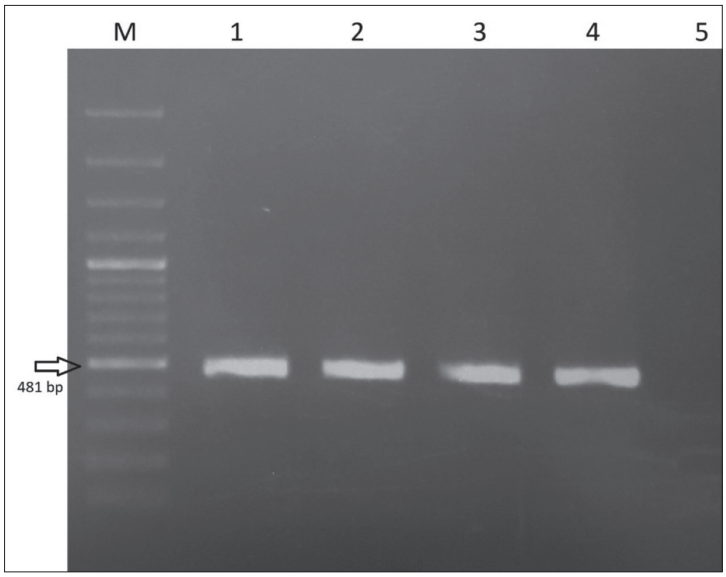

Fig 1. The results of the amplification of F gene region. M: 100 bp DNA ladder, 1: Positive control; 2,3,4: Positive samples. 5: Negative control

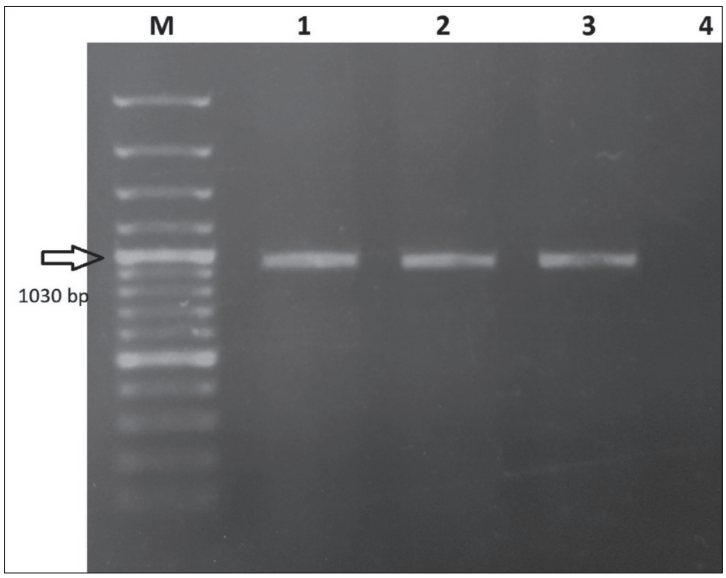

Fig 2. The results of the amplification of G gene region. M: 100 bp DNA ladder, 1: Positive control; 2,3,: Positive samples. 4: Negative control

In the phylogenetic tree *(Fig. 3)* both BRSVs were classified in the same cluster and they determined as belonging to subgroup III. Additionally, deduced amino acid sequences of G protein of two Turkish BRSVs were compared to the other deduced amino acid sequences of G protein of BRSVs belonging to different countries and vaccines *(Fig. 4)*. The partial nucleotide and amino acid sequences of the G protein gene of two BRSVs were compared with each other and with the sequences of BRSVs from other countries acquired from GenBank. The identities of the nucleotide sequences and also predicted amino acid sequences of two Turkish BRSVs were 100%. They shared the closest genetic relationship with the USA strain 236-652 (94.51%). The nucleotide sequence and deduced amino acid sequence similarities of the G protein regions between our field virus and other BRSVs identified as subgroup III, ranged from 85.4 to 100% and from 79.61-93.33%, respectively. The comparison of the deduced amino acid sequences of the G protein of two Turkish BRSVs with other BRSVs belonging to different countries and vaccines showed that the all four cysteine (Cys) residues (Cys 173-Cys 186 and Cys 176-Cys 182) in the immunodominant region were conserved in both Turkish BRSVs without any substitutions. However, Asn 179 and of Ala205 replaced by Ser and Iso in both strains, respectively, differing from the other BRSVs in all subgroups *(Fig. 4)*.

DISCUSSION

This study investigated viruses in bovine respiratory disorders in calves, including BRSV, BPIV3, BHV-1, BCoV, and BVDV. Results of PCRs and sequencing data showed that all of the nasal swab samples were positive for F protein gene of BRSV while they were negative for the other tested viral agents. Also, bacteriological investigations of the samples using routine diagnostic procedures in other lab did not provide evidence for any bacterial pathogens. BRSV was determined as the causative agent in severe acute respiratory system disease seen in calves, along with or without other possible agents which were not investigated in this study.

BRSV infection in calves is widespread in America [20,21], many European countries [1,8,22-24] and Turkey according to serologic [10,11] and virologic [2] evidence. There is only one study on the investigation with virological methods of BRSV and also other some viruses causing the respiratory disease in calves. Alkan et al.[2], reported that nasal swab samples from 95 cattle with signs of respiratory disease housed in eleven different herds were tested for BHV-1, BPIV 3, BRSV

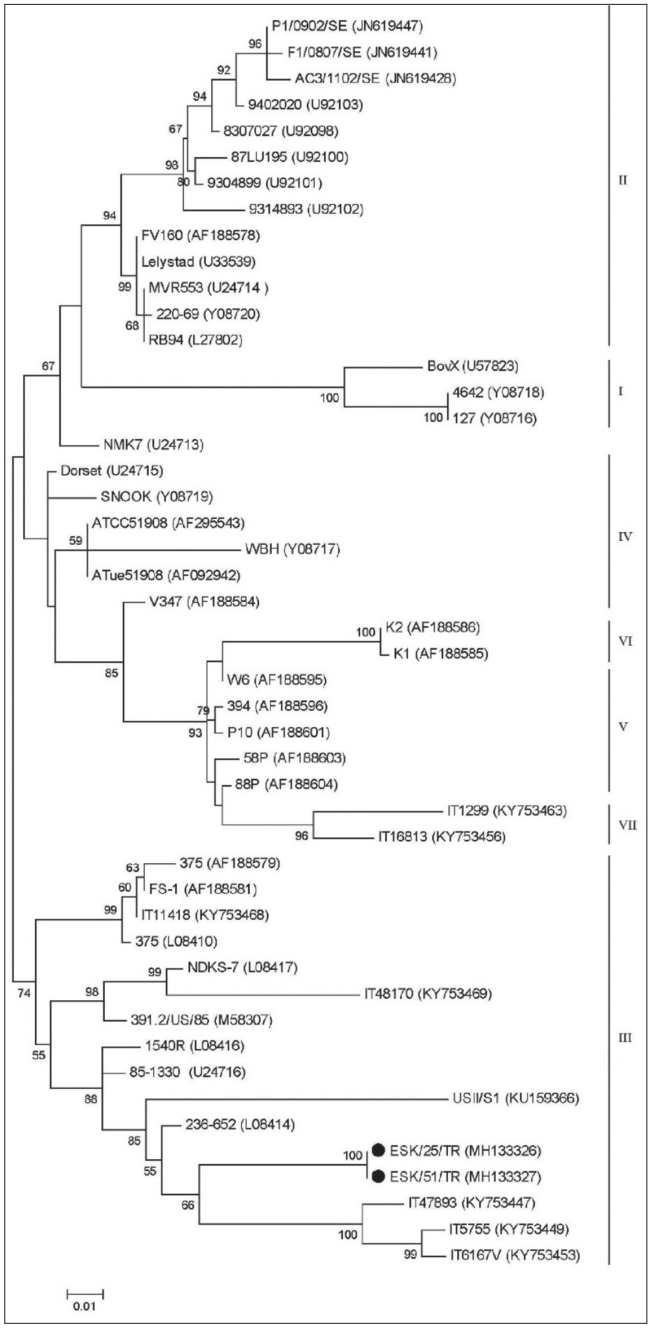

Fig 3. Phylogenetic tree of the G protein gene of BRSVs identified in this study with those of other BRSVs selected in GenBank. Our BRSVs are indicated by black dots. The statistical significance was estimated by bootstrap method (1000 pseudo-replicates) and values of <50% are omitted

and BVDV using direct immunofluorescence technique and BRSV detected as one of the causative agents especially along with BHV-1 and BPIV-3. However, there is no data about the molecular characterization of BRSV(s) in Turkey to date.

In this study, partial G protein gene region of BRSVs was successfully able to be amplified for only two nasal samples and they were sequenced. Based on the sequence analysis, both of our field viruses clustered in the genetic subgroup III, including the USA and Italian BRSVs [8,9]. Although the small number of samples was examined in this study, the obtained data suggest that subgroup III is circulating in our country.

It is suggested that there is an interaction between the genetic subgroups of BRSVs and geographic regions [23]. Thus, in the studies on the BRSV in Europe, subgroups II, V, and VI in France; subgroups II, IV and V in Belgium; subgroups III and VII in Italy; subgroup I in Switzerland; subgroup II in Poland, Norway, Sweden, and Denmark were detected [8,9,22,25,26]. Whereas Turkey is geographically close to Europe, the sequencing data of the G protein-encoding gene of BRSVs showed that they are genetically different from the most European strains but similar with the USA and Italian BRSVs [6,8,9]. The current data of Turkish BRSVs belong to the genetic subgroup III along with the USA, Italian and also Japanese strains [8,9,23] does not fully support the theory of geographical and temporal clustering of BRSV. However, due to the fact that a lot of animals were imported from the USA and some European countries to Turkey, this result needs to be investigated. It is thought that further investigation of the BRSV infection will contribute to a more precise assessment of the diversity of Turkish BRSVs and determine the possible source of viruses circulating.

The G protein is significantly important because of its immunodominant region (cysteine-rich and between 174 and 188 aa position) in the central conserved domain which is externally accessible for the neutralizing antibodies [5]. In this study, deduced amino acid sequences of G protein of two Turkish strains were compared to the other deduced amino acid sequences of G protein of BRSVs belonging to different countries and vaccines. Based on the sequencing data of the immunodominant region, all 4 cysteines (Cys) residues (Cys 173-Cys 186 and Cys 176-Cys 182) were conserved in our BRSV strains with no substitutions, however, one of the quite important amino acid for the antibody binding, Asn 179, was replaced by Ser in both strains differing from the other strains in all subgroups *(Fig. 2)*. A previous study [27] reported that the role of Asn is to stabilize helix and type I turn and also the role of Asn could also be performed by Ser which is the only residue exists as frequently as Asn at N-cap position of the helix, based on the statistical analysis. The point mutations at 180 and 205 aa determines BRSV subgroups [27]. At the central conserved region, the mutation of Ala205 to Thr determines the subgroup and distinguishes the subgroup I and II from those in the other subgroups [9]. It is demonstrated that only the mutation of Ala205 to Thr allowed escaping from antibody binding [27]. Amino acid residue 205 of the Turkish strains is an Iso, while the other isolates in subgroup III Thr are present. It is not known, this situation (Thr205 to Iso) could change the protein structure and alter the biological function of the G protein.

The biological importance of antigenic subgroups is not clear. It is reported that the mutations in the immuno-

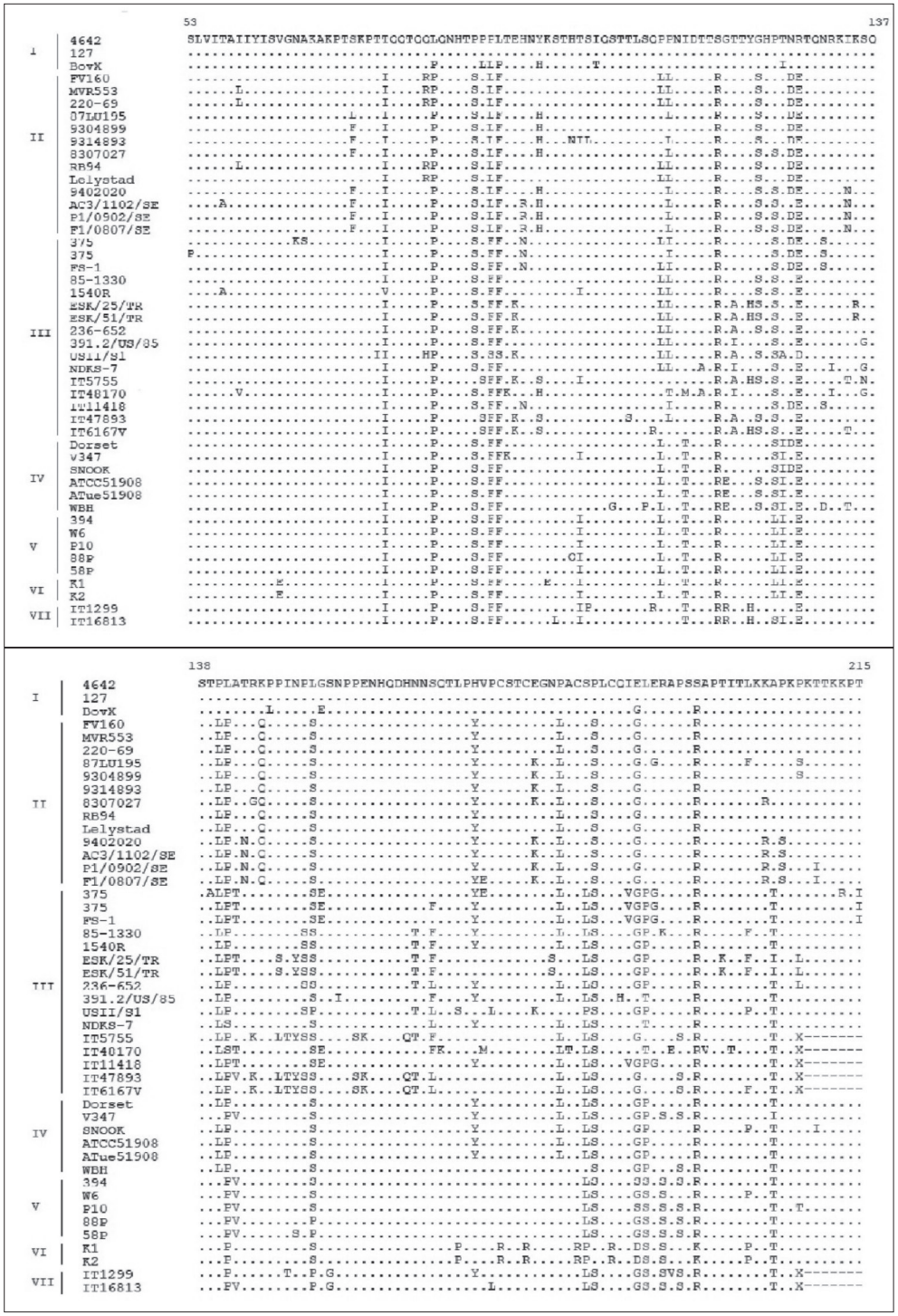

Fig 4. Sequences of amino acids 53 to 215 of the G protein of BRSVs. Designations on the left indicate the codes and the subgroup designations of BRSVs

dominant region of the G protein may contribute to the lack of cross-protection between vaccine and field isolates [5]. The sequence and structure of the G protein should be considered when designing vaccination strategies and choosing viral strains for the construction of the vaccine. Furze et al.[28] showed that polyclonal sera obtained from calves vaccinated with the BRSV G protein from subgroup A virus recognized a different subgroup A BRSV, less recognized subgroup AB, but not subgroup B or an untyped isolate. We did not investigate the antigenic features of the field strains by using monoclonal or polyclonal antibodies. However, based on our phylogenetic analysis and the results of other studies on BRSV subgroup III we could indicate that our strains belong to subgroup A. Unfortunately, it was not possible to know the BRSV strain included in a commercially inactivated vaccine had been

used in this herd although we made an effort to contact the company. Although BRSV could also reinfect seropositive calves [29], it is noted that the level of the possible maternal immunity to BRSV in sampled calves is not tested in this study. Thus, it was not possible to make an interpretation of the antigenic similarity between the vaccine and field virus, and also to speculate on the efficacy of vaccination.

This study provides an information on the molecular characterization of BRSV in Turkey which may have important implications for the planning the future studies on the epidemiology of this infection and the development and/or choice the effective vaccines. The genetic analysis and evolution of BRSVs should be monitored regularly in calves and adult animal populations for the choice of vaccine to control the infection.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Research Article

Investigation of the Effects of Storage Period for Frozen Bull Semen on *In Vitro* Embryo Production

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Abstract

The aim of this study was to investigate some spermatological parameters of frozen Brown Swiss bull semen 32 years ago by flow cytometry and to determine how storage time in liquid nitrogen affects *in vitro* embryo production ratios. For this purpose, early necrotic, necrotic, viable and apoptotic spermatozoa concentrations were analyzed using the Flow Cytometry and then the *in vitro* fertilization abilities of these sperms were investigated. AnnexinV/PI-FITC® was used to determine the apoptotic changes with flow cytometric analysis. Oocytes were obtained from slaughtered cows in a local abattoir. Brown Swiss semen, frozen in the last two years, were used for the control group. Early necrotic spermatozoa levels in semen frozen 32 years ago were lower but necrotic spermatozoa levels were higher than in the control group, but the opposite result was encountered in the control group (P<0.01) according to flow cytometry findings. The cleavage ratio in vintage spermatozoa was found to be lower than in the control group (P<0.0z), the blastocyst ratio was also lower than in the control group (P<0.01). As a result, it was observed that some spermatological parameters can be changed negatively and it can be said that a long storage period may lower the fertility capabilities of Brown Swiss bull semen.

Keywords: *Brown Swiss bull, Spermatozoa, Long term storage, Flow cytometry, In vitro fertilization*

Dondurulmuş Boğa Sperması Muhafaza Süresinin *İn Vitro* Embriyo Üretimi Üzerine Etkilerinin İncelenmesi

Öz

Bu çalışmanın amacı, 32 yıl önce dondurulmuş olan Esmer ırkı boğa spermalarının akım sitometri yoluyla spermatolojik parametrelerini araştırmak ve sıvı nitrojen içerisinde saklama süresinin *in vitro* embriyo üretim oranlarını nasıl etkilediğini araştırmaktır. Bu amaçla erken dönem nekrotik, nekrotik, canlı ve apoptotik spermatozoa yoğunlukları akım sitometri yöntemi ile incelendi ve in vitro fertilizasyon yetenekleri araştırıldı. Akım sitometri cihazında apoptotik değişimlerin analizi için AnnexinV/PI-FITC® kullanıldı. Mezhaba materyali yumurtalıklarından elde edilen oositler *in vitro* embriyo üretimi için kullanıldı. Kontrol grubu olarak, son iki yılda donmuş aynı cinse ait spermler kullanıldı. 32 yıl önce dondurulmuş spermalarda erken nekrotik spermatozoa düzeylerinin kontrol grubuna göre daha düşük ancak nekrotik spermatozoa düzeylerinin ise daha yüksek olduğu (P<0.01) gözlenmiştir. Dolayısıyla eski ile yeni dondurulmuş olanlarda, erken nekrotik ve nekrotik spermatozoa düzeylerinde aksi yönde sonuçlar alınmıştır. Eski spermatozoonlardaki ilk bölünme oranları kontrol grubuna göre daha düşük bulundu (P<0.05), blastosiste ulaşma oranı da kontrol grubuna göre daha düşüktü (P<0.01). Sonuç olarak, spermaların sıvı azot içerisinde uzun süre muhafaza edilmesi ile bazı spermatolojik parametrelerin olumsuz olarak etkilenebildiği ve uzun muhafaza sürecinde Esmer boğa spermasının fertilite kabiliyetlerinin azaldığı düşünülmüştür.

Anahtar sözcükler: *İsviçre Esmeri boğa, Spermatozoa, Uzun süreli muhafaza, Akım sitometri, In vitro fertilizasyon*

INTRODUCTION

Due to sperm freezing techniques, the storage, transport and handling of sperm are greatly facilitated. In addition, gene banks have been established to overcome infertility and the sperm of breeds facing the danger of extinction can be stored and maintained in such banks for many years [1,2].

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The discovery of glycerol was a revolutionary development in sperm storage technologies enabling successful cryopreservation of long-term frozen semen [3]. This important development has allowed the preservation period of semen to be prolonged and sperm freezing procedures have been successfully used for artificial insemination [4]. The goal of freezing sperm is to increase the number of animals with high genetic capability in population and to contribute to the protection of endangered animals [5]. Another goal of sperm freezing is to store a live cell or tissue at a very low temperature for a long time with a minimal chance of defect or without loss of function [6]. It has been estimated that male gametocytes could be frozen in liquid nitrogen and stored under suitable conditions for 500 to 3400 years [7].

Freezing spermatozoa in liquid nitrogen significantly increase the chances of long-term storage of cells [8]. Free radicals, superoxides, osmotic changes and the formation of intracellular-extracellular ice crystals have been reported to have significant negative effects on sperm integrity after short-term freezing [9]. The use of high quality sperm and the development of freezing-thawing methods and some additives contribute to the vitality of spermatozoa [10,11]. Liquid nitrogen (-196°C) turns the water into crystal form and inhibits all chemical reactions in cells and limits cellular activities. The freezing process can cause some chemical events in cells to slow down or stop, while others can accelerate. The survival of cells in the freezing process is not due to their ability to withstand extremely low temperatures. If cells survive the lethal intermediate zone temperatures (-15/-60°C) during freezing and thawing with minimal damage, they can survive [12]. Following the crystallization of the liquid, the physical properties of the unfrozen fraction change, and the crystallized gas increases the medium viscosity and makes significant changes to pH. The stress factor created by crystallization leads to osmotic shrinkage and polymerization in the cell and this causes some structural changes in the membrane lipid phase [13,14].

Cryopreservation causes fragmentation, overcondensation of DNA and sperm apoptosis. All these changes contribute to the overall decrease in fertility observed after freezing. As measured by the Annexin V/PI, the number of apoptotic sperm, characterized by the translocation of the PS in the outer leaflet of the plasma membrane, increased by up to 40% during cryopreservation in bull semen [15]. *In vitro* assessment of bull sperm using various fluorescent staining methods and CASA parameters can be a useful tool to predict the fertility of semen samples for artificial insemination [16]. Studies concerning the effect on the sperm of long-term storage in liquid nitrogen are limited. Some researchers have reported that long time storage may cause fertility losses as time progresses. Leboeuf et al.[17] noted that fertility declines due to semen dilution in the long-term storage of goat sperm, but that further studies are needed so that this can be explained more clearly.

The aim of this study was to investigate some spermatologic parameters of spermatozoa of Brown Swiss bulls frozen 32 years ago by flow cytometry and to determine how storage time in liquid nitrogen affects *in vitro* embryo production rates and thus the fertility abilities of spermatozoa.

MATERIAL and METHODS

Animal Maintenance

The study was carried out on frozen Brown Swiss semen in the International Livestock Research and Training Center (39°58'07.49"N, 33°06'29.86"E-Altitude: 1079 m). Semen from six bulls were used in the study, Group 1 (G1/n=3) was semen frozen 32 years ago (vintage group), Group 2 (Control/n=3) was semen frozen in the past two years. The selection of candidate bulls is made according to the milk yields of their mothers and optimum body condition score in the center. The six bulls were used had similar spermatological parameters and fertility capabilities in this study.

Sperm Collection and Freezing

The semen in both groups were collected by artificial vagina and frozen using the same extender and protocol. The semen was extended by dilution with Laiciphos (IMV/France), egg yolk and 7% glycerol in all groups. The extended semen was cooled to 25°C over a period of 30 min, and the semen was slowly mixed with a cold extender containing 7% (v/v) glycerol over three h. The semen samples were put into 0.25 mL French straws, and then they were left in liquid nitrogen vapor for about 10 min. After this, the straws were plunged into liquid nitrogen. The $20x10^6$ spermatozoa/mL in the straws were frozen in the laboratory's routine freezing protocol and stored in liquid nitrogen. The straws were determined to consist of spermatozoa with at least 55% subjective motility after thawing by random sampling in all groups. The spermatozoa were thawed for 30 sec at 37°C in a water bath for at least eight straws. Then semen was divided into two groups after pooling.

Spermatological Analysis

One part of the semen was used for analysis by flow cytometry, while the other part was used for *in vitro* fertilization procedures at the same time. All treatments were repeated at least twice for each bull. Ovaries used for *in vitro* fertilization were obtained from the local abattoirs.

Flow Cytometry Analysis

A commercial annexin-V/PI-FITC® (BD-USA) was used for flow cytometric analysis. The semen was diluted in PBS and then centrifuged two times at 2500 rpm for 10 min.

After removing the supernatant, the binding solution was added and the concentration was $2x10^7$ spermatozoa/mL. One hundred µL of this mixture was placed in a flow cytometry tube and 5 µL of fluorescein isothiocyanate (FITC/Annexin V®) and 5 µL of propidium iodide (PI) was added. This was vortexed delicately and incubated for 15 min at room temperature in the dark. After dyeing, 900 µL of the binding solution was added to the sperm to obtain a final concentration of $2x10^5$ spermatozoa/mL, which was then analyzed in the flow cytometry (BD-FacsAria II, USA) within an h. In the flow cytometry device, spermatozoa were firstly detected without fluorescence staining for each sample, then the total cell population was observed by fluorescence staining and the calibration was performed. Later, the analysis was completed by creating separate gradients for early necrotic (Q1), necrotic (Q2), live spermatozoa (Q3) and apoptotic (Q4) cell populations. At least 10,000 spermatozoa were examined each time [18,19].

In Vitro Embryo Production

The *in vitro* embryo production process was carried out in accordance with the method suggested by Kanagawa et al.[20]. TCM-199+10% Fetal Calf Serum (FCS) was used for maturation of immature oocytes obtained by aspiration and slicing from ovaries. A BO (Bracket&Oliphant) medium for fertilization and CR1aa (Charles Rosencrans) medium for embryo culture were used. An incubator which had at least 38.5°C+5% CO_2 and over 95% relative humidity was used for oocyte and embryo cultures. The ovaries obtained from the slaughterhouse were delivered to the laboratory within a maximum of 2-3 h in a 0.9% saline solution containing 100 mg/L Kanamycin Sulfate at 25-30°C. In the laboratory, oocytes were aspirated from follicles 2-8 mm in diameter on the surface of the ovarium using a 5 mL and 21-gauge needle with phosphate buffer solution (PBS) supplemented with 5% FCS.

The collected oocytes were put in PBS containing 5% FCS in 90 mm petri dishes and evaluated in the same solution. The oocytes of quality A and B were carried over into the maturation process. 10% FCS+5 µg/mL Follicle Stimulating Hormone (FSH/Folltropi-V, Canada) in TCM-199 was used as a maturation medium. Oocytes were incubated for 20-22 h with about 20 oocytes in each of the droplets in a volume of 100 µL. At the end of this period, oocytes with cumulus expansion were considered mature. Oocytes with expanded cumulus cells were removed by pipetting and continued on to the fertilization process in a BO medium for 5-6 h. Five U/mL heparin and 2 µM caffeine were used for spermatozoa capacitation. The straws were thawed in a 37°C water bath, washing solution was added and then they were centrifuged at 1800 rpm for 5 min. The supernatant was removed and the procedure was repeated once more to obtain the proper concentration of spermatozoa for the fertilization media, carefully arranged at 6.25×10^6 spermatozoa/mL. At the end of this period, spermatozoa were placed with oocytes in 100 µL fertilization droplets at $25\text{-}30x10^3$ spermatozoa per oocyte and incubated in the fertilization medium for 5-6 h. The *in vitro* culture period was completed in a 100 µL of CR1aa embryo culture media in 35 mm petri dishes. The cleavage rates were determined after 44-48 h and the blastocyst, degenerated and unfertilized oocyte (UFO) rates were determined after 7 days.

Statistical Evaluation

SPSS (13.0 Windows version - SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The data are shown as arithmetic mean and standard error. One-way analysis of variance (One way ANOVA) was used for data analysis.

RESULTS

As shown in *Table 1*, although there was no difference between G1 (vintage, frozen 32 years ago) and G2 (frozen in the last 2 years) spermatozoa in terms of viability in flow cytometry, the G2 early necrotic spermatozoa ratio was lower compared to the vintage frozen semen ($P<0.01$) but the necrotic spermatozoa ratio was higher than G1 ($P<0.01$). It can also be seen that the levels of apoptotic spermatozoa of the semen frozen 32 years ago are higher than those of the recently frozen sample ($P<0.05$).

As seen in *Table 2*, although there was no difference in terms of mature oocyte and degenerated oocyte ratios for the *in vitro* fertilization trials, significant differences were found between G1 and G2 in terms of cleavage ($P<0.05$) and blastocyst rates ($P<0.01$), whereas G1 UFO ratios were higher than the G2 ratios ($P<0.05$).

There was a linear correlation between blastocyst formation and early necrotic spermatozoa concentration $r^2 = 0.58$ ($P=0.04$) and necrotic spermatozoa $r^2 = 0.47$ ($P=0.01$). These values indicate that blastocyst formation correlates considerably with necrotic and early necrotic spermatozoa concentrations.

Table 1. *Flow cytometry analysis of sub populations for survival and dead spermatozoa*

Groups	Early Necrotic (%)	Necrotic (%)	Alive (%)	Apoptotic (%)
G1 (Vintage)	16.97±2.77	37.43±3.79	44.75±2.87	0.85±0.08
G2 (Control)	43.31±3.83	7.50±1.77	48.68±4.47	0.50±0.09
P	0.01	0.01	0.48	0.02

Table 2. *Fertilization of in vitro-matured oocytes by vintage spermatozoa frozen in 1986*

Groups	Oocytes (n)	Matured Oocytes (%)	Cleaved (%)	Blastocyst (%)	Degenerated (%)	UFO (%)
G1 (Vintage)	240	83.92±1.26	53.57±2.77	12.36±0.67	10.05±1.21	18.82±1.48
G2 (Control)	221	86.64±1.15	61.63±0.99	20.34±1.67	9.92±0.83	11.82±2.78
P	-	0.14	0.02	0.01	0.94	0.01

Controls consisted of semen samples frozen within the past 2 years

DISCUSSION

In this study, data were compared, focusing on the possible negative effects of long term storage in sperm banks. In similar studies, spermatological parameters were generally evaluated using conventional methods. In addition, details of the cell death process were observed by flow cytometry and the results obtained with *in vitro* fertilization were interpreted in terms of spermatozoa fertility.

In Malik et al.[21], one year and six year old bull spermatozoa with the same characteristics and stored under the same conditions were investigated and the former were found to be better in terms of life span and motility than the latter even though no changes were observed in their concentrations. In a study conducted with fresh buffalo spermatozoa frozen 1 day, 2 weeks, 1 month, 3 months, 6 months, 9 months, 1 year, 2 years and 3 years ago, life span and motility were decreased in the spermatozoa after 6 months due to acrosomal damage and loss of membrane integrity [22]. In another study, sperm from 3 Holstein bulls with the same individual characteristics were stored in liquid nitrogen for 4, 8, 12, 16, 20 and 24 months and diluted with the same extender. The result was less than 50% motility for one bull, while the other two bulls were reported to have over 50% motility after thawing up to the 16th month [23].

Although Salamon and Maxwell [24] reported in their study that there was no problem in the rate of fertility in ram sperm stored in liquid nitrogen for a long time, Leboeuf et al.[17] noted that fertility can decline with long-term storage of goat semen depending on the semen extender used, but that further work is needed so that this can be explained more clearly. Akyol et al.[25] noted that the likelihood of *in vitro* fertilization is highly variable between bulls and even between different ejaculates of the same bull. There is conflicting information about the fertility abilities of bull spermatozoa that have been stored for long periods in liquid nitrogen. In the present study, it was observed that there was no difference in terms of live spermatozoa between spermatozoa frozen 32 years ago (G1) and those frozen recently (G2-Control). However, the level of early necrotic spermatozoa was remarkably higher in recently frozen semen (P<0.01) than in G1 while the necrotic spermatozoa level observed in vintage semen was significantly higher than in the control group (P<0.01). The apoptotic spermatozoa levels of G1 were also found to be significantly higher than G2 (P<0.05).

According to the findings of this study, a great number of early necrotic spermatozoa advance to the necrotic phase over time although reports indicate that enzymatic activities were stopped in cells stored in liquid nitrogen. The conversion of early necrotic spermatozoa into necrotic spermatozoa shows that necrosis or apoptosis-causing factors are not completely inhibited at -196°C, while the viability rates of spermatozoa retained in liquid nitrogen for 32 years remain unchanged statistically. However, it is not clear how these factors react in liquid nitrogen. Necrotic or apoptotic factors may accumulate slowly in the long storage period, affects on spermatozoa or the long storage period may be causes rapidly affecting on the spermatozoa in thawing process. Therefore, it may be possible to elucidate this situation by further detailed studies.

It is possible to obtain a large number of embryos from abattoir materials through IVF techniques but *in vivo* is more difficult than *in vitro* for producing embryos and it is a quite costly procedure for scientific studies on farm animals, especially cattle. Therefore, the IVF technique is widely used to overcome the loss of fertility capabilities in spermatozoa [26]. Today, different IVF methods are used to achieve a 70-80% cleavage rate [27,28]. The findings obtained in the present study show that *in vitro* maturation occurs without problems. The first cleavage can be seen within approximately 28 to 44 h after fertilization. In one study, the first cleavage was seen at 28 h [29-32]. In a cow-based study, 55.7-64.5% fertilization rates were obtained [30]. Akyol et al.[30] found 72.0% at the 48th h, Wiemer et al.[33] got 73.3%, and Arias et al.[34] reported 88.5% cleavage ratios. The IVF study performed by Leibo et al.[35] with vintage bull semen frozen 37 years ago used highly motile spermatozoa at a concentration of >1x10^{6}/mL and achieved a 36.7% cleavage, 20.7% blastocyst rate, whereas a 50.7% cleavage and 25.4 blastocyst rate was obtained from recently frozen semen. In the present study, the cleavage rate was 53.57% for vintage semen and 61.63% for the control group. The ratios found in the vintage semen group were lower than in the control group (P<0.05). In the present study, the cleavage ratios were considered satisfactory and these findings were consistent with Leibo et al.[35].

The blastocyst ratio in many studies vary between 5%

and 40% depending on the method used [32,33,36,37]. In the present study, it is understood that there is no problem in terms of the rates of reaching total blastocyst. Leibo et al.[35] reported a 20.4% blastocyst rate for vintage semen and 25.4% with semen frozen a short while ago. In this study, the blastocyst rate was 12.36% in G1 and 20.34% in G2, using recently frozen semen. The blastocyst rate was significantly lower in vintage semen ($P<0.01$) than in the control group. Our findings regarding blastocyst ratios are parallel to those of Leibo et al.[35]. The UFO ratio was significantly higher in G1 than in the control group ($P<0.05$). Even though there is no change in the live spermatozoa ratio in the flow cytometry analysis, some transitions among to necrotic and early necrotic spermatozoa or an increase in the number of apoptotic sperm cells may be encountered.

As a result, some spermatological parameters have been adversely affected over time in bull sperm frozen 32 years ago and it can be observed that a long storage period may lower the fertility capabilities of Brown Swiss bull semen. However, it is thought that more detailed studies are needed to reveal the effects of the storage period and the details regarding changes in spermatologic parameters.

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Research Article

Isolation of Ampicillin and Vancomycin Resistant *Enterococcus faecium* from Dogs and Cats [1][2]

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Abstract

In this study, it was aimed to determine the occurence of ampicillin and vancomycin resistant enterococci (ARE and VRE) species in dogs and cats, antimicrobial susceptibility and virulence genes (*asa*1, *esp, gel*E, *hyl, cyl*A) of the isolates. Minimal inhibitor concentration (MIC) values of ampicillin and vancomycin were determined by macro dilution method and E-test, respectively. For this purpose, 531 rectal swabs collected from dogs (n=276) and cats (n=255) from three different cities (İstanbul, Ankara and Mersin) were examined. ARE was detected in 60 (21.7%) of dogs and in 47 (18.4%) of cats. VRE was detected in one dog and two cats. All ARE and VRE isolates were identified as *Enterococcus faecium* by polymerase chain reaction (PCR), and showed multi-drug resistance (MDR) phenotype. A small number of ARE*fm* isolates (4.7%) carried virulence gene. To the authors' knowledge, the study is first reporting *van*A gene harboring VRE*fm* in dogs in Turkey. The results indicated that both dogs and cats were frequent carriers of ARE*fm*. Due to close contact with humans, dogs and cats may play an important role in the spread of these nosocomial pathogens in the community. Therefore, further molecular studies are needed to elucidate the possible role of animal originated ARE*fm* and VRE*fm* strains in human nosocomial infections.

Keywords: *Ampicillin resistance, Cat, Dog, Enterococcus faecium, Vancomycin resistance*

Köpek ve Kedilerden Ampisilin ve Vankomisin Dirençli *Enterococcus faecium* İzolasyonu

Öz

Bu çalışmada, köpek ve kedilerde ampisilin ve vankomisine dirençli enterokokların (ARE ve VRE) izolasyonu, izolatların antimikrobiyal duyarlılıklarının ve virülans genlerinin (*asa*1, *esp, gel*E, *hyl, cyl*A) belirlenmesi amaçlandı. Ampisilin ve vankomisin dirençli izolatların minimal inhibitör konsantrasyonları (MİK) sırasıyla makrodilüsyon metodu ve E-test ile belirlendi. Bu amaçla üç farklı ildeki (İstanbul, Ankara ve Mersin) köpeklerden (n=276) ve kedilerden (n=255) toplanan 531 rektal svab örneği çalışmaya dahil edildi. Köpeklerin 60'ında (%21.7) ve kedilerin 47'sinde (%18.4) ARE tespit edildi. VRE bir köpek ve iki kedide saptandı. Tüm ARE ve VRE izolatları, polimeraz zincir reaksiyonu (PZR) ile *Enterococcus faecium* olarak identifiye edildi ve bu izolatlar çoklu ilaç direnç (MDR) fenotipi gösterdi. Az sayıdaki izolatta (%4.7) virülans geni saptandı. Yazarların bilgisine göre, bu çalışma ile ilk olarak Türkiye'de köpeklerden *van*A geni taşıyan VRE*fm* izolasyonu bildirilmektedir. Sonuçlar, hem köpeklerin hem de kedilerin ARE*fm* ile yüksek oranda kolonize olduklarını göstermektedir. Köpekler ve kediler yakın fiziksel temaslarından dolayı, insanlara bu nozokomiyal patojenlerin yayılmasında önemli bir rol oynayabilir. Bu nedenle, hayvanlardan izole edilen ARE*fm* ve VRE*fm* suşlarının insan nozokomiyal enfeksiyonlarındaki olası rolünü aydınlatmak için ileri moleküler çalışmalara ihtiyaç vardır.

Anahtar sözcükler: *Ampisilin direnci, Enterococcus faecium, Kedi, Köpek, Vankomisin direnci*

INTRODUCTION

Enterococci, for many years, have been considered as commensal inhabitants of the gastrointestinal tract of animals and humans. However, during last three decades, *Enterococcus* spp., especially *E. faecium*, has emerged one of the important nosocomial pathogens worldwide due to the acquired high resistance profiles such as amino-glycosides, ampicillin and vancomycin, making therapy options very limited [1,2]. Of these resistance mechanisms,

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high level ampicillin resistance (>256 µg/mL) is important problem, especially when associated with high level aminoglycoside and glycopeptide resistance. The most common mechanism for high level ampicillin resistance is multiple mutations in the active site of the penicillin binding protein (PBP5) [3-5].

The first isolation of vancomycin resistant *E. faecium* (VRE*fm*) from humans was first reported in Turkey by Başustaoğlu et al.[6] and the first VRE*fm* outbreak was announced in a tertiary hospital in Ankara by Çolak et al.[7]. Subsequently, hospital-acquired infections and outbreaks caused by VRE*fm* have been reported [8,9]. According to the 2015 and 2016 national hospital infections surveillance network reports, VRE isolation rates were reported as 14.03% and 13.33%, respectively [10,11]. In contrast to human studies, there is a paucity of studies on the isolation and molecular characterization of VRE*fm* from dogs and cats. VRE*fm* was first reported in a 3-year-old male cat with urinary system problem by Bağcıgil et al.[12]. Similarly, there is only one study of isolation and molecular characterization of ampicillin resistant *E. faecium* (ARE*fm*) from cats and dogs, in which occurence of ARE*fm* was found in 20.9% of dogs and in 25.4% of the cats [13].

Enterococci have the ability to produce a number of virulence factors**,** playing important role in their pathogenesis such as aggregation substance *(asa*1), gelatinase *(gel*E), cytolysin *(cyl*A), enterococcal surface protein *(esp)*, hyaluronidase *(hyl)* [14]. Of these factors, *esp* was reported to be more frequently related with infections and nosocomial infections caused by ARE*fm* and VRE*fm*. The reason for this has been shown as increased ability of adherence to epithelial surfaces and biofilm formation of *esp* carrying isolates.

Dogs and cats are close contact with humans, and may transmit resistant bacteria to their owners. The data on the occurence of ARE*fm* ve VRE*fm* in dogs and cats have remained largely unknown in Turkey. Therefore, current study was conducted to investigate the occurence of ARE*fm* and VRE*fm* in pet animals to elucidate possible public health implications.

MATERIAL and METHODS

Ethical Statement

The study was approved by the Animal Ethical Committee of Hatay Mustafa Kemal University 2018/3-7

Sample Collection

Rectal swab samples were collected from dogs (n=276) and cats (n=255) from three different provinces (İstanbul, Ankara and Mersin) between March 2018 and April 2018. The rectal swabs were taken from both healthy and sick pet animals.

Isolation and Identification

For the presence of ARE and VRE, the rectal swab samples were inoculated into two different Enterococcosel broth (BD, UK), one with 32 µg/mL ampicillin to detect ARE isolates, and the other with 6 µg/mL vancomycin to detect VRE isolates. Both were incubated for 48 h at 37°C. In the case of growth in the Enterococcosel broth for ARE detection, a loopfull of culture was inoculated on VRE agar (Oxoid, UK) plates supplemented with 32 µg/mL ampicillin. In the case of growth in the Enterococcosel broth for VRE detection, a loopfull of culture was inoculated on VRE agar plates supplemented with 6 µg/mL vancomycin. Both plates were incubated for 48 h at 37°C. Subsequently, one putative colony from each plate was randomly selected and identified by a species-specific multiplex polymerase chain reaction (mPCR) method [15]. mPCR assays confirming the presence of the genus *Enterococcus* and identifying *E. faecalis* and *E. faecium* were performed in a total volume of 25 µL, consisting of 10× PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 1.5 mM $MgCl_2$, 200 µM each dNTP, 20 pmol of E1-2 and FMB1-2 primer, 32 pmol of FL1-2 primer, 10 µL template DNA and 2 U Taq DNA polymerase. PCR amplification was carried out in following thermal cycling conditions: initial denaturation at 94°C for 3 min and 30 cycles of amplification consisting of denaturation at 94°C 1 min, annealing at 55°C 1 min, and extension at 72°C 1 min, with a final extension step at 72°C for 7 min. The presence and size of the amplified products were analyzed by electrophoresis in 1x TBE buffer on 1.5% agarose gels. The species-specific primers for mPCR of *E. faecium* and *E. faecalis* are given in *Table 1*.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the isolates were tested for eight antimicrobials using disc diffusion method following Clinical and Laboratory Standards Institute (CLSI, 2012) criteria [16]. The antimicrobials used were: ampicillin (AM, 10 µg), ciprofloxacin (CIP, 5 µg), erythromycin (E, 15 µg), gentamicin (CN, 120 µg), rifampin (RA, 5 µg), tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg) and vancomycin (VA, 30 µg). MIC values of ARE and VRE isolates were determined by macrodilution method and E-test (Bioanalyse, Turkey), respectively. The isolates, which were resistant to three or more antimicrobials from different classes, were evaluated as multiple resistance (MDR).

Determination of Vancomycin Resistance Genes

The isolates found to be phenotypically as vancomycin resistant, resistance genes mediating vancomycin resistance were investigated by multiplex PCR as previously described by Depardieu et al.[17]. Briefly, PCR reaction was carried out in a total volume of 50 µL, consisting of 10× PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 2 mM $MgCl_2$, 200 µM each dNTP, 40 pmol of

Table 1. *PCR primers used for E. faecium and E. faecalis identification in this study*

Primer Name	Sequence (5′-3′)	Target Taxon	Target Gene	Amplicon Lenght (bp)
E1	TCA ACC GGG GAG GGT	*Enterococcus* spp.	16S rRNA	733
E2	ATT ACT AGC GAT TCC GG			
FL1	ACT TAT GTG ACT AAC TTA ACC	*E. faecalis*	sodA	360
FL2	TAA TGG TGA ATC TTG GTT TGG			
FM1B	ACA ATA GAA GAA TTA TTA TCT G	*E. faecium*	sodA	214
FM2B	CGG CTG CTT TTT TGA ATT CTT CT			

Table 2. *Primers used for detection of the vancomycine resistance genes*

Primer Name	Sequence (5′-3′)	Gene	Amplicon Lenght (bp)
EA1	GGGAAAACGACAATTGC	*van*A	732
EA2	GTACAATGCGGCCGTTA		
EB3	ACGGAATGGGAAGCCGA	*van*B	647
EB4	TGCACCCGATTTCGTTC		
EC5	ATGGATTGGTAYTKGTAT	*van*C1/C2	815/827
EC8	TAGCGGGAGTGMCYMGTAA		
ED1	TGTGGGATGCGATATTCAA	*van*D	500
ED2	TGCAGCCAAGTATCCGGTAA		
EE1	TGTGGTATCGGAGCTGCAG	*van*E	430
EE2	ATAGTTTAGCTGGTAAC		
EG1	CGGCATCCGCTGTTTTTGA	*van*G	941
EG2	GAACGATAGACCAATGCCTT		

each primer, 10 µL template DNA and 2 U Taq DNA polymerase. PCR amplification was carried out in following thermal cycling conditions: initial denaturation at 94°C for 3 min and 30 cycles of amplification consisting of denaturation at 94°C 1 min, annealing at 54°C 1 min, and extension at 72°C 1 min, with a final extension step at 72°C for 7 min. The presence and size of the amplified products were analyzed by electrophoresis in 1xTBE buffer on 1.5% agarose gels. Primers used for the detection of the vancomycine resistance genes are shown in *Table 2*.

Detection of Virulence Genes

Presence of virulence genes *(asa*1, *gel*E, *cyl*A, *esp*, and *hyl)* were investigated by mPCR [14]. PCR reactions were performed in a total volume of 50 µL containing 10× PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 2 mM $MgCl_2$, 200 µM each dNTP, 20 pmol of *asa1*, *gelE* and *hyl* primers, 40 pmol of *cylA* and *esp*, 10 µL template DNA and 2 U Taq DNA polymerase. After initial denaturation at 95°C for 5 min, 30 cycles of amplification were performed with denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min, followed by one cycle of a final extension at 72°C for 10 min. The amplified products were detected by electrophoresis 1× TBE buffer on 1.5% agarose gels.

Pulsed Field Gel Electrophoresis (PFGE) Analysis

Clonal relationship of the VRE isolates were determined by PFGE, which was performed in Public Health Institution of Turkey (Ankara) as described previously by Morrison et al.[18]. Briefly, bacterial cells (approximately 2×10^9 cells/mL) were mixed with an equal volume of low-melting-point agarose. The plugs were lysed with lysozyme and proteinase K, and then chromosomal DNA was digested with 40 U *Sma*I (Fermentas). Fragmented DNA samples were electrophoresed in 1% pulsed-field certified agarose (Bio-Rad) using a CHEF-DR II system(Bio-Rad) with 5-30 s pulse times, for 20 h at 14°C at 6 V cm^{-2}. The gel was stained with ethidium bromide (5 mg mL^{-1}), visualized under UV light, and photographed using a gel logic 2200 imaging system (Resolution: 1708×1280 pixel; Kodak). The DNA band profiles were analysed with GelCompar software (version 3.0; Applied Maths). Band tolerances of 1.5% and 1% normalization were used for comparison of DNA profiles.

Statistical Analysis

Differences in frequencies of isolation rates according to cities, different age groups and genders were evaluated using Pearson's chi-square test. SPSS 14.01 was used for statistical analysis. Any P value equal to/or less than <0.05 was considered as statistically significant.

RESULTS

Ampicillin resistant enterococci was detected in 60 (21.7%) of dogs and in 47 (18.4%) of cats *(Table 3, 4)*. All ampicillin resistant isolates were identified as *E. faecium* by PCR *(Fig. 1)*. Isolation rates between cities were found statistically significant (P<0.001). But, no statistically significant differences was observed among age groups and genders. VRE was isolated from two cats and one dog in Mersin. No VRE was isolated from other cities. All VRE isolates were identified as vancomycin resistant *E. faecium* (VRE*fm)* and positive for *van*A gene by PCR *(Fig. 2)*.

All ARE*fm* and VRE*fm* isolates were MDR phenotype *(Table 5)*. Ampicillin MIC values was between 64 and ≥256 µg/mL. Sixty isolates showed ≥256 µg/mL, 31 isolates 256 µg/mL, 13 isolates 128 µg/mL and three showed 64 µg/mL. All VRE*fm* isolates showed a MIC value of ≥256 µg/mL for vancomycin.

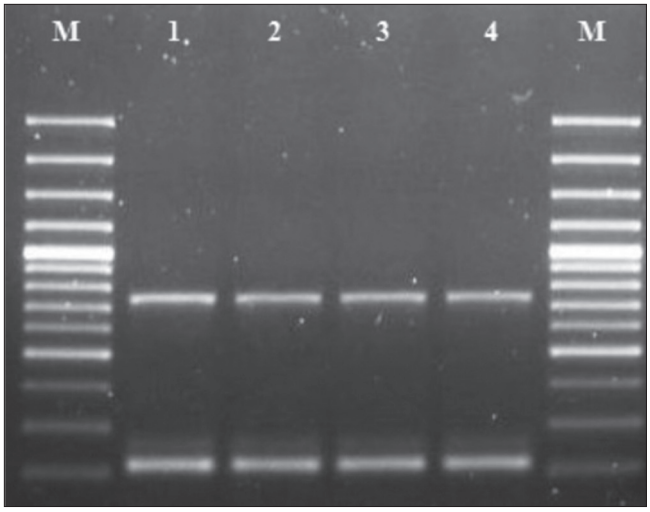

Fig 1. Agarose gel electrophoresis of *E. faecium* isolates. Lane M: 100 bp plus molecular marker, Lane 1-4: *E. faecium* (214 bp) plus *Enterococcus* spp. (733 bp)

Table 3. *Distribution of AREfm isolates according to age groups, genders and cities among dogs*

Variables	Mersin					Ankara					İstanbul					Total				
	Ex.[a] (n)	Neg.[b] (n)	Pos.[c] n (%)	X² Value	P Value[d]	Ex. (n)	Neg. (n)	Pos. n (%)	X² Value	P Value	Ex. (n)	Neg. (n)	Pos. n (%)	X² Value	P Value	Ex. (n)	Neg. (n)	Pos. n (%)	X² Value	P Value
Sex																				
Female	41	38	3 (7.3)	0.240	0.624	47	37	10 (21.3)	1.447	0.229	21	4	17 (81)	11.803	0.01	109	79	30 (27.5)	3.542	0.06
Male	59	53	6 (10.2)			63	55	8 (12.7)			45	29	16 (35.6)			167	137	30 (18)		
Age																				
<1	27	25	2 (7.4)	3.392	0.183	24	19	5 (20.8)	0.909	0.635	11	5	6 (54.5)	0.121	0.941	62	49	13 (21)	0.872	0.647
1-3	20	16	5 (25)			60	52	8 (13.3)			33	18	16 (54.5)			113	84	29 (25.7)		
>3	34	32	2 (5.9)			26	21	5 (19.2)			22	11	11 (50)			82	36	16 (19.5)		

[a] Examined, [b] Negative, [c] Positive, [d] Probability level

Table 4. *Distribution of AREfm isolates according to age groups, genders and cities among cats*

Variables	Mersin					Ankara					İstanbul					Total				
	Ex.[a] (n)	Neg.[b] (n)	Pos.[c] n (%)	X² Value	P Value[d]	Ex. (n)	Neg. (n)	Pos. n (%)	X² Value	P Value	Ex. (n)	Neg. (n)	Pos. n (%)	X² Value	P Value	Ex. (n)	Neg. (n)	Pos. n (%)	X² Value	P Value
Sex																				
Female	42	41	1 (2.4)	2.156	0.142	44	41	3 (6.8)	0.060	0.806	39	15	24 (61.5)	9.785	0.002	125	97	28 (22.4)	2.569	0.109
Male	40	36	4 (10)			49	45	4 (8.2)			41	30	11 (26.8)			130	111	19 (14.6)		
Age																				
<1	47	45	2 (4.3)	4.894	0.087	22	18	4 (18.2)	5.108	0.078	14	6	8 (57.1)	1.834	0.400	83	69	14 (26.4)	0.622	0.733
1-3	18	15	3 (16.7)			58	55	3 (5.1)			52	32	20 (38.5)			128	102	26 (20.3)		
>3	17	17	0 (0)			13	13	0 (0)			14	7	7 (50)			44	37	7 (15.9)		

[a] Examined, [b] Negative, [c] Positive, [d] Probability level

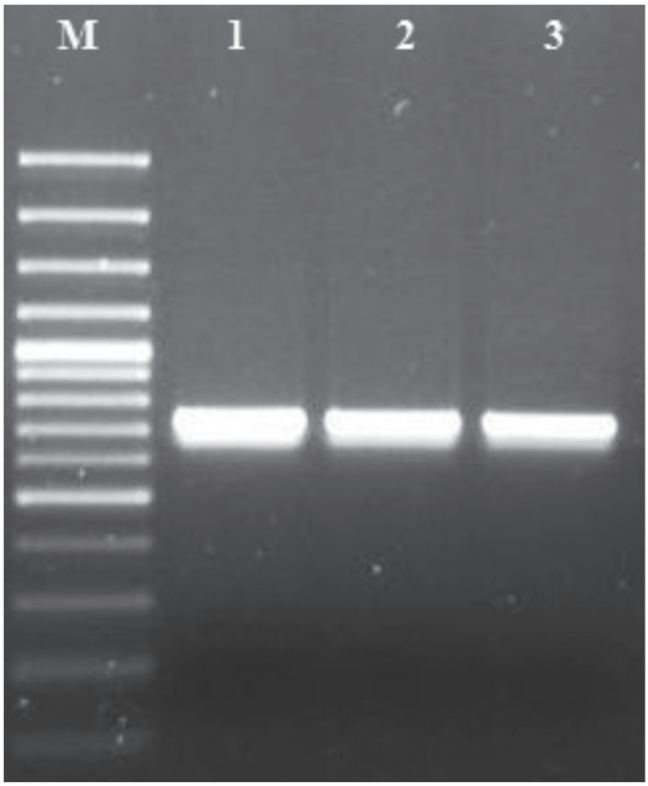

Fig 2. PCR amplification of vanA gene of VREfm isolates. Lane: 100 bp plus molecular marker, Lane 1-3: vanA gene (732 bp) positive isolates

Table 5. *Antimicrobial resistance phenotypes of AREfm isolates*

Resistance Phenotype	Species	
	Dog (n=60)	Cat (n=47)
AM, RA, CN, CIP, TE, E	25	18
AM, CN, CIP, TE, E	7	4
AM, RA, CIP, TE, E	5	6
AM, RA, CN, CIP, E	1	-
AM, RA, CN, TE, E	3	4
AM, RA, CN, TE	-	1
AM, CN, CIP, TE	-	1
AM, RA, CIP, TE	2	-
AM, CN, TE, E	1	-
AM, CIP, TE, E	2	2
AM, RA, TE, E	3	2
AM, CN, CIP, E	1	-
AM, CN, TE, E	-	2
AM, CIP, TE	2	-
AM, TE, E	7	6
AM, RA, TE	1	1

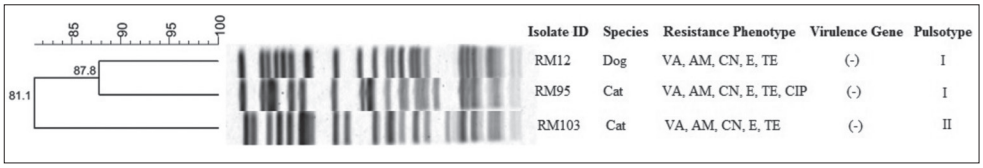

Fig 3. Dendogram showing the results of SmaI PGFE VREfm isolates. Pulsotypes are indicated as Roman numerals. Based on a similarity coefficient ≥85%, the isolates indicated two pulsotypes

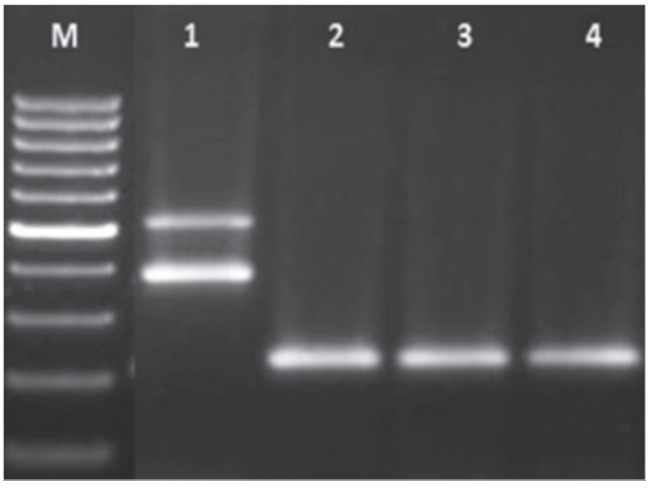

Fig 4. Agarose gel electrophoresis of virulence genes detected in ARE*fm* isolates. Lane M: 100 bp molecular marker, Lane 1: *esp* (510 bp) + *asa*1 (375 bp), Lane 2-4: *hly* (276 bp)

A shown in *Fig. 3*, PFGE typing of three VRE*fm* isolates showed two distinct PGFE pulsotypes based on a similarity coefficient of ≥85.

A small number of isolates (4.7%) carried virulence genes among ARE*fm* isolates. Among the isolates, *esp*, *asa*1 and *hly* genes were only virulence genes detected, but *gel*E and *cyl*A were not detected in any of the isolates tested *(Fig. 4)*. None of VRE*fm* isolates were positive for virulence genes tested.

DISCUSSION

The present study revealed a high intestinal carriage rate of ARE*fm* in dogs (21.7%) and cats (18.4%) in Turkey. In a previous study, Çelik et al.[13] reported comparable colonization rate in dogs and cats (20.9% and 25.4%, respectively) in İstanbul. In a countrywide population-based study in Netherland, de Regt et al.[19] reported that prevalence of intestinal carriage of ARE*fm* was 25.6% in dogs and 5.1% in cats. In a cross-sectional study carried out in the United Kingdom and Denmark, the prevalence rates of ARE*fm* in dogs were reported as 23% and 76%, respectively [20].

The *van*A carrying VRE*fm* was isolated from 0.13% of dogs and 0.8% of cats in this study. In Japan, Kataoka et al.[18] did

not detect VRE in dogs and cats subjected to different antibiotic regimens. In contrast, Devriese et al.[22] investigated the presence of faecal carriage of VRE in 87 dogs treated at the Animal Hospital of the School of Veterinary Medicine in Madrid, Spain, detected 11 (12.6%) *van*A carrying VRE*fm*. Since no information on the living conditions, contact with different animal species and eating habits, previous treatment records was available in this study, it was not possible to determine the origin of VRE transmission to cats and dogs. Guardabassi et al.[23] suggested that VRE isolates was generally resistant to different classes of antimicrobials such as macrolides, aminoglycosides, tetracyclines, the use of such antimicrobials in pet animals might lead the co-selection of VRE. Although carriage rate of MDR VRE was found to be very low in this study, it should be ruled out that this microorganism might emerge as a nosocomial pathogen in veterinary medicine, might play role as a source of VRE for humans, and might be able to promote the horizontal dissemination of resistance genes among strains of animals and humans [24].

A small number of ARE*fm* isolates were positive for virulence genes, and any VRE*fm* isolates carried virulence genes in this study. Similar observation was reported by Çelik et al.[13], who detected only *efa*A (13.8%) and *gel*E (11.1%) as virulence genes among ARE*fm* isolates. Leavis et al.[25] reported that the *esp* gene carrying ARE*fm* isolates are generally epidemic and cause severe nosocomial infections in hospitals. In this study, *esp* gene was detected only in one isolate from a cat together with *asa*1. Similarly, Damborg et al.[20] also didn't detect *esp* gene in any ARE*fm* isolate. Leavis et al.[25] explained this with two different views: (i) the dog ARE*fm* isolates might be evolved by acquiring virulence genes such as *esp* and *hyl* and adapted to hospital settings, (ii) human ARE*fm* strains might be ancestors of dog strains and lost their virulence factors outside hospital settings.

In conclusion, to the authors' best knowledge, this is first report of VRE*fm* carrying *van*A in dogs in Turkey. MDR bacteria including ARE*fm* and VRE*fm* in pet animals should be monitored by national surveillance programs. To elucidate the possible role of these bacteria in human nosocomial infections, the isolates from both pet animals and human nosocomial infections should be compared using advanced molecular techniques.

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Short Communication

Molecular Screening and Characterization of Shiga Toxin-Producing *Escherichia coli* By Multiplex PCR Assays for stx_1, stx_2, *eae*A, H7 in Raw Milk

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Abstract

The presence of Shiga-toxin producing *Escherichia coli* (STEC) was evaluated in bulk milk from dairy cattle farms in Bulgaria. From 20 regional farms only 4 were selected as a source for milk sampling on the basis of their annual STEC shedding in feces. All the samples were PCR analyzed after selective enrichment. Positive samples were detected in subsequent molecular testing as: two of samples (9.08%) were positive for stx_1, one sample (4.54%) for stx_2, 10 samples (45.54%) for *eae* and 12 (54.54%) for H7 coding genes.

Keywords: *Escherichia coli O157: H7, Dairy farms, Bulk milk, Enrichment, PCR*

Çiğ Sütte Shiga-Toksin Üreten *Escherichia coli*'nin stx_1, stx_2, *eae*A ve H7 Bakımından Multipleks PCR ile Moleküler İncelenmesi ve Karakterizasyonu

Öz

Bulgaristan'da süt sığırı işletmelerinden toplanan çiğ sütlerde Shiga-toksin üreten *Escherichia coli* (STEC) varlığı değerlendirildi. Toplam 20 bölgesel çiftlikten sadece 4'ü yıllık dışkıyla STEC yayma miktarı temel alınarak süt toplama kaynağı olarak seçildi. Tüm örnekler selektif zenginleştirme sonrasında PCR ile analiz edildi. Pozitif örnekler daha sonra moleküler test ile değerlendirildi. İki örnek (%9.08) stx_1 için, 1 örnek (%4.54) stx_2 için, 10 örnek (%45.54) *eae* için ve 12 örnek (%54.54) H7 kodlayan genler için pozitif bulundu..

Anahtar sözcükler: *Escherichia coli O157: H7, Süt çiftliği, Çiğ süt, Zenginleştirme, PCR*

INTRODUCTION

Shiga-toxin producing *Escherichia coli* O157: H7 is a dangerous food pathogen that could induce a wide range of illnesses in human - from mild transient to haemorrhagic diarrhoeas accompanied with abdominal pain. It could be also the causative agent of the life-threatening haemolytic uremic syndrome (HUS) [1]. Other possible sources could be fresh, RTE (Ready to eat) products of plant or animal origin, contaminated with bovine faeces. Large ruminants have no specific receptors for toxins, they do not exhibit clinical disease but are considered asymptomatic carriers [2]. Consequently, STEC are commonly encountered as faecal contaminants of foods of animal origin as meat, milk and dairy products. As a result, STECs are often found to be fecal contaminants of foods of animal origin such as meat, milk and dairy products.

It is no surprise that raw milk has been identified as a source for contamination with STEC with potential sequels for public health from its consumption, particularly in line with the recent trend for consuming raw, non-thermally processed milk [3].

The transmission of STEC to humans is realized by various

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pathways, e.g. consumption of undercooked meat, vegetables, water contaminated with faeces of carriers, less frequently from man to man or from the contaminated environment. In 2016, Li et al.[4] have performed a large survey in China on fresh meats and vegetables and reported that out of 35 isolates, 27 had virulence genes for *eae* (77.1%), while 7 *E. coli* O157 strains were positive for stx_2. The cytotoxic isolates also carried *eae* in addition to *stx* and belonged to the H7 serotype (11%) [4].

Dairy farms are a specific source of *E. coli* (STEC), *Listeria, Staphylococcus aureus, Bacillus cereus*, *Campylobacter* and *Salmonella* [5]. The risk of human infection with STEC originating from dairy farms could emerge after consumption of raw milk, dairy products whose technology does not imply thermal processing of milk or contaminated meat from dairy cattle [6].

The severity of disease, high lethality rates and the lack of sufficiently efficient treatment requires protection of consumers from the risk of exposure to occasional low-level contamination of foods with *E. coli* O157. This further entails compliance to high standards of control in production enterprises in order to prevent cross-contamination [7].

In the period from 2008 to 2012, at least six more cases of haemolytic anaemia, HUS and renal failure which required dialysis and life-saving kidney transplantation were reported again in the USA in people aged 2-27 years [8].

In our country, there are no in-depth research data on the carriership rates in calves, cattle, milk, meat, fodders etc. as well as on the virulence profile, Shiga-toxin production and seasonal shedding variations. That is why, the present study was designed to investigate the genetic virulence profile STEC and presence of amplicons for the *eae* gene coding for intimin production in bulked raw milk.

MATERIAL and METHODS

Sampling Conditions

A total of 48 dairy farms form 20 of 28 administrative districts of Bulgaria were previously investigated by our team in the period 2014-2016 for the presence of *E. coli* O157:H7, and only in 4 of these farms have been found Shiga toxin-producing *E. coli* in fecal samples of cows [9]. These 4 farms have been selected as the subject of this study. Each of the selected farms has over 100 dairy cows and at least 2 tanks for bulk milk storage after milking. A total of 56 samples of raw milk in a volume of 50 mL were collected during the summer months (July, August, September) in 2017. Milk samples are collected once a week, from each storage tank for raw bulk milk, after morning and after evening milking at the farm. The samples are chilled to a temperature of 6-8°C and transported under refrigerated conditions to the Laboratory of the Department Contagious Diseases at the Trakia University.

Enrichment of E. coli O157:H7 in Raw Milk

In a laboratory conditions, each milk sample was homogenized by shaking and 10 mL was transferred to a sterile Falcon test tube with a cap. Each milk test tubes were centrifuged for 10 min at 4000 rpm-1 for cell structure sedimentation. Then the formed milk cream was carefully removed by sterile cotton swab and liquid milk was discarded. The sediment was suspended in 5 mL sterile physiological saline and centrifuged once again under the same conditions. The supernatant was discarded and the pellet was resuspended by vortexing in 9 mL sterile selective enrichment broth (Tryptone Soya Broth Modified (mTSB) supplemented with Novobiocin, Oxoid, UK (SR01181) Enrichment procedure was similar to enrichment described in ISO 16654:2001 for STEC O157 in all food types (ISO, 2001).

All the test tubes were incubated aerobically at 37°C for 10-12 h for selective STEC *E. coli* enrichment. After incubation the samples were centrifuged (Rotofix 32A, D-78532) again at 4000 rpm^{-1} for 10 min and the pellet containing bacterial cells was double washed in 1 mL sterile physiological saline. In the final centrifugation the bacterial cells were suspended in 500 µL deionized MilliQ DNA free pure water (GenPure Ultrapure Water System) and transferred in sterile 1.5 mL microtubes.

Bacterial DNA Extraction and Identification of STEC by Multiplex PCR

Bacterial DNA was extracted by boiling method as each 1.5 mL microtube with bacterial cell suspension were capped with parafilm and heated in boiling water for 10 min over sealing rack. Then followed by a centrifugation at 14000 rpm^{-1} for 10 min at room temperature and the supernatant was separated from the pellet by pippeting 250 µL in sterile 1.5 mL lock caps microtube. DNA concentration was measured by (GeneQuant 1300, GE) and only samples with DNA yield between 200 and 500 ng/µL were considered as positive enriched and this samples were further evaluated by multiplex PCR.

PCR Amplification

PCR was performed in a volume of 20 µL with primer sequences (Eurofins Genomics) for virulence genes encoding *eae*; shiga-like toxin1 *(stx_1)*, shiga-like toxin 2 *(stx_2)* and fliCh7 of pathogenic *E. coli*. PCR reaction mixture contains 1xPCR buffer, 1.5 mM $MgCl_2$; 200 µM each dNTP, 0.5 µM each primer; 2.0 U Taq polymerase (Fermentas, Lithuania) and 1 µL bacterial DNA. Thermocycler (Quanta Biotech QB-96 thermocycler) was programmed for: initial incubation step of 3 min at 94°C; 35 cycles: 60 sec at 94°C, 90 sec at 60°C, and 90 sec at 72°C and a final extension step of 7 min at 72°C followed by final 4°C. Genomic DNA (IRMM-449, No 0242) of STEC *Escherichia coli* was used as positive control

and PCR mix with deionized water for negative control. *Table 1* presents primers used for target genes of *E. coli* are synthesized in the following sequence.

The PCR products (10 µL with 2 µL gel loading) from amplification were separated by electrophoresis (APELEX ps304, minipac2) on 2% agarose gel in 1xTBE buffer for 90 min at 120 V, stained with ethidium bromide (0.5 mg/mL) and photographed using a gel documentation system (Image Quant 150, ATIR6D, GE Healthcare). The positive PCR was realized as positive bands in size of 775, 302, 516 and 625 for primers eae; stx_1, stx_2 and fliCh7, respectively. DNA ladder 100 bp was used in each agarose gel.

RESULTS

Efforts from our investigation resulted in positive PCR reaction in 28 h after milk samples receiving in a laboratory conditions, which time include sample processing, incubation for enrichment, sedimentation and pellet destruction for DNA extraction and PCR reaction with visualization. The investigation time from milk sampling at farm level to the positive or negative results was nearly 34 h (33-37 h depending the kilometers distances of the farm from the laboratory).

In the milk samples we evaluated more than 39.2% of the samples a positive (22 of 56 milk samples) for *E. coli*, by detection of eae genes. Negative samples were 34 of 56 (60.7%). The most common gene in the milk samples was virulence gene H7, which were detected in 12 (54.5%) of positive milk samples after enrichment. One sample was positive for stx_2 (4.54%) and another two - to stx_1 (9.09%).

Table1. *Primers used for target genes of E. coli*

Gene	Primer Sequence	Reference
eae	< F-5`-TCAATGCAGTTCCGTTATCAGTT-3'; R-5'-GTAAAGTCCGTTACCCCAACCTG-3'>	[10]
stx_1	< F-5'-CAGTTAATGTGGTGGCGAAGG-3'; R-5'-CACCAGACAATGTAACCGCTG-3'>	[10]
stx_2	< F-5'-ATCCTATTCCCGGGAGTTTACG -3'; R-5'-GCGTCATCGTATACACAGGAGC-3'>	[10]
fliCh7	< F-5'-ATCCTATTCCCGGGAGTTTACG-3'; R-5'-GCGTCATCGTATACACAGGAGC-3'>	[11]

Table 2. *Virulence factors detected in milk samples after cell sedimentation, washing and selective broth enrichment*

Milk Samples and *E. coli* Virulence Profile	Detected Genes by Primers Pair			
	stx_1	stx_2	*eae*	H7
Type 1 (n=5)	-	-	+	+
Type 2 (n=5)	-	-	+	+
Type 3 (n=5)	-	+	+	+
Type 4 (n=5)	+	-	+	+
Type 5 (n=5)	+	+	-	+
Type 6 (n=34)	-	-	-	-

On the basis of the presence of each of the target gens we classify the milk in 6 different types. Negative samples are type 6 (n=34) in which no of the genese was detected. In type 1 are samples with genes eae and H7. In types 3 and 4 are samples with detection of one of the *stx* genes. Most unusual are samples (n=2) in the type 5 where no intimin genes was detected *(Table 2)*.

The results on *Fig. 1* present the gene identification in 11 samples (No. 1-11) from two farms.

The data of gene identification demonstrate the presence of amplicons with size 348 bp in strains 4 and 5 and a 482 bp product in milk sample 1, 2, 4 and 7, which corresponded to stx_1 and *eae* A genes. The other isolates did not exhibit presence of amplicons coding both genes.

The negative control (NTC) did not detect any amplification product or other carrier of genetic information. *Fig. 2* presents the gene identification of 11 milk samples from three farms.

The results demonstrated amplicons with size of 584 bp

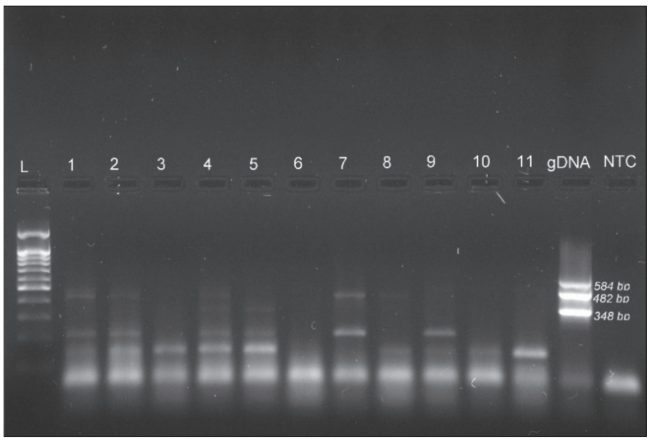

Fig 1. Molecular identification through PCR amplification of virulence genes *eae* A, stx_1, stx_2, in *E. coli* isolates from milk samples No. 1-11

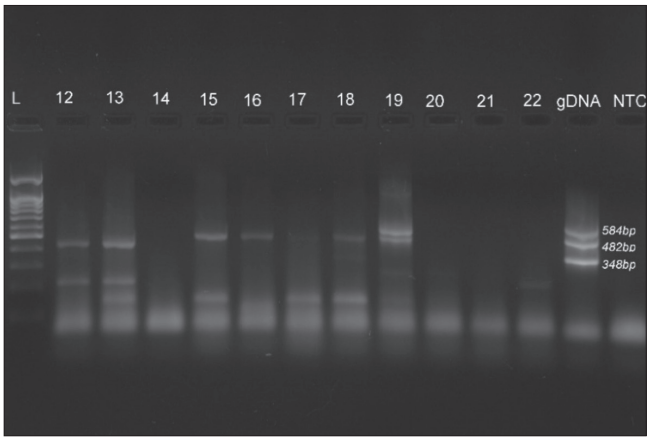

Fig 2. Molecular identification through PCR amplification of virulence genes *eae* A, stx_1, stx_2, in *E. coli* isolates from milk samples No. 12-22

in strain 19 and size of 482 bp in strains 12, 13, 15, 16, 18, 19, corresponding to *stx*$_2$ and *eae* A genes, respectively. The 348 bp product corresponding to stx$_1$ was absent in all milk samples.

The negative control (NTC) did not detect any amplification product or other carrier of genetic information.

Fig. 3 presents the genetic identification of 11 strains (No. 1-11). Except for strains 6, 10 and 11, all other tested isolated showed a 625 bp product equivalent to the H7 gene. The fragments' size was read against a 100 bp DNA ladder and through amplification of a positive DNA control from reference strain ATCC (genome DNA) with primer pairs specific for the H7 gene.

The genetic identification of 11 strains (No.No 12-22) depicted on *Fig. 4* showed amplicons with size of 625 bp, corresponding to H7 gene in 4 milk samples (isolates 12, 15, 18 and 21). The fragment size was evaluated with the help of a DNA ladder and amplification of positive DNA control from reference strain ATCC (genome DNA) with primer pairs specific for the H7 gene.

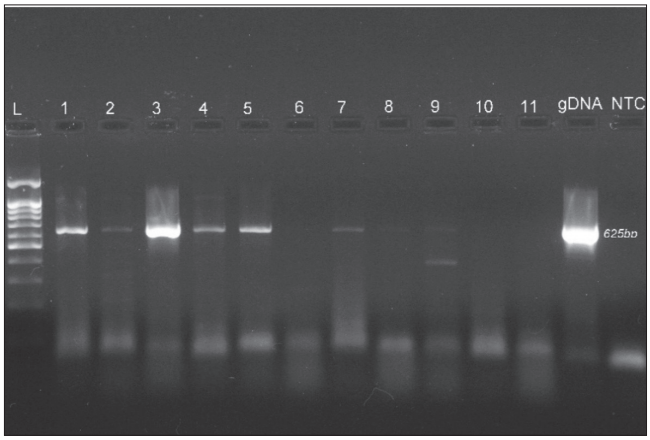

Fig 3. Molecular identification through PCR amplification of virulence gene H7 in *E. coli* isolates from milk samples No. 1-11

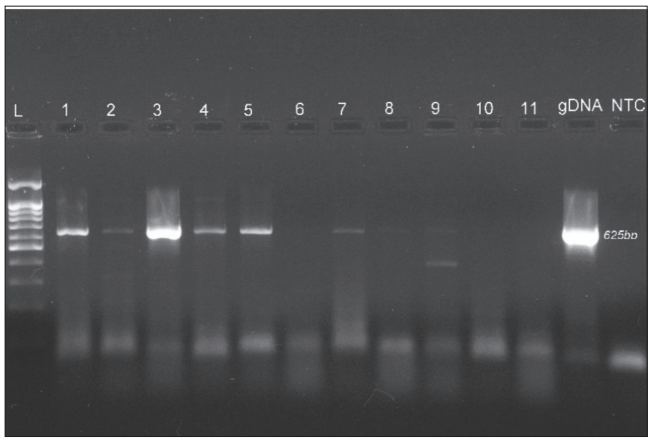

Fig 4. Molecular identification through PCR amplification of virulence gene H7 in *E. coli* isolates from milk samples No. 12-22

DISCUSSION

Unlike the most *E. coli* O157:H7 screening studies, our experimental design was different, as we investigated the presence of genes coding for toxin production directly in milk samples from bulk milk tanks. Dairy farms used as a sampling sources where preliminarily confirmed as positive presence of *E. coli* O157: H7 in milking cows. From the study results we were targeting shorter time for STEC bacterial detection and reliable and faster identification and virulence fingerprinting in the same time of the positive milk samples.

Hlavsa et al.[12] demonstrated that the direct consumption of whole milk obtained under non-hygienic conditions and its use as a source in the production of cheeses increases the risk for infections along the food chain and hence, the risk from a severe disease or death caused by *E. coli* O157: H7 [12].

Our results showing low prevalence of STEC are similar to those reported by Caro et al.[1] from screening sheep milk samples: out of seven cases suspicious for STEC only three *E. coli* O157: H7 isolates have been confirmed. Genetic profiles of the three isolates showed that all were *stx*$_2$-positive, and one was *stx*$_1$-positive. Furthermore, all possessed the *eae* virulence gene coding for intimin synthesis. It should be noted that the medium used for selective enrichments at the time of the initial identification of milk samples was modified tryptone soya broth supplemented with novobiocin in line with our research [1].

McKee et al.[13] screened 420 milk samples from two dairy enterprises in North Ireland and found out that 9 (2.14%) STEC-positive samples, four among which carried only the *stx*$_2$ gene, four carrying both *stx*$_2$ and eae genes, one sample positive for both *stx*$_1$ and *eae* genes - a profile, very similar to that in the present study [13].

A similar experiment with 130 milk samples was investigated by Brenjchi et al.[14]. After selective enrichment, 8 sorbitol-non-fermenting isolates were identified, and using bio-chemical markers, only one was confirmed as *E. coli*. The latter was identified as *E. coli* O157: H7 by means of PCR and virulence gene profiling showed presence of the gene coding from *stx*$_2$ [14].

In a similar research in Libya [15], 108 cow, camel and goat raw milk and soft cheese samples were analysed. Three *E. coli* O157 isolates were confirmed in raw milk - one from cow milk and 2 from goat milk.

Comparable results have also been found in Rahimi et al.[16] in Iran the highest prevalence of *E. coli* O157 was found in samples of water buffalo milk (5.5%), followed by cattle (3.6%) and all 3 isolates from *E. coli* O157: H7 were positive for stx$_1$, stx$_2$, as well as genes [16].

This study emphasised the important of STEC for milk

and dairy products by revealing the potential risk from contamination of the milk with *E. coli* O157. The role of dairy cows in the epidemiology of STEC-induced human pathology and especially the role of serotype O157:H7 was confirmed. This necessitates strict biosecurity measures and good hygiene practices on farms to prevent the risk from contamination of milk at the time of its production and storage. Additionally, measures aimed at reduction of the possibility for transfer of strains among the farms are also needed.

Conflict of Interests

None of the authors had any conflict of interests in the writing of this paper.

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Case Report

Insect Bite Hypersensitivity (Sweet Itch) in a Non-Descript Riding Local Breed Mare

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Abstract

Summer itch or insect bite hypersensitivity is common problem during summer in all animals. Some horses are allergic to insect saliva. A 13 year old mare kept for riding purpose at livestock farm FVS, BZU, Multan was examined with a condition characterized by little bumps, papules and abrasions on neck, abdomen, thighs and tail, anaemic mucous membranes, concentrated urine, partial anorexia and generalized emaciation. There was a history of chronic lesion of about more than one year old and many therapies were tried but all non-responsive. A mixed biting infection of *Tabanus* spp. and *Stomoxys* spp. was identified. On the basis of clinical sign(s), history and fly identification a diagnosis of insect bite hypersensitivity was detected. Treatment protocol was done in two rounds i.e. first round of treatment was done with dexamethasone along & vitamin E administrated through intramuscular route for 5 days and followed by 10 mg dexamethasone orally for one month. Animal respond the treatment and there was full recovery from condition and on follow up no relapse of the condition was observed.

Keywords: *Tabanus, Stomoxys, Mare*

Yerel Irk Bir Kısrakta Böcek Sokmasına Bağlı Aşırı Duyarlılık (Tatlı Kaşıntı)

Öz

Yaz kaşıntısı veya böcek sokması aşırı duyarlılığı tüm hayvanlarda yaz aylarının yaygın bir problemidir. Bazı atlar böcek salyasına alerjiktir. FVS, BZU, Multan'da bir çiftlikte binek amaçlı tutulan 13 yaşlı bir kısrakta boyun, abdomen, uyluk ve kuyrukta küçük şişkinlikler, papüller ve abrazyonlar, anemik mukoz membranlar, konsantre idrar, kısmi iştah kaybı ve generalize zayıflama bulguları gözlemlendi. Yaklaşık bir yıldan daha uzun süredir devam eden kronik lezyon tablosu olup birçok tedavi uygulanmış ancak hepsi cevapsız kalmıştı. *Tabanus* spp. ve *Stomoxys* spp.'nin her ikisinin sokmasına bağlı enfeksiyon belirlendi. Klinik bulgular, hastalık hikâyesi ve sinek tespit edilmesine dayanarak böcek sokması aşırı duyarlılığı tanısı koyuldu. Tedavi iki basamaklı olarak gerçekleştirildi. Birinci basamakta 5 gün dekzametazon ile birlikte kasiçi vitamin E uygulaması yapılırken ikinci basamakta 10 mg dekzametazon oral yolla bir ay uygulandı. Hayvan tedaviye yanıt vererek hastalığı tamamen atlattı ve takipte hastalık tekrarı gözlenmedi.

Anahtar sözcükler: *Tabanus, Stomoxys, Kısrak*

INTRODUCTION

Insect bite or arthropod bite causes an allergic response in the body due to provoking of allergic response in the skin [1]. This allergy is mostly called allergic dermatitis allegedly caused by *Culicoides* spp. by the evoking of T cells (Th2 and T reg) [2]. As the horses are very sensitive to various salivary proteins and Airway hyperactivity sensitive [3]. The disease like Insect bite Hypersensitivity (IBH) may find its some clues in horse genetics too [4]. This is a case report of hypersensitivity in teaching hospital a 13 year old local breed mare kept for riding purpose at Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan Pakistan which is caused by *Tabanus* spp. and *Stomoxys* spp. as unusual one.

CASE HISTORY

A 13 year old mare kept for riding purpose at Livestock farm FVS, BZU, Multan was examined with a condition

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characterized by little bumps, papules and abrasions *(Fig. 1)* on neck, abdomen, thighs and tail, anaemic mucous membranes, concentrated urine, partial anorexia and generalized emaciation. There was a history of chronic lesion of about more than one year old and many topical therapies including Betnovit ointment at local area and ivermectin injection were tried but all non-responsive. A mixed biting infection of *Tabanus* spp. and *Stomoxys* spp. was identified on the basis of their specific biting site(s) and pattern. On the basis of clinical sign(s), history and fly identification a diagnosis of insect bite hypersensitivity was detected. Treatment protocol was done in two rounds i.e. first round of treatment was done with dexamethasone along & vitamin E administrated through intramuscular route for 5 days and in round two treatments by 10 mg dexamethasone orally for one month. The blood parameters (WBC, Hb, PCV, RBC) were analyzed through hematology analyzer NIHON KOHDEN MEK 6450K and physical parameters like TPR (body temperature, pulse and respiration rate) were recorded. The different blood and physical parameters were in normal limit before and after treatment. After the due treatment animal responded the treatment and there was full recovery from condition(s) *(Fig. 2)* and on follow up no relapse of the condition was observed even after a year. Repeated Topical application with trichlorfon 0.2% solution proved effective to avoid the recurrent attacks of biting flies.

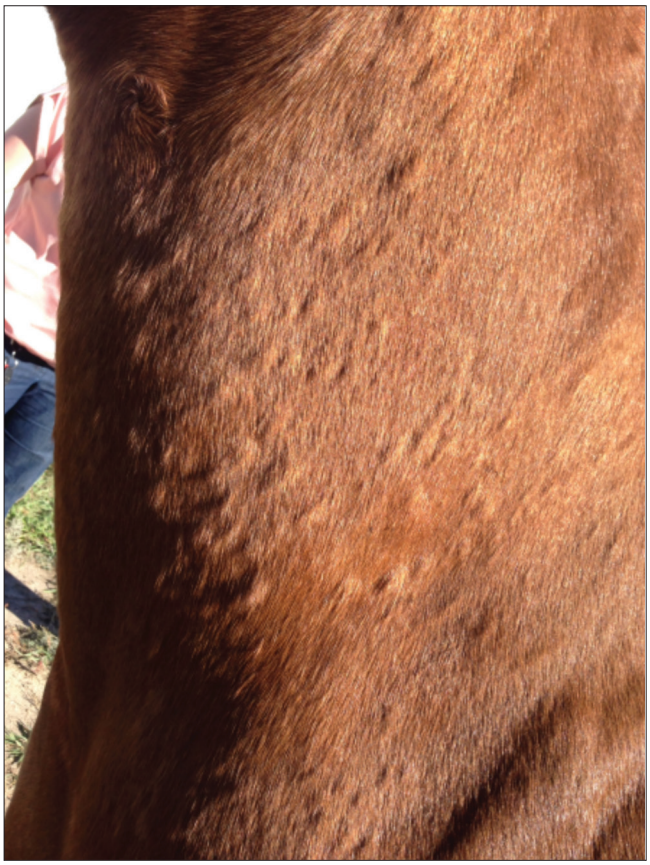

Fig 1. Before treatment

Fig 2. After treatment

DISCUSSION

Insect bite hypersensitivity (IBH) is very common in horses reared in hot and temperate climate where biting flies are commonly observed like *culicoides*, *tabanus* and *stomoxys* spp. it is very hard to find the exact modus operandi that how the response was provoked but involvement of certain helper cells (Th cells and T reg), interleukins (IL-25 and IL-33) along with innate cytokines of epithelium and epidermis with the collaboration of Thymic Stromal Lymphopoietin (TSLP) play a vital role in dermatitis specially in actopic dermatitis and asthma [5]. ITLN knockdown cells are prerequisite for the induction of phosphorylation for epidermal growth factor receptor (EGFR) and extracellular-signal regulated kinase (ESRK) required by interleukins and suppresses IL-33, Tslp and Th2 along with eosinophilic involved inflammation [5] which clearly demonstrates the contribution of ITLN1 induced hypersensitivity in horses and humans and the similar phenomenon has been observed in pollen allergy [6]. It has been widely accepted that allergic response occurs in all domestic animal along with humans which is mediated by IgE Abs which binds to mast cells and help to release or synthesise a progeny of potent mediators (PM) [7]. It is main objective of this study to treat such filed problems which are commonly observed in horses. Regarding the therapy against urticarial or allergy either insect bite hypersensitivity, food borne allergies, dust mite allergies or pollen they are multifactorial even including genes [7,8] and the ever best option is avoidance of that factor(s) to suppress or meltdown IgG and IgE Abs [9] but the other options like topical application of pesticides can be a good option [10]. After a follow-up of a year post-treatment there was no sign of reoccurrence of the problem in local breed mare despite of unavailability of any specific treatment for IBH [11]. The available treatment with cortisones suppressed the factors involved in sensitivity along with histamine release factors controlled the exaggerating situation proved already in a study [12,13]. The vitamin E

is now routinely used in the treatment of skin allergies along with other problems. Vitamin E plays a major role in provoking immune system, anti-oxidant, anti-allergic, actopic dermatitis and cell function. It is lipid soluble non enzymetic vitamin. It is first report from Pakistan on IBH or Summer Itch here we can suggest about genetic analysis [14] of various horses before purchasing and possible available treatment regime with cortisones and antihistaminic substances.

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Letter to the Editor

An Extraordinary Fetal Death: Inguinal Hernia in a Terrier Dog [1]
(Sıradışı Bir Fötal Ölüm: Terrier Bir Köpekte İnguinal Herni)

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Dear Editor,

Congenital or acquired inguinal hernias occur as a result of subcutaneous involvement of abdominal organs due to defects in the inguinal region [1,2]. The inguinal canal is anatomically open in carnivors [3]. The inguinal canal is shorter and wider in female dogs than male [4]. Young dogs have processus vaginalis in the inguinal canal. The only animal species that have processus vaginalis are female dogs. The inguinal canal extends backwards from down the processus vaginalis. Ligamentum teres uteri adheres to the distal end of this processus. In pregnancy, this ligament is stretched, causing the uterus to be drawn into the vaginal pouch and it causes hernia [3]. In addition to anatomic factors, metabolic and hormonal factors play a role in the formation of inguinal hernia. The vaginal processus dilates as the lipoidosis increases around the ligament, and the inguinal canal allows herniation [2]. Although it is not known exactly, it is mostly seen in middle-aged and non-castrated female dogs [1]. Uterus, urinary bladder, colon, omentum and spleen are also herniated into inguinal canal [1,2,4]. Pregnant uterus is mostly confined to this canal [4]. Uterus hernia is chronic [5] and usually has no clinical symptoms until pregnancy [4,6] or pyometra development [5].

The case presented to Kafkas University, Faculty of Veterinary Science, Obstetrics and Gynecology Clinics was about a Terrier dog (age: 4 year, bw: 8 kg) brought with the complaint of swelling in the left inguinal breast. In medical history it was inquired that there was a small, droopy, non-painful bulk in inguinal region of this dog which was adopted about 6 months ago but this bulk grew rapidly in the last 20 days. The dog was found to copulate over a month ago. It was determined that the dog had anorexia but the general condition was not yet affected. Clinical examination revealed normal body temperature, respiratory rate and pulse rate of the dog. No abnormal structure (pyometra, pregnancy, etc.) was found in the ultrasonographic examination of the abdominal region. However, pain was determined in palpation of the left inguinal breast lobe and the bulk was examined by ultrasonography. Amniotic fluid, fetus and fetal heart beats were viewed on USG. An appointment was given to perform the operation on the next day to ensure that the dog became suitable for operation conditions. However, the owner did not bring the dog on the appointment date but about a month later. In the second examination, it was determined that Amniotic fluid had decreased and there was no heart beat. The dog was immediately taken into operation. After premedication (0.04 mg/kg, Atropine sulfat, i.m., Vetas Atropine®, Vetas, Turkey), dissociative anesthesia [1-2 mg/kg, xylazine HCl (Rompun®, Bayer, Turkey), 10 mg/kg, ketamine HCl (Ketasol® 10%, Interhas, Turkey)] were applied. The patient was placed on her back and the ventral abdomen was prepared according to the standard operating conditions under aseptic conditions. From the anterior part of the left inguinal breast incision was made and the hernia sac was reached. It was seen that her left cornu was herniated. It was determined that there was no abnormal temperature and color change in this cornu. It was found that the right cornu was in the abdominal cavity, there was no pregnancy and ovario-hysterectomy was performed *(Fig. 1)*. Postoperative 5-day antibiotic treatment (250 mg, i.m., Iespor®, I.E. Ulugay, Turkey) was applied. In the first week following the operation, the general condition of the dog was found to be good and within 2 months after the operation there was no recurrence.

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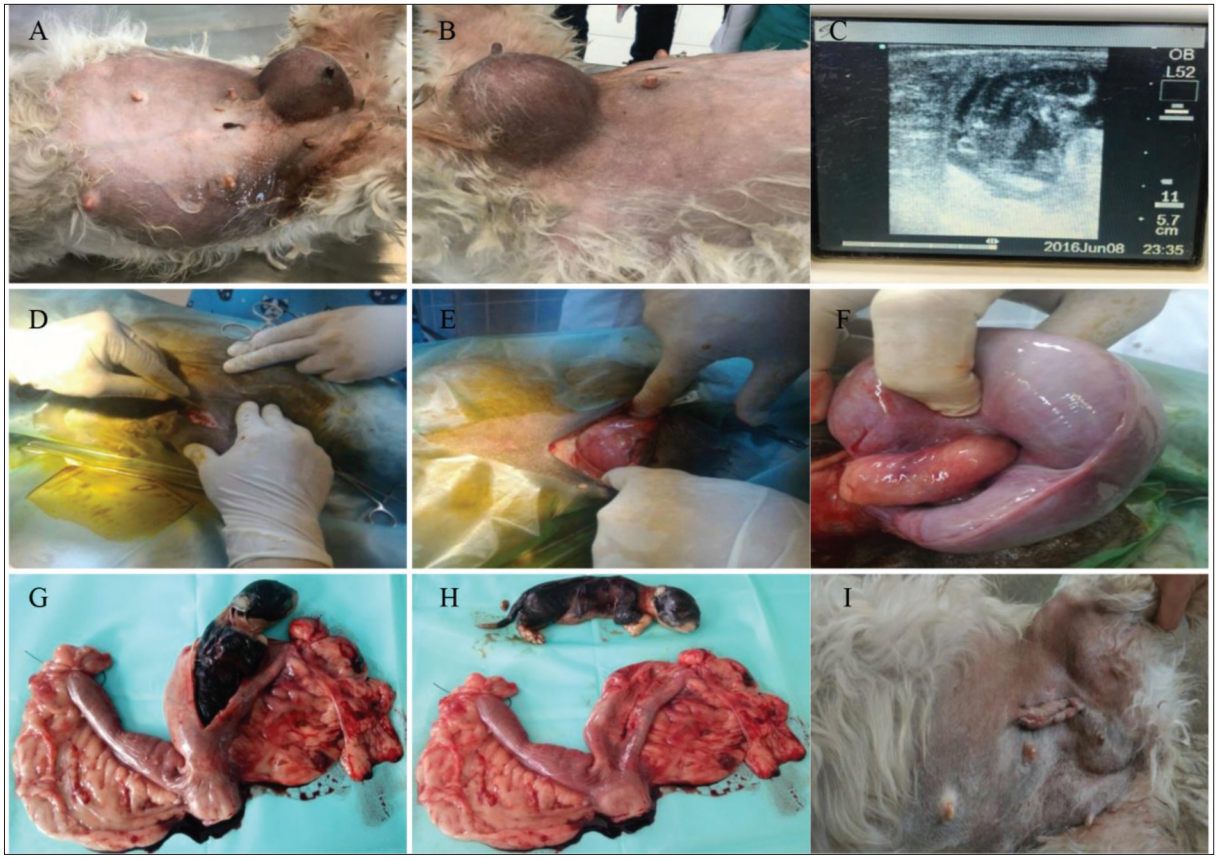

Fig 1. Ultrasonographic image (C) of a pregnant dog of Terrier breed brought to our clinic with complaint of left inguinal breast swelling (A-B), operative intervention (D-F,I) and findings of fetus and uterus from herniated region (G-H)

In conclusion, despite the growth of the fetus in inguinal hernia of uterine horn, the inability of the inguinal ring to expand will lead to a lack of adequate blood flow in the region and the formation of incarceration. This may lead to a reduction in the chance of survival and endanger the mother's life. Ovariohysterectomy was found to be the most appropriate treatment option in such cases.

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