ISSN 1300 - 6045
e-ISSN 1309 - 2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Journal of the Faculty of Veterinary Medicine, Kafkas University

Published Bi-monthly

http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Volume: 25 | Issue: 5 SEPTEMBER - OCTOBER | Year: 2019

ISSN: 1300-6045
e-ISSN: 1309-2251

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JOURNAL OF THE FACULTY OF VETERINARY MEDICINE, KAFKAS UNIVERSITY

(SEPTEMBER - OCTOBER)

Volume: 25 Number: 5 Year: 2019

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ELEKTRONİK BASKI (ELECTRONIC EDITION)
http://vetdergikafkas.org

ONLINE MAKALE GÖNDERME (ONLINE SUBMISSION)
http://submit.vetdergikafkas.org

Bu dergi Kafkas Üniversitesi Veteriner Fakültesi tarafından iki ayda bir yayımlanır
This journal is published bi-monthly, by the Faculty of Veterinary Medicine, University of Kafkas

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Kafkas Univ Vet Fak Derg
25 (5): 589-596, 2019
DOI: 10.9775/kvfd.2018.21183

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

Research Article

Study on the Antibody Level Differences of PED IgG and IgA, TGE IgG and PoR IgG After Immunization with Different Porcine Viral Diarrhea Vaccine Combinations

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Article ID: KVFD-2018-21183 Received: 15.10.2018 Accepted: 29.04.2019 Published Online: 09.05.2019

How to Cite This Article

Chen QY, Chen RL, Wu XM, Che YL, Hou B, Wang CY, Wang LB, Zhou LJ: Study on the antibody level differences of PED IgG and IgA, TGE IgG and PoR IgG after immunization with different porcine viral diarrhea vaccine combinations. *Kafkas Univ Vet Fak Derg,* 25 (5): 589-596, 2019. DOI: 10.9775/kvfd.2018.21183

Abstract

To prevent porcine viral diarrhea, a vaccine combination that can provide good antibody levels needs to be determined. In this study, we screened 30 pregnant sows divided into six experimental groups, namely, five immunized groups and one control group, to investigate the antibody level differences of different vaccine combinations on porcine epidemic diarrhea (PED), porcine transmissible gastroenteritis (TGE), porcine rotavirus (PoR) IgG, and PED IgA. The antibody level was detected by ELISA. Results showed that the antibody levels of PED and TGE IgG in serum and PED IgA in breast milk of the "PT+PT*" vaccine combination group were higher than those of the other groups, and vaccine combination including "PTR" could stimulate the sows to produce PoR IgG antibody. These findings revealed that the vaccine combination of "PT+PT*" is optimal for preventing porcine viral diarrhea, and "PTR+PT*" may be an alternative option in areas under PoRV infection risk. This study suggested that pig farms should select suitable immunization on the basis of the local epidemic situation of porcine viral diarrhea.

Keywords: *Antibody level differences, Porcine epidemic diarrhea IgG and IgA, Porcine transmissible gastroenteritis IgG, Porcine rotavirus IgG, Vaccine combination, ELISA detection*

Farklı Domuz Viral Diyare Aşı Kombinasyonları İle İmmunizasyon Sonrası PED IgG ve IgA, TGE IgG ve PoR IgG Antikor Seviyelerindeki Farklılıkların Araştırılması

Öz

Domuz viral diyareyi önlemek için iyi antikor seviyesi sağlayan bir aşı kombinasyonunu belirlemeye ihtiyaç bulunmaktadır. Bu çalışmada, farklı aşı kombinasyonlarının domuz epidemik diyare, domuz bulaşıcı gastroenteritisi ve domuz rotavirus IgG seviyeleri ile domuz epidemik diyare IgA seviyesine etkilerini araştırmak amacıyla 30 gebe domuz 5'i immunize grup 1'i kontrol olmak üzere altı gruba ayrıldı. Antikor seviyeleri ELISA ile belirlendi. Elde edilen sonuçlar, domuz epidemik diyare ve domuz bulaşıcı gastroenteritisi serum IgG seviyeleri ile meme sütünde domuz epidemik diyare IgA seviyesinin "PT+PT*" aşı kombinasyonu grubunda diğerlerinden daha yüksek olduğunu ve "PTR"yi içeren aşı kombinasyonunun domuzları domuz rotavirus IgG antikoru üretmek üzere stimüle ettiğini gösterdi. Bu sonuçlar "PT+PT*" aşı kombinasyonunun domuz viral diyareyi önlemede en iyi olduğunu ve "PTR+PT*"in domuz rotavirus enfeksiyon riski bulunan bölgelerde bir alternatif olabileceğini gösterdi. Çalışma sonucunda domuz viral diyarenin bölgesel epidemik durumuna göre domuz çiftliklerinin immunizasyon seçiminde bulunması önerilir.

Anahtar sözcükler: *Antikor seviyesi farklılığı, Domuz epidemik diyare IgG ve IgA, Domuz Bulaşıcı Gastroenteritisi IgG, Domuz rotavirus IgG, Aşı kombinasyonu, ELISA tespiti*

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV), porcine transmissible gastroenteritis virus (TGEV), and porcine rotavirus (PoRV) are the three main pathogens causing viral diarrhea in pigs. They can infect pigs of all ages and causes watery diarrhea, vomiting, dehydration, and gradual weight loss [1]. In recent years, porcine viral diarrhea diseases have shown mixed infections with multiple pathogens. Additionally, considering new problems, such as the epidemics of variant PEDV, the protective rate of porcine viral diarrhea vaccines has decreased. The immune protection effect of

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many immune pig farms has not reached the desirable expectation [2,3], causing huge economic losses to the pig breeding industry in China.

Porcine epidemic diarrhea virus is an enveloped, single-stranded, positive-sense RNA virus belonging to the order Nidovirale, the family Coronaviridae, subfamily Coronavirinae, and genus *Alphacoronavirus* [4]. Sequencing and genotyping based on the S gene is suitably used for molecular epidemiology analysis and vaccine development of PEDV [5,6]. Phylogenetic analysis of the full-length S gene inferred by a neighbor-joining method indicates that PEDV could be genetically divided into two groups, which include GI and GII. GI and GII can be further divided into subgroups Ia and Ib, and IIa and IIb [3].

Transmissible gastroenteritis virus is a member of the enteropathogenic *alpha-coronavirus* family, with a large positive-stranded RNA genome [7]. And it is currently divided into two distinct genogroups: the Miller cluster and the Purdue cluster, most of strains isolated since 2010 from China has a close relationship with the Purdue strain and is more distant evolutionarily from the Miller strains group [8]. PoRV is double-stranded RNA (dsRNA) viruses with 11 genomic segments encoding 6 structural viral proteins (VP1-VP4, VP6, VP7) and 5 or 6 nonstructural proteins, is a member of *Rotavirus* genus, within the *Reoviridae* family [9].

Vaccination has been used for many years in China, and vaccines used include inactivated and live-attenuated vaccines. There are three main commercial vaccines include: A genotype Ia strain CV777-based attenuated trivalent vaccine was licensed by the Harbin Veterinary Research Institute, Chinese Academy of Agriculture Sciences in 2014. A genotype II a strain ZJ08-based attenuated bivalent vaccine was licensed by the Beijing Dabeinong Technology Group Co., Ltd. in 2015. A genotype II b strain AJ1102-based inactivated bivalent vaccine developed by Wuhan Keqian Biology Co., Ltd. in 2016.

At present, A consensus of commercial vaccines for preventing and controlling porcine viral diarrhea has been reached regarding the immunization time of commercial vaccines, but the differences of antibody levels of different vaccine combinations have not been further studied. In this study, pregnant sows were immunized with different vaccine combinations. The blood samples and breast milk were collected at different stages, and relevant specific antibody levels were obtained to analyze the differences in antibodies between different vaccine combinations. Results of this study provided an experimental basis for viral diarrhea vaccine immunization in pig farms.

MATERIAL and METHODS

Ethical Statement

All experimental procedures involving pigs were performed in accordance with the regulations of the Administration of Affairs Concerning Experimental Animals, approved by Laboratory Animal Bioethics Committee of Institute of Animal Husbandry and Veterinary Medicine in accordance with animal ethics guidelines and approved protocols. The approval numbers of the ethics committee are IAHV-AEC-2018-0126.

Experimental Sows and Sites

A total of 30 "Landrace ×Yorkshire" gestation sows with similar gestational ages were screened for this study. The sows' feces and blood samples were collected to confirm that pregnant sows were PEDV, TGEV, and PoRV pathogen negative by RT-PCR, and serological antibody levels were consistent via ELISA before immunization. The experimental sows and sites were provided by the Farm of Fujian Academy of Agricultural Sciences.

Experimental Vaccines and Main Reagents

PEDV-TGEV-PoRV (CV777 + H + NX-G5) trivalent attenuated vaccine (PTR for short), PEDV-TGEV (ZJ08 + HB08) duple attenuated vaccine (PT for short), and PEDV-TGEV (AJ1102 + WH-1) duple inactivated vaccine (PT* for short) were employed. The PED, TGE, and PoR ELISA antibody detection kits (batch numbers: 20170526, 20170713, 20170526) were purchased from Harbin Animal Biological Products National Engineering Research Center Co., Ltd., China. The PED IgA ELISA antibody detection kit was purchased from BIONOTE Biotechnology Co., Ltd., Korea.

Experimental Sow Groups and Immunization Procedures

The 30 gestational sows were randomly divided into six experimental groups, with five gestational sows in each group. Five immunization groups and one control group were established. The sow immunization schedule is shown in *Table 1*. All sows were vaccinated by injection at Houhai acupoint twice at 40 days and 20 days before farrowing. In group A, the sows were vaccinated PTR and PT*, respectively. In group B, the sows were vaccinated PTR and PTR, respectively. In group C, the sows were vaccinated PT and PT*, respectively. In group D, the sows were vaccinated PT and PT, respectively. In group E, the sows were vaccinated PT* and PT*, respectively. And the sows in control group were injected with 4 mL sterile 0.9% NaCl.

Collection of Serum and Breast Milk Samples from Immunized Sows

About 5 mL of blood samples were collected from each experimental group through the ear vein at 0, 21, 35, and 49 days post-first immunization. The collected blood samples were centrifuged to obtain the supernatant, which was stored at -20°C before use. Approximately 2 mL of breast milk was collected from each experimental group at 1, 3, 7, and 14 days post-delivery, centrifuged

Table 1. *Sow immunization schedule*

Groups	The First Immunization Time	Vaccine Species	Immunization Dose	The Second Immunization Time	Vaccine Species	Immunization Dose	Immunization Pathways
A	Prenatal 40d	PTR	Attenuated vaccine 1 dose	Prenatal 20d	PT*	Inactive vaccine 4 mL	Houhai acupoint injection
B	Prenatal 40d	PTR	Attenuated vaccine 1 dose	Prenatal 20d	PTR	Attenuated vaccine 1 dose	Houhai acupoint injection
C	Prenatal 40d	PT	Attenuated vaccine 1 dose	Prenatal 20d	PT*	Inactive vaccine 4 mL	Houhai acupoint injection
D	Prenatal 40d	PT	Attenuated vaccine 1 dose	Prenatal 20d	PT	Attenuated vaccine 1 dose	Houhai acupoint injection
E	Prenatal 40d	PT*	Inactive vaccine 4 mL	Prenatal 20d	PT*	Inactive vaccine 4 mL	Houhai acupoint injection
F	Prenatal 40d	0.9% NaCl	0.9% NaCl 4 mL	Prenatal 20d	0.9% NaCl	0.9% NaCl 4 mL	Houhai acupoint injection

to remove the cream to obtain whey, and stored at -20°C before use.

ELISA Antibody Detection Method

Detection of PED, TGE, and PoR IgG antibodies in serum were carried out according to the instructions of each ELISA antibody detection kit. PED IgA antibody in breast milk was detected following the kit instructions of BIONOTE Biotechnology Co., Ltd., Korea. S/P values for PED, TGE, and PoR IgG antibodies were calculated as follows: (Mean OD_{450nm} of sample - Mean OD_{450nm} of the standard negative control)/(Mean OD_{450nm} of the standard positive control - Mean OD_{450nm} of the standard negative control). The judging criteria were as follows: PED, TGE, and PoR IgG antibody S/P value ≥0.4 is positive; PED IgA antibody cut off value = 0.35 + mean OD_{450nm} of the standard negative control, mean OD_{450nm} of the sample above the cut off value is positive, and mean $OD_{450nm}$of the sample less than the cut off value is negative. The rest of the operating procedures and conditions for establishment were performed in accordance with corresponding kit instructions.

Statistics

We used SPSS16.0 and Excel 2010 for data statistics and charting. Data are presented as mean ± standard deviation (SD). Statistical significance was calculated using a one-way analysis of variance (ANOVA) that was applied for multiple comparisons between the groups. The significance was considered as significant at $P<0.05$ and highly significant at $P<0.01$.

RESULTS

The blood samples of the six groups of experimental sows were obtained at different times after immunization, and the PED IgG antibodies were detected by ELISA. The results showed *(Fig. 1A)* that the PED IgG antibody levels of sows varied at 21 days post-first immunization. The antibody levels increased with different degrees in groups A, B, C, and D and decreased in groups E and F. The PED IgG antibody levels in groups C and D were significantly different from those in group E and control group F ($P<0.05$) *(Table 2)*. Moreover, the IgG antibody levels of groups C and D were higher than those of groups A and B, but those of groups C and D were not significantly different from those of groups A and B ($P>0.05$) .

After secondary immunization, the PED IgG antibody levels of sows increased at 35 days post-first immunization in groups A, C, and E due to PT* *(Fig. 1A)*. The three groups reached the peak value, and the order of S/P value from highest to lowest was groups C, A, and E. Unfortunately, after sows were boosted by PTR in group B and PT in group D, the PED IgG antibody level of the sows decreased. Group B was significantly different from groups A ($P<0.05$), C ($P<0.01$), and E ($P<0.05$), but no significant difference was observed among A, C, and E ($P>0.05$). Thus, the immunization of secondary booster with attenuated vaccines resulted in the decrease in PED IgG antibody levels. The PED IgG antibody level of sows in the control group F showed a downward trend.

At 49 days post-first immunization, the PED IgG antibody levels of sows in all groups showed a decreasing trend. The order of S/P values from highest to lowest was C, D, A, E, B, and F. The above results showed that different vaccine combinations and PEDV vaccine strains exerted certain effects on the PED IgG antibody level in the sows.

On the basis of PED IgG antibody growth and decline after immunization *(Fig. 2A)*, we found that the PED IgG antibody level showed a change law of "rise, rise, and decline" in groups that used the vaccine combination of "attenuated vaccine + inactivated vaccine" (groups A and C). In groups that used the vaccine combination of "attenuated vaccine + attenuated vaccine" (groups B and D), the PED IgG antibody level showed a change law of "rise, decline, and decline". With the vaccine combination of "inactivated vaccine + inactivated vaccine" (group E), the PED IgG antibody level showed a change law of "decline,

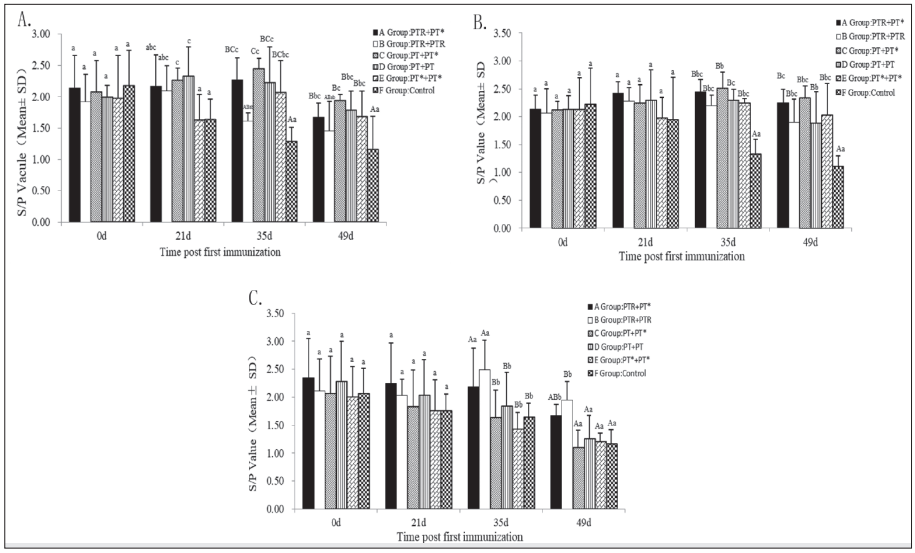

Fig 1. Specific IgG antibody responses in sows serum. The sows (n=5 per group) were vaccinated with five vaccine combinations or sterile 0.9% Nacl. Serum samples were collected at 0, 21, 35, and 49 days post-first immunization. ELISA antibody titers were measured by an indirect ELISA. Each bar represents mean + SD. Statistical significance was calculated using a one-way analysis of variance (ANOVA). Different uppercase letters on the S/P value bars indicate that the difference is extremely significant (P<0.01), and different lowercase letters indicate significant difference (P<0.05). (A) PED, (B) TGE, (C) PoR

Fig 2. Growth and decline of specific antibody in sows after vaccinated with five vaccine combinations or sterile 0.9% Nacl. (A) PED-specific IgG antibodies in serum. (B) TGE-specific IgG antibodies in serum. (C) PoR-specific IgG antibodies in serum. (D) PED-specific IgA antibodies in breast milk

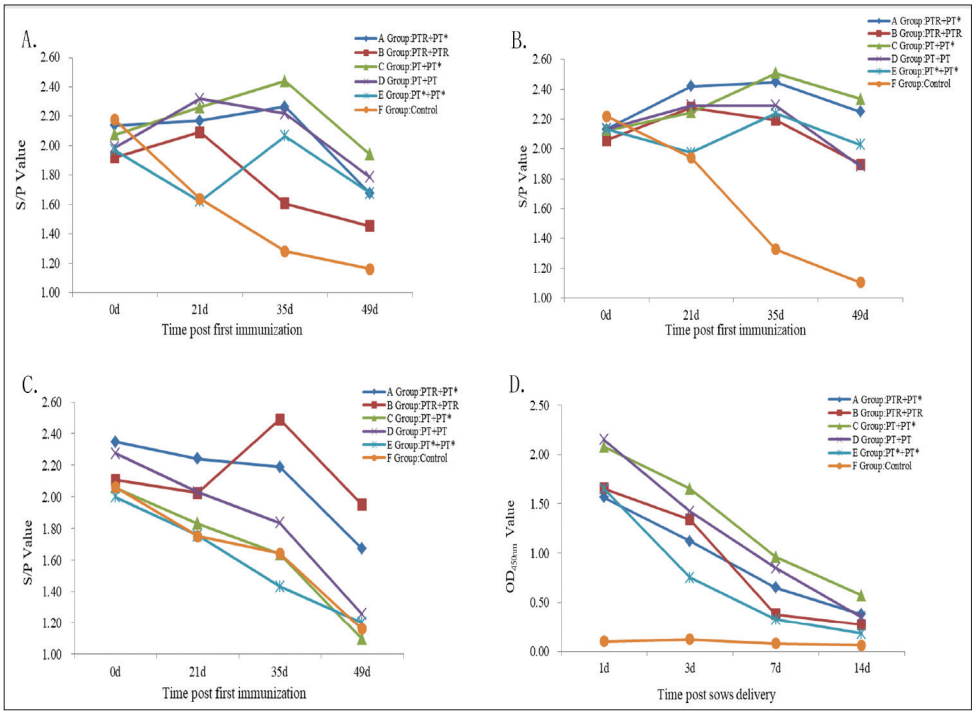

Table 2. *The detection results of PED IgG antibody in different vaccine combinations*

Groups	S/P Value (Mean±SD)/Time			
	1d	21d	35d	49d
A	2.14±0.52^{a}	2.17±0.50^{abc}	2.27±0.35^{BCc}	1.68±0.22^{Bbc}
B	1.92±0.44^{a}	2.09±0.40^{abc}	1.61±0.14^{ABab}	1.45±0.47^{ABab}
C	2.08±0.50^{a}	2.26±0.19^{c}	2.44±0.17^{Cc}	1.94±0.10^{Bc}
D	1.99±0.20^{a}	2.32±0.48^{c}	2.22±0.57^{BCc}	1.79±0.30^{Bbc}
E	1.97±0.69^{a}	1.62±0.41^{a}	2.07±0.51^{BCbc}	1.68±0.41^{Bbc}
F	2.18±0.56^{a}	1.64±0.33^{a}	1.28±0.24^{Aa}	1.66±0.53^{Aa}

Different uppercase letters on the S/P or OD450nm value columns indicate that the difference is extremely significant (P<0.01), and different lowercase letters indicate significant difference (P<0.05)

Table 3. *The detection results of TGE IgG antibody in different vaccine combinations*

Groups	S/P Value (Mean±SD)/Time			
	1d	21d	35d	49d
A	2.13±0.25^{a}	2.42±0.21^{a}	2.45±0.22^{Bbc}	2.25±0.24^{Bc}
B	2.06±0.44^{a}	2.28±0.24^{a}	2.19±0.19^{Bbc}	1.90±0.41^{Bbc}
C	2.12±0.15^{a}	2.24±0.33^{a}	2.51±0.29^{Bb}	2.33±0.22^{Bbc}
D	2.13±0.25^{a}	2.29±0.55^{a}	2.29±0.20^{Bc}	1.88±0.57^{Bb}
E	2.13±0.56^{a}	1.98±0.37^{a}	2.32±0.09^{Bbc}	2.03±0.56^{Bbc}
F	2.22±0.65^{a}	1.94±0.76^{a}	1.33±0.26^{Aa}	1.11±0.19^{Aa}

Different uppercase letters on the S/P or OD450nm value columns indicate that the difference is extremely significant (P<0.01), and different lowercase letters indicate significant difference (P<0.05)

rise, and decline" whereas the control group (group F) showed a trend of "decline, decline, and decline". These results indicated that the vaccine combination of "attenuated vaccine + inactivated vaccine" showed better immune effects than the other combinations, and group C demonstrated improved PED IgG maternal antibody for suckling piglets.

The TGE IgG antibody detection results showed that the TGE IgG antibodies levels in groups A, B, C, and D increased at 21 days post-first immunization *(Fig. 1B)*, and the order of S/P values from highest to lowest was A, D, B, and C. Inversely, the TGE IgG antibody levels in groups E and F decreased. However, the difference among six experimental groups was not significant (P>0.05) *(Table 3)*.

At 35 days post-first immunization, the results of TGE IgG antibody levels showed that the groups that used PT* booster immunization for the second immunization (groups A, C, and E) had higher TGE IgG antibody levels than groups B and D, which used PTR or PT, respectively. The order of peak S/P values of TGE IgG antibody in each group from highest to lowest was C, A, E, D, and B. The difference between groups C and D was significant (P<0.05), whereas groups A and C showed no significant difference (P>0.05).

The antibody levels of TGE IgG decreased in all groups at 49 days post-first immunization. The above results indicated that the difference in the antibody level of TGE IgG between the groups was mainly caused by the combination of attenuated vaccine or inactivated vaccine.

After immunization with different vaccine combinations, the results of the growth and decline law of TGE IgG antibody levels showed that the groups showed a trend of "rise, rise, and decline" *(Fig. 2B)*, which used the "attenuated vaccine + inactivated vaccine" combination (groups A and C). In the groups that used the combination of "attenuated vaccine + attenuated vaccine" (groups B and D), the growth and decline of TGE IgG antibody levels showed a law of "rise, fall (or plateau), and decline". Moreover, the TGE IgG antibody level of group E that used "inactivated vaccine + inactivated vaccine" showed a law of "decline, rise, and decline" but a decreasing trend in control group F. These results showed some differences in the growth and decline law of TGE IgG antibody among vaccine combinations, and the vaccine combination of groups A and C was suitable in practice. However, the difference between groups A and C was not significant (P>0.05), thereby indicating that the TGE IgG antibody level was not significantly correlated with the TGEV vaccine strain. Concurrently, the results showed that the second booster immunization could reduce the TGE IgG antibody levels of sows by using the "attenuated vaccine + attenuated vaccine" combination.

The PoR IgG antibody detection results showed that the PoR IgG antibody levels of sows in each group decreased to some extent at 21 days post-first immunization *(Fig. 1C)*. In the groups that used the triplex attenuated vaccine containing PoRV (groups A and B), the PoR IgG antibody levels of the sows decreased to a lesser extent than in the other groups. The antibody level of the sows in group F decreased the most, and the decline in the antibody level of the sows in groups A and B did not significantly differ (P>0.05) *(Table 4)*.

The PoR IgG antibody levels of the sows in groups C, D, E, and F remained decrease at 35 days post-first immunization but peaked in group B, which showed an extremely significant difference (P<0.01) from groups C, D, E, and F. The PoR IgG antibody levels of the sows in group A decreased, similar to those in other groups (groups C, D, E, and F) at 49 days post-first immunization. However, the reduction in group A was lower (P>0.05) than that in group B and significantly different compared with that in groups C, D, E, and F (P<0.05). The PoR IgG antibody level of group B was highest compared with those of group A and other groups. Thus, the triplex attenuated vaccine that included PoRV had a certain immunizing effect in stimulating the production of PoR IgG antibodies in the sows.

After immunization with different vaccine combinations, the PoR IgG antibody levels in the group immunized with the "PTR+PTR" vaccine combination (group B) demonstrated a change trend of "decline, rise, and fall" *(Fig. 2C)*. In the other immune combinations (groups A, C, D, and E), the

Table 4. *The detection results of PoR IgG antibody in different vaccine combinations*

Groups	S/P Value (Mean± SD)/Time			
	1d	21d	35d	49d
A	2.35±0.71^{a}	2.24±0.72^{a}	2.19±0.69^{Aa}	1.67±0.20$^{A\,Bb}$
B	2.11±0.57^{a}	2.02±0.29^{a}	2.49±0.53^{Aa}	1.95±0.33^{Bb}
C	2.06±0.68^{a}	1.83±0.65^{a}	1.64±0.49^{Bb}	1.10±0.31^{Aa}
D	2.27±0.72^{a}	2.03±0.64^{a}	1.83±0.61^{Bb}	1.25±0.42^{Aa}
E	2.00±0.55^{a}	1.75±0.56^{a}	1.43±0.30^{Bb}	1.20±0.16^{Aa}
F	2.06±0.45^{a}	1.75±0.30^{a}	1.64±0.25^{Bb}	1.16±0.26^{Aa}

Different uppercase letters on the S/P or OD450nm value columns indicate that the difference is extremely significant (P<0.01), and different lowercase letters indicate significant difference (P<0.05)

Table 5. *The detection results of PED IgA antibody in different vaccine combinations*

Groups	OD450nm (Mean±SD/Time			
	1d	3d	7d	14d
A	1.56±0.33^{Bb}	1.12±0.30^{BCc}	0.65±0.21^{Cc}	0.38±0.15^{BCbc}
B	1.65±0.39^{Bbc}	1.34±0.31^{Bcd}	0.38±0.11^{Bb}	0.27±0.18^{ABb}
C	2.08±0.13^{Bcde}	1.65±0.28^{Bd}	0.96±0.16^{Dde}	0.57±0.17^{Cc}
D	2.15±0.49^{Be}	1.42±0.29^{Bcd}	0.85±0.15^{CDd}	0.35±0.18^{BCb}
E	1.65±0.40^{Bbcd}	0.75±0.30^{Cb}	0.33±0.14^{ABb}	0.18±0.14^{ABab}
F	0.10±0.05^{Aa}	0.12±0.05^{Aa}	0.08±0.06^{Aa}	0.06±0.04^{Aa}

Different uppercase letters on the S/P or OD450nm value columns indicate that the difference is extremely significant (P<0.01), and different lowercase letters indicate significant difference (P<0.05)

PoR IgG antibody levels showed a "decreasing" trend, and the declining degree in each test group differed. Moreover, the decline in the PoR IgG antibody levels of the "PTR+PT*" vaccine combination (group A) was lower than those of the other test groups (groups C, D, and E). The PoR IgG antibody growth and decline trends showed that the level of PoR IgG antibody produced in the sows that used the "PTR + PTR" vaccine combination (group B) was superior to that in the sows with the "PTR + PT*" vaccine combination (group A) and other vaccine combinations.

The specific PED IgA antibody detection results showed that IgA antibody was positive in all experimental groups at 1-3 days post-delivery, except control group F *(Fig. 2D)*. As expected, the PED IgA antibodies showed a downward trend at 1-14 days post-delivery in all the experimental groups, but they changed differently in experimental groups. In particular, the PED IgA antibody in groups A and D turned negative at 14 days post-delivery, whereas that in group B and E turned negative at 7 days post-delivery. Fortunately, the PED IgA antibody remained positive at 14 days post-delivery in group C.

Moreover, group C showed significant different form group F (P<0.01) *(Table 5)*, and from groups A (P<0.05) at 1 days after post-delivery *(Table 5)*. As the days increase after delivery, more groups showed significant different from group C. The groups E and F had extremely significant different (P<0.01) from group C at 3 days after post-delivery, and the groups A, B, E and F (P<0.01) showed extremely significant different from group C at 7 days after post-delivery. At 14 days post-delivery, group C also showed significant different from group D (P<0.05) and groups B, E and F (P<0.01). The results abovementioned revealed that group C, which was immunized with PT+PT*, exhibited more long-lasting PED IgA antibody than the other groups.

DISCUSSION

In recent years, an outbreak of porcine virus diarrhea created an epidemic in much of China [10]. The disease is one of the main causes of growth retardation and high mortality in suckling piglets, causing huge economic losses in the pig industry [11]. Therefore, the change trends of IgG antibody in the serum and IgA antibody levels in the breast milk of sows should be understood, and effective vaccine combinations are necessary to immunize sows for the prevention and control of porcine viral diarrhea in pig farms.

IgG drawn in the serum of sows mainly exists in the colostrum, from which suckling piglets obtain passive immunoprotection by sucking. This result indicates that IgG in serum plays an important role in the immune protection of suckling piglets [12,13]. By monitoring the PED

IgG antibodies in serum, we found that the IgG antibody levels continued to increase in groups A and C after immunization at 0-35 days, and their S/P values were higher than those in other groups. Therefore, the vaccine combination of groups A and C may be an alternative for sows to enhance antibody protection and prevent the suckling piglets from PEDV infection. Moreover, the regeneration of intestinal epithelial cells in suckling piglets is slow, and the development of mucosal immune system is imperfect, so hosts cannot produce effective mucosal immune responses due to the inoculated vaccines. Thus, IgA antibody in breast milk plays the most important role to protect suckling pigs from PEDV infection [14]. Therefore, the level of IgA antibodies in breast milk is important for the immunity of suckling piglets [15,16]. The results of this study showed that the IgA antibodies in the different vaccine combinations presented varying growth and decline rules. The PEDV IgA antibody level started to decrease to the negative level at 7 days post-delivery *(Fig. 2D, Table 5)*. Moreover, group C showed significant different with A, B, E and F ($P<0.05$, $P<0.01$) from 1 to 7 days after post-delivery *(Table 4)*. At 14 days post-delivery, except for group C, the antibody levels in the other groups dropped to a negative level, which was consistent with findings of previous report [17]. The results of PEDV IgA antibody monitoring revealed that the vaccine combination of "PT+PT*" led to longer positive antibody levels compared with the other vaccine combinations. Thus, the vaccine combination of "PT+PT*" (group C) demonstrated good effects in stimulating the production of specific PED antibodies in sows, and this result may be related to vaccine type and strain.

Previous studies have shown that single attenuated or inactivated vaccine inoculation is far less effective than the alternate use of attenuated and inactivated vaccines [17]. In general, attenuated vaccines can elicit cellular immune responses in a short time period after inoculation, but their disadvantages include low antibody levels and rapid reduction. Moreover, inactivated vaccines are generally an oil emulsion adjuvant vaccine, which functions as an antigen reservoir and can stimulate the host to continuously produce antibodies. In this study, groups A and C adopted the alternate use of attenuated and inactivated vaccines get better results than that in groups B, D, and E, whose inoculated single attenuated or inactivated vaccine. These vaccines play a good role in immunity precisely because of the combination of attenuated vaccine and inactivated vaccine. In addition, the PEDV gene sequence has shown certain variations in recent years, and its genetic distance indicated that the current PEDV variant strain is far from the classical strain used to develop vaccines [18,19]. In this study, the vaccine strain CV777 in groups A and B is belong genotype GIa, the other strain ZJ08 in groups C and D and AJ1102 in groups C and E were belong genotype GIIa and IIb, respectively. Results of this study demonstrated the alternate use of genotype GIIa strain ZJ08-based attenuated bivalent vaccine and genotype GIIb strain AJ1102-based inactivated bivalent vaccine (group C) produced best immune antibody levels. Therefore, difference vaccine strain may be an important factor that influences vaccine immune antibody levels.

The TGE IgG antibody monitoring results showed that the vaccine combination in groups A and C could stimulate the sows to produce higher immune antibody levels compare with groups B, D, and E. The Miller cluster TGEV vaccine strain H in groups A and C is distant from the Purdue cluster strain WH-1 or HB08 in other experiment groups. But statistical analyses of group A showed no significant difference with groups B, C, and E, also no difference showed in group C with groups A, B, and E. Moreover, groups A and C employed an alternate pattern of attenuated and inactivated vaccines, indicating that the combination of vaccine types was the main factor that affected the production of specific TGE IgG antibodies in the sows, less related to the vaccine strain. It may have a certain relationship with the genetic conservation of TGEV in gene evolution [8,20,21], but further experiments are need to study. PoRV infection is highly common in pig herds and has a high positive rate of serology, but PoRV was the least frequent viral agent detected in the diarrheal samples [22,23]. Moreover, single infection with PoRV occurred in only 0.4% of the population in a previous report in China, whereas most cases involved mixed infections [24]. Therefore, PoRV may be a follow-up agent for PEDV or various diarrheal viruses. Thus, on the whole, prevention and control of PEDV has become particularly important. In this study, we found that the PTR attenuated vaccine could stimulate the sows to produce specific IgG antibody after 15-20 days of immunization, and it can be an alternative option in areas under PoRV infection risk.

The level of IgA antibody in breast milk of sows directly affects the acquired protective antibody effects of suckling piglets, but its detection has a certain time lag and cannot be widely used in practice. However, a previous study found that IgG antibody levels in serum are positively correlated with IgA antibody levels in breast milk [25]. Similarly, we found that the level of PEDV IgA antibody in the sows at 7 days post-delivery was correlated with the level of PEDV IgG in the serum of the corresponding vaccine combination sows at 49 days post-first immunization. This finding suggested that the IgG antibody level in serum may be a reference indicator for IgA antibodies in breast milk. Clinically, the immune antibody level could be monitored by detecting IgG antibody levels in serum, but additional clinical sample data are needed for further validation and analysis.

In conclusion, this report is the first on the special antibody differences of different vaccine combinations that are often used to prevent porcine viral diarrhea in China. Our findings proved that the vaccine combination of "PT+PT*" is optimal for preventing the disease without PoRV infection risk, and "PTR+PT*" may be an alternative option for cases with PoRV infection risk.

Conflict of Interests

The authors declare no competing financial interests.

Acknowledgments

This work was funded by The Public Welfare projects of Fujian Province, China (no. 2018R1023-17); Fujian Academy of Agriculture Science Innovative Research Team Project (STIT2017-1-5, STIT2017-3-10); Fujian Academy of Agriculture Science Research Project(A2018-12).

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Kafkas Univ Vet Fak Derg
25 (5): 597-602, 2019
DOI: 10.9775/kvfd.2018.21335

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

Research Article

Estimation of Parametric Single Index Ordered Logit Model on Milk Yields

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Article Code: KVFD-2018-21335 Received: 12.11.2018 Accepted: 17.04.2019 Published Online: 30.04.2019

How to Cite This Article

Akkuş Ö, Sevinç V, Takma Ç, İşçi Güneri Ö: Estimation of parametric single index ordered logit model on milk yields. *Kafkas Univ Vet Fak Derg,* 25 (5): 597-602, 2019. DOI: 10.9775/kvfd.2018.21335

Abstract

This article aims to determine some important factors affecting the milk yield of Holstein Friesian cows and introduce the use of single index ordered logit model in milk yield studies. Considering the three-level ordered structure of the dependent variable of milk yield, a single index ordered logit model is applied to the data set. The data set used in this study is consisted of the 305-day milk records of the Holstein Friesian cows that calved between 2001-2011 years. 14487 records, obtained from 1840 herds belonging to the members of Cattle Breeders Association in Isparta province, Turkey, are analyzed. The direct effects of the variables: parity, lactation length, first year of calving and calving season on milk yield are investigated and comprehensive interpretations are presented. Results show that the cows having longer lactation lengths produce more milk than the cows having middle lactation lengths. The highest amount of milk yield is produced by the cows calving for the first time in their middle ages and in autumn season. The amount of milk obtained after a birth occurring in spring season is higher than the one reached after a birth in summer. Cow being on its 1st parity decreases the milk yield compared to the 6th parity. This result is consistent with the finding in literature in that cows reach their highest amount of milk on their 4th parities.

Keywords: *Milk yield, Holstein Friesian, Lactation, Single index ordered logit model*

Süt Veriminde Parametrik Tek İndeks Sıralı Lojit Model Tahmini

Öz

Bu makale, Holstein Friesian ineklerinin süt verimini etkileyen bazı önemli faktörleri belirlemek ve süt verimi çalışmalarında tek indeks sıralı lojit modelin kullanımını tanıtmayı amaçlamaktadır. Süt verimi bağımlı değişkeninin üç düzeyli sıralı yapısını göz önünde bulundurarak, veri setine tek indeks sıralı lojit model uygulanmıştır. Bu çalışmada kullanılan veriler 2001-2011 yılları arasında buzağılayan Holstein Friesian ineklerinin 305 günlük süt kayıtlarından oluşmaktadır. Isparta ilinde bulunan Sığır Yetiştiricileri Birliği üyelerine ait 1840 sürüden elde edilen 14487 kayıt analiz edilmiştir. Parite, laktasyon süresi, ilk buzağılama yılı ve buzağılama mevsimi değişkenlerinin süt verimi üzerindeki doğrudan etkileri araştırılmış ve sonuçların ayrıntılı yorumlarına yer verilmiştir. Sonuçlar daha uzun laktasyon süresine sahip olan ineklerin, orta laktasyon süresine sahip olan ineklerden daha fazla süt ürettiğini göstermektedir. Orta yaşlarında ve sonbahar mevsiminde ilk kez buzağılayan inekler en yüksek düzeyde süt üretmektedir. İlkbahar mevsiminde meydana gelen bir doğumdan sonra elde edilen süt miktarı, yazın doğumdan sonra ulaşılan miktardan daha yüksektir. Birinci paritesindeki ineklerin süt verimi 6. pariteye göre azalır. Bu sonuç, ineklerdeki en yüksek süt miktarının 4. paritede elde edildiği yönündeki literatürde yer alan bulguyla tutarlıdır..

Anahtar sözcükler: *Süt verimi, Holstein Friesian, Laktasyon, Tek indeks sıralı lojit model*

INTRODUCTION

One of the most important mistakes made in statistical studies is to construct a model without considering the data structure, statistical assumptions and variable type used. If the concerned (dependent) variable is categorical with two or more levels, using a classical linear regression model is inappropriate due to various assumption violations. In this case, an alternative and more appropriate approach is to use models involving categorical dependent variables and taking into account the ordered or nominal structure of the dependent variables. In this paper, the introduction and interpretation of the Single Index Ordered Logit Model (SIOLM) are mainly dealt with in milk yield estimation of

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Holstein Friesian cows. In the literature, although there are some binary logistic regression applications in the related area, a study using SIOLM in milk yield estimation is not included in this degree especially in terms of interpretation. Hence, we believe that the introduction of the use of the method and especially the odds ratio interpretations will be a good reference for the researchers in the area as the model is also a flexible model which can be reconstructed with new variables for further research. The advantage of the ordered model is that it also provides probabilities related to the dependent variable. In this study, differing from the former models that have been used in the area, the prediction probabilities of milk yield on the variables first year of calving, parity, lactation length and calving season are provided through the model estimated. Furthermore, the significance tests of the levels of those variables are also performed so that necessary improvements on the conditions affecting the milk yield related to those levels can be established. It is known that only logistic regression models are able to calculate odds ratio scores and give comparable interpretations.

There are various studies about milk productivity in the literature. In some of the former studies in the related area, İşçi et al.[1] and Aytekin et al.[2], investigated the factors affecting the milk yield (recorded as a continuous variable) of Holstein Friesians using path analysis. Ristevski et al.[3] examined the correlations between ultrasound measurement of thickness of fat over the tuber ischiadicum, body condition scoring and the risk of lameness developing in Holstein Friesians using correlation analysis. Öner et al.[4], investigated the polymorphisms in seven genes related to reproductive traits in dairy heifers. They employed mixed effect logistic regression in their analysis. Solano et al.[5] examined the correlation between lying behaviour and lameness in Canadian Holstein Friesians. Koçak and Ekiz [6] studied the factors affecting the milk yield and lactation curve of Holstein cows using Wood equation in the analysis of the lactation curve. In the study of Tahtalı et al.[7], the factors affecting the milk yield were determined using path analysis. In the study carried out by Verma et al.[8], the effect of various genetic and non-genetic factors on milk yield and milk constituent traits in Murrah buffaloes were investigated using 176 Murrah buffaloes over a period of 10 years. Inspired by this work, using some of the variables and variable categorization in the study, we modeled 305-day milk yield data of the Holstein Friesian cows that calved between 2001-2011 years in Isparta province in Turkey for determining the most significance factors and also their levels using the SIOLM.

MATERIAL and METHODS

Material of this study consisted of the 305-day milk record of the Holstein Friesian cows that calved between 2001-2011 years. 14487 records, obtained from 1840 herds belonging to the members of Cattle Breeders Association in Isparta province, Turkey, are analyzed. In statistical analysis, determining the true model according to the structure of the variables in the data set used is a crucial point to be taken into consideration. Categorical dependent variables with more than two levels should be analyzed by using the models given in *Table 1* by considering the model assumptions for constructing an efficient statistical model with minimum error [9]. The data collected are used to determine the important factor levels affecting the milk yield of Holstein Friesians. SIOLM is used for analyzing the data, which is one of the most popular single index models. In the phase of estimating the SIOLM, definitions of the dependent and independent variables, variable levels and base categories are given in *Table 2*. To account for differences among herds, milk yield for each cow is categorized as low, middle and high based on herdmate deviations [10] and lactation length (middle and high) and first calving year (low, middle and high) are classified on the basis of expert opinion (Çiğdem Takma).

Due to the non-separable nature of the dependent variable, data that is measured with an ordinal scale cannot easily be modelled with the classical regression. Another alternative to be taken into consideration here is Multinomial Logit Model (MLM). However, such models fail since they do not consider the ordered structure of the dependent variable and consequently do not use the available information fully [11].

Table 1. *Multilevel dependent variable models*

Model	Type of the Dependent Variable	Model Assumptions
Multinomial Logit	Nominal	*Only the characteristics of individuals are required *Strict assumption of Independence of Irrelevant Alternative (IIA) has to be satisfied
Multinomial Probit	Nominal	*Only the characteristics of individuals are required *No other assumption is necessary including IIA
Ordered Logit	Ordered	*Only the characteristics of individuals are required *Parallel Slopes Assumption (PSA) is required
Ordered Probit	Ordered	*Only the characteristics of individuals are required *Parallel Slopes Assumption (PSA) is required
Nested Logit	Nested Nominal Design	*Inclusive Value (IV) is required to be positive
Conditional Logit	Nominal	*Characteristics of the choice and individuals are both required

Table 2. *Variables in the model*

Dependent Variable: Milk Yield 1000-4000 kg (Low) 4001-7000 kg (Middle) 7001+ kg (High)	
Lactation Length (day) 100-200 (Middle) 201-305 (High) (Base category)	**Parity (P)** P1 P2 P3 P4 P5 P6 (Base category)
First Calving Year (month) <23 Low 24-27 Middle >28 High (Base category)	**Calving Season** Winter Spring Summer Autumn (Base category)

The estimation of SIOLM is made using STATA 7.0 package program. The direct effects of the variables: parity, lactation length, first year of calving and calving season on milk yield are investigated. In order to assess the model quality in SIOLM, Correct Classification Rate (CCR) is computed. Additionally, "odds ratio" values which can only be estimated by using logistic regression models are calculated by taking the exponential of the estimated b_k coefficients. These coefficients provide a comparative comparison of the effect degree of factor levels. The consistency of our finding is investigated by also comparing the results with the results of various studies.

Latent variable (Y^*) represents the quantities that are not directly measured but only inferred from the observed covariations among a set of variables [12]. The effects of some latent variables are determined by the following linear model [13,14].

$$Y^* = \sum_{k=1}^{K} \hat{b}_k X_k + \varepsilon \quad (1)$$

Here, estimated b_k and ε denote the estimated coefficients of the explanatory variables X_k and the error term, respectively. SIOLM can be obtained if the error term has mean "0" and variance $\pi^2/3$. In SIOLM, there is a certain ordering between the dependent variable-levels.

Considering that the dependent variable has J pieces of ordered categories, the relation between the observed levels and slopes could be given as follows.

$$\begin{aligned} Y_i &= 1, \quad Y^* \le \mu_1 \\ Y_i &= 2, \quad \mu_1 \le Y^* \le \mu_2 \\ Y_i &= 3, \quad \mu_2 \le Y^* \le \mu_3 \\ Y_i &= J, \quad \mu_{J-1} \le Y^*; \ (i = 1,2,\cdots,N) \end{aligned} \quad (2)$$

Here, μ represents the threshold parameter that distinguishes the unknown ordered categories. In SIOLM, the probability that the dependent variable belongs to the category "j" conditional to the explanatory variable is expressed as the following.

$$P(Y = j \backslash x_k) = F\left(\mu_j - \sum_{k=1}^{K} \hat{b}_k x_k\right) - F\left(\mu_{j-1} - \sum_{k=1}^{K} \hat{b}_k x_k\right) \quad (3)$$

In Eq. (3), F denotes the assumed distribution of the error term ε in the model. At the beginning of the analysis, it must be first checked whether the threshold parameters are statistically significant or not. If they are significant, it is determined whether the dependent variable has an ordered structure as it is assumed. The probability that the dependent variable y belongs to the category j or a lower category can be calculated using the following equation in SIOLM.

$$P(Y \le j) = P\left(Y^* \le \mu_j\right) = \frac{exp\left(\mu_j - \sum_{k=1}^{K} \hat{b}_k x_k\right)}{1 + exp\left(\mu_j - \sum_{k=1}^{K} \hat{b}_k x_k\right)} \quad (4)$$

The left side of Eq. (4) is called cumulative logit. SIOLM is achieved if the logistic distribution denoted by ψ is specifically chosen for F in Eq. (3). The probabilities that the dependent variable belongs to the relevant categories are given by Eq. (5), Eq. (6) and Eq. (7) [15,16].

$$P(Y = 1) = \Psi\left(\mu_1 - \sum_{k=1}^{K} \hat{b}_k x_k\right) = \frac{exp\left(\mu_1 - \sum_{k=1}^{K} \hat{b}_k x_k\right)}{1 + exp\left(\mu_1 - \sum_{k=1}^{K} \hat{b}_k x_k\right)} \quad (5)$$

$$\begin{aligned} P(Y = 2) &= \Psi\left(\mu_2 - \sum_{k=1}^{K} \hat{b}_k x_k\right) - \Psi\left(\mu_1 - \sum_{k=1}^{K} \hat{b}_k x_k\right) \\ &= \left[\frac{exp\left(\mu_2 - \sum_{k=1}^{K} \hat{b}_k x_k\right)}{1 + exp\left(\mu_2 - \sum_{k=1}^{K} \hat{b}_k x_k\right)}\right] - \left[\frac{exp\left(\mu_1 - \sum_{k=1}^{K} \hat{b}_k x_k\right)}{1 + exp\left(\mu_1 - \sum_{k=1}^{K} \hat{b}_k x_k\right)}\right] \end{aligned} \quad (6)$$

$$\begin{aligned} P(Y = J) &= 1 - \Psi\left(\mu_{J-1} - \sum_{k=1}^{K} \hat{b}_k x_k\right) \\ &= 1 - \left[\frac{exp\left(\mu_{J-1} - \sum_{k=1}^{K} \hat{b}_k x_k\right)}{1 + exp\left(\mu_{J-1} - \sum_{k=1}^{K} \hat{b}_k x_k\right)}\right] \end{aligned} \quad (7)$$

RESULTS

Single Index Ordered Logit Model results including the estimated coefficients of the variable levels of lactation length (middle), first calving year (low and middle), parity (P1, P2, P3, P4 and P5) and calving season (winter, spring and summer), their standard errors, Wald statistics, P-values and odds ratios are given in *Table 3*.

When *Table 3* is examined, it can be realized that the threshold parameters (μ_1) and (μ_2) are statistically significant with a level of 5% (P≤0.05). The significance of the threshold parameter indicates that the dependent variable milk yield is ordered as it is assumed at the beginning of the study. Also, it appears that SIOLM is a suitable model for the data structure.

The linear combinations of the explanatory variables

Table 3. *Single index ordered logit model results*

Dependent Variable: Milk Yield 1:1000-4000, 2:4001-7000, 3: 7000+	Estimated b_k	Std. Error	Wald	P-value	Odds-Ratio
Lactation Length					
Middle (100-200)	**-2.679**	0.0574	2185.550	**0.000**	0.069
First Calving Year					
Low (<23)	**-0.231**	0.087	7.024	**0.008**	0.794
Middle (24-27)	**0.171**	0.042	16.665	**0.000**	1.186
Parity					
P1	**-0.561**	0.152	13.710	**0.000**	0.571
P2	-0.130	0.153	0.727	0.394	-
P3	0.070	0.156	0.199	0.656	-
P4	-0.062	0.163	0.146	0.702	-
P5	-0.026	0.178	0.022	0.882	-
Season					
Winter	**-0.443**	0.055	64.235	**0.000**	0.642
Spring	**-0.197**	0.053	13.935	**0.000**	0.821
Summer	**-0.264**	0.061	18.613	**0.000**	0.768
Threshold Parameters					
$\mu_1 = -2.447$		0.154	251.416	**0.000***	
$\mu_2 = 2.019$		0.154	172.724	**0.000***	
Model Validity					
Log likelihood = 1053.125; LR chi2(11) = 2710.703; Prob>chi2 = 0.000					

** Coefficient is statistically significant at a significance level of 5%*
Base Categories: *Lactation Length (High), First Calving Year (High), Parity (6), Season (Autumn)*

can be obtained by substituting the characteristics of each cow in the model equation given below. *Table 3* shows the model constructed using the estimated coefficients b_k.

$$\sum_{i=1}^{14487}\sum_{k=1}^{11}\hat{b}_k x_{ik} = (-2.679)xMiddle - (0.231)\,x\,Low + \cdots + (-0.264)xSummer \quad (8)$$

The following probability equations are obtained depending on the expression in Eq. (8) and formulations given by Eq. (5), Eq. (6) and Eq. (7). At the different levels of all the factors affecting the milk yield, the probabilities for the given categories of the milk yield can be calculated with the following equations.

For the category 1000-4000 kg,

$$P(Y_i = "1000-4000") = \frac{exp\left(-2.447-\sum_{k=1}^{11}\hat{b}_k x_{ik}\right)}{1+exp\left(-2.447-\sum_{k=1}^{11}\hat{b}_k x_{ik}\right)} \quad (9)$$

For the category 4001-7000 kg,

$$P(Y_i = "4001-7000") = \left[\frac{exp\left(2.019-\sum_{k=1}^{11}\hat{b}_k x_{ik}\right)}{1+exp\left(2.019-\sum_{k=1}^{11}\hat{b}_k x_{ik}\right)}\right] - \left[\frac{exp\left(-2.447-\sum_{k=1}^{11}\hat{b}_k x_{ik}\right)}{1+exp\left(-2.447-\sum_{k=1}^{11}\hat{b}_k x_{ik}\right)}\right] \quad (10)$$

For the category 7000+ kg,

$$P(Y_i = "7001+") = 1 - \left[\frac{exp\left(2.019-\sum_{k=1}^{11}\hat{b}_k x_{ik}\right)}{1+exp\left(2.019-\sum_{k=1}^{11}\hat{b}_k x_{ik}\right)}\right] \quad (11)$$

The second column of *Table 3* contains the estimated coefficients of the explanatory variables. In the fifth column, the p-values are given for testing the significance of the estimated model parameters. An estimated coefficient with a negative sign indicates that the corresponding variable affects the milk yield in a decreasing way and a positive coefficient means the variable makes the milk yield increase with respect to the base category displayed in *Table 2*. The odds ratios given in the last column of *Table 3* are interpreted only for statistically significant factors in the analysis. The patterns with arrows display the increase or decrease along the categories of the milk yield, which are presented in *Table 3*, with respect to the significant categories of the variables. Detailed interpretations of the results for all variables are given below.

$$\text{Middle (100-200)} \xrightarrow[\text{High (201-305) (base)}]{\text{(low) (middle) (high)}} \text{(-) effect on odds}$$

According to the model estimated, a middle lactation length (100-200) causes the milk yield to decrease compared to the long lactation length (201-305). In other words, the

cows having longer lactation lengths produce more milk than the cows having middle lactation lengths.

The odds ratio for the middle lactation length is 0.069 which yields 14.5 when inverted. Hence, the probability that a cow having a high lactation length will produce middle or high amount of milk is 14.5 times higher than the probability that it will produce low amount of milk.

While a low first calving year (<23 month) seems to cause the milk yield to decrease compared to a high first calving year (>28 month), a middle first calving year (24-27 month) has an impact which increases the milk yield. Thus, it can be interpreted that the highest amount of milk yield is produced by the cows calving for the first time in their middle ages (24-27 month).

The odds ratio for low first calving years is calculated as 0.794. When we invert this odd ratio the result is 1.26. This means that when a cow has a high first calving year, the probability that the milk yield will be high is 1.26 times more likely than it will be low compared to low first calving year.

$$\text{Low (<23)} \xrightarrow[\text{High (>28) (base)}]{\text{(low) (middle) (high)}} \text{(-) effect on odds}$$

The odds ratio that the first calving year in middle level is 1.186. This can be interpreted as the probability that the cows having middle first calving year will produce high amount of milk is 1.186 times greater than the ones having high first calving years. Thus, we can say that the ideal first calving years for cows to produce high amount of milk are the middle ages.

The odds ratio for Parity 1 (P1) is calculated as 0.571 which yields an odds value of 1.75 when inverted. This means that cow being on its 1st parity decreases the milk yield compared to the 6th parity.

$$\text{Middle (24-27)} \xrightarrow[\text{High (>28) (base)}]{\text{(low) (middle) (high)}} \text{(+) effect on odds}$$

When calving seasons are considered, the highest amount of milk is seen in autumn season. The least amount of milk, on the other hand, is observed in winter season. The amount of milk obtained after a birth occurring in spring season is higher than the one reached after a birth in summer. The decrease in the milk yield in winter is approximately 1.68 (-0.443/-0.264) times higher than the decrease in summer season when compared to the amount of milk reached in autumn season. However, it is 2.25 (-0.443/-0.197) times higher when compared to spring season. Also, the decrease in the amount of milk yield obtained in summer season is 1.34 (-0.264/-0.197) times less than the amount that in spring compared to the autumn season.

$$\text{P1} \xrightarrow[\text{P6 (base)}]{\text{(low) (middle) (high)}} \text{(-) effect on odds}$$

When calving is in winter season, the odds ratio is calculated as 0.642 which is 1.56 when inverted. Therefore, when calving is in autumn season, the probability that the milk yield will be high is 1.56 times more likely compared to winter season.

$$\text{Winter} \xrightarrow[\text{Autumn (base)}]{\text{(low) (middle) (high)}} \text{(-) effect on odds}$$

When calving occurs in spring season, the odds ratio is calculated as 0.821 whose inverse is 1.22. Thus, when calving occurs is autumn, the probability that the milk yield will be high is 1.22 times more likely than the case when calving occurs in spring.

$$\text{Spring} \xrightarrow[\text{Autumn (base)}]{\text{(low) (middle) (high)}} \text{(-) effect on odds}$$

The odds ratio for the case of calving in summer is 0.768 which is inverted as 1.30. Hence, in the case when calving occurs in autumn, the probability that the milk yield will be high is 1.30 times more likely than the one obtained when calving occurs in summer season.

$$\text{Summer} \xrightarrow[\text{Autumn (base)}]{\text{(low) (middle) (high)}} \text{(-) effect on odds}$$

The correct classification rate given in *Table 4* indicates the quality of the model. It measures the consistency between the actual and the predicted amount of milk yield estimated by the SIOLM model. It is seen that 77.9% of the cows have been correctly assigned to the related category by the model. This means that, by using this model, it is

Table 4. *Correct classification rate*

Actual	Predicted			
	1000-4000	4001-7000	7001+	Total
1000-4000	1236*	1500	0	2736
4001-7000	616	10061*	0	10677
7001+	23	1051	0*	1074
Total	1875	12612	0	14487

** Number of cows that have been correctly assigned to the related category by the model*

possible to predict the milk yield of any animal, in terms of the first year of calving, lactation length, parity and calving season with a 77.9% level of success rate.

DISCUSSION

The classical linear methods give statistically incorrect results when the concerned dependent variable is categorical with more than two levels. This study is about the determination of the important factors affecting the milk yield of Holstein Friesian cows using SIOLM. The most important advantage of SIOLM compared to other models is that it is the only model that can be used when the dependent variable has an ordered structure. Using a SIOLM to determine the significant variables and variable levels affecting the 305-day milk yield of Holstein Friesians enabled us to test the significance of all the categories of the variables separately as well as their positive or negative effects on the milk yield. Another advantage of SIOLM is that it also enables the researcher calculate odds ratios. Therefore, we are also able to compare the effects of the categories with each other through the bilateral odds ratio values calculated among them.

The present study is concluded as milk yield is affected by all the factors concerned at different levels of importance. The probability that a cow having a high lactation length will produce middle or high amount of milk is 14.5 times higher than the probability that it will produce low amount of milk. This result is in accordance with the findings of Vijayakumar et al.[17] who recorded that Holstein cows reach the highest milk yield in greater lactation length periods. Additionally, Lateef et al.[18] stated that the maximum milk yield is obtained from the Holstein Friesian cows having the highest lactation lengths (greater than 400 days). When a cow has middle first calving year, the probability that the milk yield will be high is 1.186 times more likely than it will be low. That is cows having middle first calving years produce the most amount of milk. This result is partly supported by the findings of Eastham et al.[19] stating that lower ages of first calving cause more milk yield. Cow being on its 1st parity produces less milk compared to the one on its 6th parity. This finding is also supported by M'hamdi et al.[20] who show in a tabular form that milk yield increases as the first calving year increases. Finally, the most amount of milk is obtained in autumn season. When calving is in spring season, the probability that the milk yield will be high is 2.25 times more likely compared to winter season. This result is supported by one of the findings of Nalubwama et al.[21] which shows that Holstein cows that calved in wet seasons have higher milk production compared to those that calved in the dry seasons.

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Kafkas Univ Vet Fak Derg
25 (5): 603-609, 2019
DOI: 10.9775/kvfd.2018.21401

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

Research Article

Effects of Exogenous Amylase in Transition Dairy Cows Fed Low-Starch Diets: 2. Total Tract Digestibility and Blood Urea Nitrogen

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Article Code: KVFD-2018-21401 Received: 01.12.2018 Accepted: 24.03.2019 Published Online: 25.03.2019

How to Cite This Article

Gençoğlu H, Kara Ç, Efil MM, Biricik H, Türkmen İİ, Deniz G, Kovanlıkaya A, Shaver RD, Kivanç RT, Yildırım R: Effects of exogenous amylase in transition dairy cows fed low-starch diets: 2. Total tract digestibility and blood urea nitrogen. *Kafkas Univ Vet Fak Derg,* 25 (5): 603-609, 2019. DOI: 10.9775/kvfd.2018.21401

Abstract

The objective of this trial was to determine the effect of exogenous amylase during the transition period on total tract digestibility, rumen pH and blood urea nitrogen in lactating dairy cows. The effect of exogenous dietary amylase supplementation on lactation diets with low starch concentration (19.5% of dry matter) and dry period diets with moderate starch concentration was evaluated (15.5% of dry matter). A total of 30 multiparus Holstein cows were randomly assigned to two groups with amylase (n=15) or control (n=15). Three cows from each group were randomly selected and ruminally cannulated for digestibility trials. The research was conducted starting at 21 d prepartum until 84 d postpartum. Digestibility of dry matter, organic matter, neutral detergent fiber, starch, and crude protein remained unaffected by treatment in postpartum. Average pre- and postpartum rumen pH concentrations were 6.25 and 6.15, respectively, and did not differ between treatments. Blood urea nitrogen (BUN) concentrations were lower in cows fed amylase supplemented diet compared to those fed diet without amylase in both pre- and postpartum periods (P<0.001). In conclusion, the dietary supplementation of amylase did may not affect the digestibility of nutrients, however, it may decrease the BUN concentration in pre- and postpartum period for cows fed amylase. Therefore, it may offer potential for improving nitrogen efficiency in dairy cows.

Keywords: *Amylase, Starch, Total tract digestibility, Blood urea nitrogen, Dairy cows*

Düşük Nişastalı Rasyonlarla Beslenen Geçiş Dönemindeki İneklerde Amilaz Enziminin Etkisi: 2. Toplam Sindirilebilirlik ve Kan Üre Azotu

Öz

Bu araştırmanın amacı geçiş dönemindeki ineklerin rasyonlarına amilaz enzimi ilavesinin toplam sindirilebilirlik, rumen pH'sı ve kan üre azotu üzerine etkisini incelemektir. Rasyonların nişasta düzeyi kuru madde esasına göre kuru dönemdeki hayvanlar için %15.5, laktasyon dönemindekiler için ise %19.5 olarak tespit edildi. Araştırmada birden fazla doğum yapmış 30 baş siyah alaca ırkı inekler rastgele amilaz (15) ve kontrol (15) gruplarına dağıtıldı. Her bir grupta üçer inek rumen kanülü mevcuttu. Deneme doğumdan önceki 21 gün ile doğumdan sonraki 84. günler arasında yürütüldü. Doğum sonrası kuru madde, organik madde, nötral deterjan fiber, nişasta ve ham protein sindirilebilirlik düzeyleri bakımından gruplar arasında bir farklılık bulunmamıştır. Ortalama doğum öncesi ve sonrası rumen pH değerleri sırasıyla 6.25 ve 6.15 olarak tespit edilmiş ve gruplar arasında fark bulunamamıştır. Hem doğum öncesi hem de doğum sonrası amilaz ile beslenen ineklerde kan üre azotu değerleri kontrol grubuna göre daha düşük bulunmuştur (P<0.001). Sonuç olarak, süt ineği rasyonlarına amilaz enzim ilavesi besin maddesi sindirilebilirliklerini etkilemez iken diğer yandan hem doğum öncesi hem de sonrası kan üre azotu konsantrasyonlarını azaltmıştır. Böylece, amilaz ile beslenen gruplarda kan üre azotunun azalması; azotun kullanım etkinliğini iyileştirmesi için bir fırsat yaratabilir.

Anahtar sözcükler: *Amilaz, Nişasta, Toplam sindirilebilirlik, Kan üre azotu, Süt ineği*

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INTRODUCTION

Dietary starch content is important to increase rumen microbial production [1]. Starch is fermented and increases propionate production in the rumen [2], and unfermented starch that escapes ruminal fermentation provides glucose that is absorbed or metabolized to lactate in the small intestine [3]. Starch level [4,5] and starch content [6,7] in ration was assessed in terms of performance in dairy cows.

There are studies in which some feed additives [8-10] and treatments [11] was used for transition dairy cows. In addition, exogenous amylase was evaluated in some trials to improve performance [12-14] and digestibility [15,16]. The supplementation of exogenous amylase in diets for dairy cows is designed to increase the utilization of starch in feeds. In some non ruminant animals, the salivary glands secrete amylase to begin breaking down starch when food enters the mouth. Ruminants do not have salivary amylase [17]; therefore the microbial population in the rumen is largely responsible for the degradation of starch.

The inclusion of exogenous amylase to the diet of the lactating cows can increase ruminal starch digestibility [18] and stability in ruminal fluid [19]. Increased ruminal starch availability may increase ruminal microbial yield and feed efficiency by intake regulation induced by increased liver oxidation of propionate [20]. However, some starch sources are rapidly fermented; excessive ruminal fermentability can decrease ruminal pH and alter ruminal biohydrogenation pathways, reducing milk fat concentration and yield.

Based on starch ruminal degradation rate, grains can be ranked from fastest to slowest degradations and, thus, it is possible to infer the respective potential for acidification in the following order: oats, wheat, barley, high-moisture corn, steam-flaked corn, dry-rolled corn, whole corn grain and whole sorghum grain [21-23].

Dietary alterations that increase ruminal digestibility have been demonstrated to affect the morphology of the rumen papillae [24] and may be valuable for providing more energy from the transition dairy cow's diet through increased volatile fatty acids (VFA) production and absorption. The VFA influence papillae growth in the rumen [24]. Increasing propionate concentration in the rumen favors elongation of papillae. Greater DMI increases passage rate through the gastrointestinal tract reducing the time for starch hydrolysis, thereby it limits starch digestibility [25] in rumen and intestine.

This paper is companion papers (1 of 2) [26] from an experiment designed to examine the effects of exogenous amylase in transition dairy cows fed low-starch diets: Lactation performance, total tract digestibility, rumen and blood parameters.

Low starch diets may be an economic alternative when grain prices are high. However, the effect of amylase addition to diets with a too low starch concentration (19.5%) has not been evaluated for total tract digestibility and blood urea nitrogen. Therefore, the objective of the trial was to determine the effect of exogenous amylase during the transition period on total tract digestibility, rumen pH, and blood urea nitrogen in dairy cows fed low starch diet. The energy supply supported by starch may affect negatively milk urea nitrogen by completing the deficiencies of protein metabolism [27] and milk urea nitrogen (MUN) is also positively correlated with blood urea nitrogen (BUN) [28,29].

MATERIAL and METHODS

The experiment was conducted from January 2011 through August 2011 at Omer Matli Research Center (Karacabey, Bursa Turkey). All the procedures were approved by the Bursa Uludag University, Animal Experiments Local Ethics Committee (Committee Number and Date: 2010-07/02 and 02.11.2010). Thirty (30) multiparous Holstein cows were randomly assigned to with or without (control) exogenous amylase groups in a completely randomized design. Three of the fifteen cows in each group were ruminally cannulated with soft plastic cannulae of 10 cm internal diameter (Ankom, pliable rumen cannula #29.4 inches, NY, USA) to determine rumen parameters. Current lactation numbers for control and amylase cows were 2±0.3 and 2±0.4, respectively. Previous lactation 305-d milk yields for control and amylase cows were 8289±1322 kg and 8332±1779 kg, respectively. Disease incidences for control and amylase cows, respectively, were retained placenta (1 vs. 0), milk fever (2 vs. 1), ketosis (4 vs. 5), dystocia (0 vs. 2), and mastitis (2 vs. 3).

The research was conducted starting at 21 d prepartum until 84 d postpartum. Cows were housed in a free-stall barn and fed diets as a total mixed ration (TMR) with an automatic feeding door system. At 35 d prior to the expected calving date, cows were assigned to their respective diets and housed for adaptation to the automatic feeding door system 2 week before initiation of the experimental period. Cows were housed in individual maternity pens from parturition until 4 days in milk and then cows were moved to free-stall housing equipped with a automatic feeding door system. Cows were fed individually the TMR once daily (0800 h) to allow for *ad libitum* consumption and animals were allowed access to feed at all times, except during milking times. Ingredient composition of the experimental diets, nutrient composition and particle size of diets and all feedstuffs are shown in first companion paper [26].

The control diet did not contain exogenous amylase. The amylase diet was fed with exogenous amylase addition to the concentrate mixtures. A granular amylase formulation, Ronozyme RumiStar (Lot number: 600 (CT) AU360001) with an amylase activity of 600 Kilo Novo Units (KNU) per g provided by DSM Nutritional Products (Basel, Switzerland) was used for this study. The targeted dosage of 300

KNU/kg of the TMR dry matter (DM) in amylase diet was achieved by adding 1 g of Ronozyme RumiStar per kg of concentrate mixture (as-fed basis). The control and amylase concentrate mixtures were prepared as pelleted feed (pelleting temperature 65°C) by Matli Feed Co. (Karacabey, Turkey). The pelleted concentrates of control and amylase were sampled every 4 week, stored at -20°C, and then sent to DSM Nutritional Products Analytical Services Center (Basel, Switzerland) for analysis of amylase activity [30]. Determined amylase activities for control and amylase pelleted concentrates mixtures were 0±0, and 606.9±53.4 KNU/kg (as-fed basis), respectively. The treatment TMR for lactating cows averaged 303.4±27 KNU/kg of DM, which was similar to the targeted dosage of 300 KNU/kg of DM recommended by DSM Nutritional Products and the dosage used in the trials of Klingerman et al.[19], Ferraretto et al.[31], and Gencoglu et al.[32].

Ruminal fluid was collected from rumen-cannulated cows immediately before feeding (0 h) and at 2, 4, and 8 h after feeding. Target day and actual day of prepartum ruminal fluid sampling before calving were 21 and 22.3 (SD=2.6), 7 and 8.0 (SD=2.8) for control, 21 and 21.3 (SD=3.3), 7 and 7.1 (SD=1.6) for amylase. Postpartum ruminal fluid samples were taken at 1, 3, 5, 7, 10, and 12 weeks after calving. Samples were collected from multiple sites in the ventral rumen via the cannula using a metal filter probe. Samples were immediately squeezed through 2 layers of cheesecloth and pH was measured using a pH meter (Inolab pH, serial no: 00200018, pHElectrode SenTix 41, D-82362, Weilheim, Germany). The filtered duplicate rumen fluid samples of 1.5 mL were were acidified in 30 µL of 50% TCA solution and immediately frozen in microfuge tubes until prepared and analyzed for ammonia-N (NH_3-N) as described by Bal et al.[33].

Fecal grab samples were collected from each cow twice daily to cover 0400, 0800, 1200, 1600, 2000, and 2400 h time points over the 3-d sampling period in week 8 of the lactation period. The TMR and ort samples were collected from each cow daily during the 3-d sampling period. Fecal and ort samples were dried and ground as described previously and composited by cow within period; composite samples were analyzed for DM, organic matter (OM), crude protein (CP), neutral detergent fiber (NDF), and starch. Total-tract nutrient digestibilities were determined using 120-h indigestible NDF as an internal marker. Composite fecal, ort, and TMR samples were incubated ruminally in Dacron bags in triplicate for 120-h at the end of experimental period. In situ Dacron bags were made from nitrogen-free polyester, with a pore size of 50 microns (Ankom, R1020-10×20 cm, forage bags, 14502, NY, USA). Composite fecal, ort, and TMR samples were dried at 60°C for 48 h and ground through 2 mm screen, the samples were weighed (5 g sample) into the bags, and incubated in the rumen of each cow for 120 h. After incubation, bags were withdrawn from the rumen, and plunged into cold water for 10 min to stop fermentation. Bags were then washed for approximately 120 min in cold water and dried in a forced-air oven at 60°C for 48 h, and then analyzed for NDF content. The NDF content of the bag residues was determined in triplicate using α-amylase and sodium sulfite [34]. Total-tract nutrient digestibilities were calculated from 120 h indigestible NDF and nutrient concentrations in the orts-adjusted diet and feces [35].

Target day and actual day of prepartum blood sampling before calving were 21 and 21.7 (SD=3.8), 7 and 6.9 (SD=2.5) for control, and 21 and 21.3 (SD=2.8), 7 and 6.5 (SD=2.0) for amylase. Postpartum blood samples were taken 1, 3, 5, 7, 10, and 12 weeks after calving. Before feeding, blood samples were taken from coccygeal vessel into evacuated tubes. Serum harvested for analyzed BUN, non-esterified fatty acids (NEFA), glucose, Ca, P, albumin, alkaline phosphatase (ALP), bilirubin, creatine, Na, K, total protein and globulin (Comprehensive profile kit, VetScan classic Abaxis, CA, USA). Approximately 4 h after feeding, blood was sampled from a coccygeal vessel into 1 evacuated tube and plasma was harvested and analyzed for beta-hydroxybutyric acid (BHBA) using KetoSite diagnostic kit (Stanbio Laboratory Boerne, Texas, 78006, USA) with STAT-Site Meter (GDS Diagnostic, 25235 Leer Drive Elkhart, 46514, IN).

Data were analyzed as a completely randomized design using the Linear Mixed Model of SPSS (SPSS 13.0, 2004). The model included treatment, time (week for prepartum and early lactation measurements and day for blood measurements), and treatment × time interaction as Fixed effects and cow within treatment as a Random effect. The REML (Restricted Maximum Likelihood) was the chosen estimation method. Means were determined using the least squares means statement. Statistical significance and trends were considered at $P \leq 0.05$ and $P > 0.05$ to $P < 0.10$, respectively.

RESULTS

Treatment effects on least squares means for apparent total-tract nutrient digestibilities are in *Table 1*. Dietary addition of exogenous amylase did not affect digestibility of DM, OM, CP, NDF, or starch ($P > 0.10$).

Treatment effects on least squares means for blood plasma parameters are presented in *Table 2*. The concentrations of serum NEFA, BHBA, albumin, bilirubin, Ca, P, creatinine, glucose, Na, K, protein and globulin unaffected by treatment in both prepartum and early-lactation cows. Dietary addition of exogenous amylase did not affect BHBA concentration, but was numerically 1 mg/dL greater for amylase than control during the postpartum period. BUN concentration ranged from 18.5 to 21.6 mg/dL across the treatments lactation period, and BUN concentration were reduced ($P < 0.001$) for cows fed amylase compared to control in both prepartum and lactation periods (Fig. 1).

Table 1. *Effect of treatment on least squares means for apparent total-tract nutrient digestibilities[1,2]*

	Item	Control	Amylase	SEM[3]	P
Digestibility, %	DM[4]	76.2	75.8	1.6	NS[5]
	OM[6]	76.9	77.1	1.6	NS
	CP[7]	82.9	82.0	1.3	NS
	NDF[8]	59.0	58.9	3.5	NS
	Starch	96.8	97.4	0.4	NS

[1] Treatments were pelleted concentrate mixture without amylase (Control) and with amylase (Amylase); [2] Determined using 120 h indigestible NDF as an internal marker; [3] Standard error of the mean; [4] Dry matter; [5] Non significant; [6] Organic matter; [7] Crude protein; [8] Neutral detergent fiber

Table 2. *Effect of treatment on least squares means for plasma concentrations of BHBA, albumin, ALP, bilirubin, BUN, Ca, P, creatinine, glucose, Na, K, protein and globulin prepartum and early lactation cows[1]*

Parameter		Control	Amylase	SEM[2]	P
NEFA[3] mEq/L	Prepartum	367.17	409.04	53.98	NS[4]
	Lactation	605.39	608.97	31.16	NS
BHBA[5] mg/dL	Prepartum	9.70	8.32	1.67	NS[4]
	Lactation	14.13	15.18	0.97	NS
Albumin g/dL	Prepartum	2.11	2.18	0.06	NS
	Lactation	2.44	2.40	0.04	NS
ALP[6] U/L	Prepartum	69.58	55.62	7.43	0.01
	Lactation	62.76	46.90	4.29	0.01
Bilirubin mg/dL	Prepartum	0.32	0.37	0.03	NS
	Lactation	0.37	0.35	0.02	NS
BUN[7] mg/dL	Prepartum	16.54	13.79	0.75	0.001
	Lactation	21.65	18.46	0.53	0.001
Ca mg/dL	Prepartum	9.46	9.49	0.14	NS
	Lactation	9.23	9.39	0.11	NS
P mg/dL	Prepartum	6.72	5.91	0.28	0.06
	Lactation	6.19	6.10	0.19	NS
Creatinin mg/dL	Prepartum	1.10	1.04	0.05	NS
	Lactation	0.93	0.85	0.05	NS
Glucose mg/dL	Prepartum	73.02	73.42	2.03	NS
	Lactation	63.99	64.92	1.62	NS
Na mmol/L	Prepartum	137.09	137.04	0.77	NS
	Lactation	133.03	133.25	0.47	NS
K mmol/L	Prepartum	4.87	4.90	0.07	NS
	Lactation	4.88	4.97	0.05	NS
Protein g/dL	Prepartum	7.03	7.04	0.15	NS
	Lactation	7.82	8.18	0.13	0.06
Globulin g/dL	Prepartum	4.92	4.87	0.18	NS
	Lactation	5.38	5.79	0.16	0.08

[1] Treatments were pelleted concentrate mixture without amylase (Control) and with amylase (Amylase); [2] Standard error of the mean; [3] Non-esterified fatty acids; [4] Non significant; [5] Beta-hydroxybutyric acid; [6] Alkaline phosphatase; [7] Blood urea nitrogen

Least squares mean for rumen NH_3N and pH are presented in *Table 3*. The addition of exogenous amylases to the diet did not affect these parameters (P>0.10). Cows fed amylase compared with cows fed control in lactation tend to have lower average daily rumen pH (6.07 vs 6.22), but not significant as statistically.

Table 3. *Effect of treatment on least squares means for rumen NH_3N and pH in prepartum and postpartum cows[1]*

Parameter		Control	Amylase	SEM[2]	P
NH_3N[3] mg/dL	Prepartum	16.66	12.91	2.87	NS[4]
	Lactation	16.88	20.68	1.68	NS
pH	Prepartum	6.27	6.23	0.10	NS
	Lactation	6.22	6.07	0.60	NS

[1] Treatments were pelleted concentrate mixture without amylase (Control) and with amylase (Amylase); [2] Standard error of the mean; [3] Ammonia-N; [4] Non significant

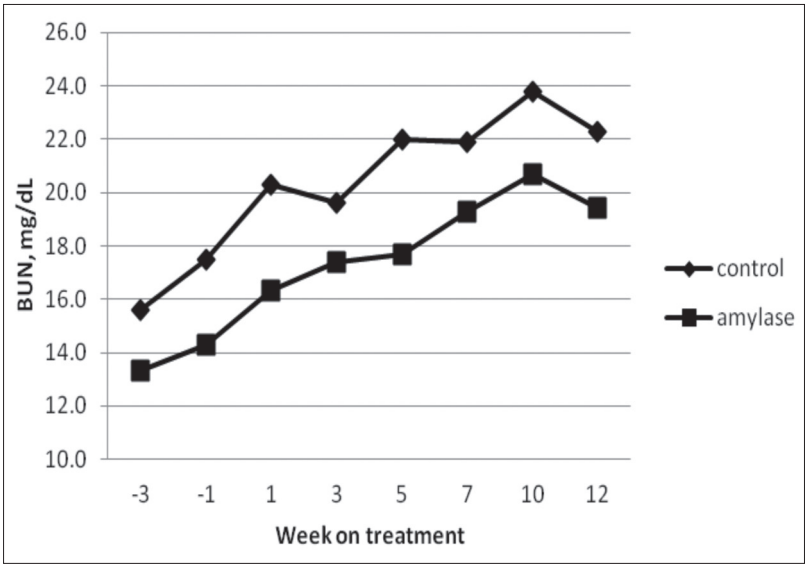

Fig 1. Effect of treatment on BUN (mg/dL) least squares means by week on treatment. Treatments were pelleted concentrate mixture without amylase (Control) and with amylase (Amylase). Week and week x treatment interaction ($P<0.001$)

DISCUSSION

The results of the current study revealed that addition of exogenous amylase did not affect ruminal digestibility of nutrients. Gencoglu et al.[32] reported an increase in DM and OM digestibility for cows fed reduced starch with amylase compared to reduced starch without amylase. In contrast, Weiss et al.[36] reported no effect of exogenous amylase on total-tract digestibility of DM, OM, energy or starch. McCarthy et al.[37] reported no differences among treatments for apparent total-tract starch digestibility. Several researchers have reported increased NDF digestibility with addition of exogenous amylase [19,32,36]. Noziere et al.[18] reported that exogenous amylase supplementation increased the true ruminal digestibility of OM by an average of 4%, but the lower ruminal digestibility of OM and starch in the control diet without exogenous amylase addition was compensated for postruminally, and as result no differences were observed for total-tract digestibility measurements.

Similarly to Gencoglu et al.[32], we have several possible explanations for the lack of effect on starch digestibility when exogenous amylase was added to a reduced-starch diet: 1) starch digestibility was not affected ruminally or postruminally, 2) starch digestibility was increased ruminally [19] but small intestine compensatory starch digestion resulted in similar total-tract starch digestibilities for the treatments, or 3) starch digestibility was increased ruminally, but hindgut fermentation resulted in similar total-tract starch digestibilities for the treatments.

The increase in BHBA for cows fed amylase versus control could be related to the greater ruminal butyrate concentrations. Plasma samples for BHBA were collected 4 h post-feeding to capture peak BHBA concentrations [38]. Addition of exogenous amylases increased serum concentrations of BHBA in the trials of Tricarico et al.[39] and DeFrain et al.[40]. Duffield [41] and Oetzel [42] identified 14.4 mg/dL BHBA as the cut-point for significant subclinical ketosis. Excess amounts of butyric acid from ruminal production are easily converted to BHBA in the wall of the rumen. Huhtanen et al.[43] reported increases in blood BHBA and decreases in blood glucose concentrations. However, amylase supplementation did not change serum glucose concentrations in this study.

The results of the current study revealed that addition of exogenous amylase decreased the BUN concentration when compared with control cows in both prepartum and lactation periods. Greater ruminal starch digestibility could explain the reduced BUN for amylase compared to control [44]. In addition, the reduction in BUN for cows fed amylase versus control was likely related to greater

ruminal propionate concentrations [45]. The MUN is an indicator of protein intake and utilization in dairy cows [46,47], and MUN has a high correlation with BUN [48]. Gencoglu et al.[32] reported reduced MUN for cows fed reduced starch with amylase compared to reduced starch without amylase. Reduced MUN coupled with greater milk protein concentrations for reduced starch with amylase compared to reduced starch without amylase coincides with the suggestion of Voelker and Allen [49] that lower ruminal amylase activity for a reduced starch diet may reduce the rate of starch digestion ruminally and thus microbial protein production. This effect may be enhanced by addition of exogenous amylase to reduced starch diets. The NH_3-N is converted to microbial protein in rumen but a portion of NH_3-N is absorbed through the rumen wall. The NH_3-N that reaches the systemic circulation can be toxic, but in most physiological conditions it is converted in the liver to urea which is less toxic. The NH_3-N which enters the portal circulation may exceed the rate of hepatic metabolism. Murondoti et al.[50] reported that diets containing high concentrations of CP can increase the risk for fatty liver related to high concentrations of blood NH_3-N which are at toxic concentrations. Addition of exogenous amylase to normal-starch diets did not affect MUN in the trials of Klingerman et al.[19], Ferraretto et al.[31], and Weiss et al.[36] and Tricarico et al.[39]. The BUN is a useful indicator of protein status in dairy cows [47]. Ferguson et al.[51] reported that BUN exceeding 20 mg/dL was associated with reduced conception rates in lactating dairy cows. Least squares means by week on treatment for BUN are in *Fig. 1*; week and week × treatment interaction (P<0.001). The BUN values were greater than we expected, on average, based on dietary CP and increased throughout the trial for both treatment groups reaching 20 mg/dL or graeter by 3 week for control and 10 week for amylase. This could possibly be attributed to low starch intake related to the formulation of reduced-starch diets.

The addition of exogenous amylases to the diet did not affect rumen NH_3.N and pH parameters. Cows fed amylase compared with cows fed control in lactation tend to have lower average daily rumen pH (6.07 vs 6.22).

In conclusion, the supplementation of amylase did not affect the digestibility of nutrients. However, the BUN concentrations decreased in pre- and postpartum period for cows fed amylase, thus it may offer potential for improving nitrogen efficiency in dairy cows.

Acknowledgments

Partial funding was provided by DSM Nutritional Products (Basel, Switzerland) and Research Fund of Uludag of University (OUAP(V)-2013/2).

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Kafkas Univ Vet Fak Derg
25 (5): 611-618, 2019
DOI: 10.9775/kvfd.2018.21414

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

Research Article

Effect of *Inhibin-βA* Subunit Gene on Reproductive Performance of Kazakh Sheep in Non-breeding Season

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Article ID: KVFD-2018-21414 Received: 27.11.2018 Accepted: 19.05.2019 Published Online: 21.05.2019

How to Cite This Article

Zhao Z, Zhu M, Cao S, Zhai M, Yang H, Nan Y: Effect of *inhibin-βA* subunit gene on reproductive performance of Kazakh sheep in non-breeding seasons. *Kafkas Univ Vet Fak Derg,* 25 (5): 611-618, 2019. DOI: 10.9775/kvfd.2018.21414

Abstract

Inhibin-βA (INHBA) could feedback suppress synthesis and secretion of follicle-stimulating hormone (FSH), which correlates with the litter size of sheep. In this study, the *inhibin-βA* subunit was used as a candidate gene, and Kazakh sheep was used as a test object. The polymorphism of the gene was detected by PCR-SSCP method and its association with litter size was analyzed. The results showed that there were three polymorphisms in the exon 5'UTR region (primer 0-2), for which AA, AB and BB were detected in Kazakh sheep. The correlation analysis showed that genotype AA had 0.13 ($P<0.05$) lambs and 0.16($P<0.05$) lambs more than genotype AB and BB in Kazakh sheep. Vectors that interfering the *INHBA* expression including PLLU2G-shINHBA-1 (I-1), PLLU2G-shINHBA-2 (I-2), PLLU2G-shINHBA-3 (I-3) and PLLU2G-INHBA-4 (I-4) were constructed by RNA interference (RNAi) technology in the study. After the successfully separated ovarian granulosa cells were transfected with the vector, and the expression level of the gene was detected by quantitative RT-PCR. The results showed that the four vectors suppressed *INHBA* mRNA levels with a silencing efficiency of 34%, 58%, 39% and 19% respectively, with better interference efficiency of I-2. Then, we determined the contents of *INHBA*, FSH, luteinizing hormone (LH) and estradiol (E2) in serum by directing intro-ovarian injection of the I-2. The results showed that the *INHBA* level dropped and the FSH level raised in serum, while LH and E2 levels did not change, indicating the RNAi vector could successfully silence the *INHBA* expression *in vivo*. This study sets a good theoretical basis of researching the breeding and estrus properties of sheep, and the short hairpin (shRNA) vector is hopefully used in promoting the fecundity of sheep in practice.

Keywords: *Inhibin-βA (INHBA), Kazakh sheep, RNA interference (RNAi), Ovarian granular cells, Reproductive Performance*

Üreme Mevsimi Dışında Kazak Koyunlarının Üremeleri Üzerine *İnhibin-βA* Geninin Etkisi

Öz

İnhibin-βA (INHBA), folikül stimule edici hormon (FSH) sentez ve salınımını baskılayabilir ki bu durum koyunlarda yavru sayısı ile ilişkili olabilir. Bu çalışmada, *inhibin-βA* geni aday gen olarak değerlendirilerek Kazak koyunları çalışma materyali olarak kullanıldı. Genin polimorfizmi PCR-SSCP metodu ile belirlendi ve yavru sayısı ile ilişkisi analiz edildi. Elde edilen sonuçlar, Kazak koyununda ekzon 5'UTR bölgesinde AA, AB ve BB için üç polimorfizmin bulunduğunu gösterdi (primer 0-2). Korelasyon analizi, Kazak koyununda genotip AA'da 0.13 kuzu bulunduğunu ($P<0.05$) ve AA genotipinde AB ve BB genotiplerinden 0.16 daha fazla ($P<0.05$) kuzu bulunduğun gösterdi. Bu çalışmada INHBA ekspresyonuna etkiyen PLLU2G-shINHBA-1 (I-1), PLLU2G-shINHBA-2 (I-2), PLLU2G-shINHBA-3 (I-3) ve PLLU2G-INHBA-4 (I-4)'ü içeren vektörler, RNA interferans (RNAi) teknolojisi ile oluşturuldu. Başarıyla ayrılan ovaryum granuloza hücreleri vektörler ile transfekte edildi ve gen ekspresyon seviyesi kantitatif RT-PCR ile belirlendi. Sonuçlar dört vektörün, sırasıyla %34, %58, %39 ve %19 gen susturma etkinlikleri ile *INHBA* mRNA seviyelerini baskıladığını ve daha iyi interferans verimi I-2 olduğunu göstermiştir. *INHBA* ve serum FSH, luteinize edici hormon (LH) ve östradiol (E2) seviyeleri I-2'nin intraovaryan enjeksiyonu sonucu ölçüldü. Sonuçlar, *INHBA* seviyesinin düştüğünü ve serum FSH seviyesinin arttığını, LH ve E2 seviyelerinin ise değişmediğini ve böylece RNAi vektörünün *in vivo INHBA* ekspresyonunu başarıyla susturabildiğini gösterdi. Bu çalışma koyunların üreme ve östrus özelliklerini araştırma amacıyla iyi bir teorik temel oluşturmuştur. Kısa saç tokası (shRNA) vektörü, koyun veriminin arttırılması amacıyla uygulamada kullanılabilir.

Anahtar sözcükler: *İnhibin-βA (INHBA), Kazak koyunu, RNA interferans (RNAi),Ovaryum granular hücreleri, Üreme performansı*

INTRODUCTION

Inhibins were firstly discovered in the testicular extracts in 1932 [1]. Robertson et al.[2] isolated the inhibin molecular weigh of 56.000 which contained two subunits 44.000 and 14.000 Daltons from bovine follicular fluid, inhibin A. At the same year, Ling et al.[3] isolated a 32.000 weighing protein with inhibin activity from porcine follicular fluid which

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contained two subunits 18.000 and 14.000 Daltons, inhibin B. Activin and inhibin proteins are dimeric polypeptide that belong to the TGF-β superfamily of growth and differentiation factors. Activins are formed by the hetero-dimeric combinations of the two subunits (activin A =βA-βA, activin AB =βA-βB and activin B =βB-βB) [3]. Inhibin inhibits FSH secretion from the anterior pituitary and in turn regulates gonadal function and development [4]. And the mutations of *INHBA* gene effects on litter size in sheep significantly [5], and plasma *inhibin* concentrations was related to higher ovulation rates in pigs during follicular phase [6]. Ovaries were recovered from six adult female *Lezhi black goats* and Tibetan goats at 12-24 h after onset of estrus, and used to collect follicles to study cDNA sequence and mRNA expression of *INHA* and *INHBA* genes, and the result showed that base changes in *INHA* and *INHBA* genes resulting in amino acid substitutions may be important in regulating the differential fecundity of these goat breeds as molecular mechanism [7].

In sheep, the length of the oestrus cycle from 13 to 19 days and averages 17 days. Estrus also known as heat period when the ewe is receptive to the ram and will stand for mating. It lasts approximately 24 to 36 h which is influenced by the breed and age of the ewe, the onset of puberty, the presence of the male, and the season [8]. Interestingly though, *INHBA* has a certain relationship with the litter size of sheep, and also probably affect on the estrous cycle through the negative feedback mechanism by suppressing synthesis and secretion of FSH [9]. Several groups have developed transgenic RNAi mice that can produce a gene knockdown phenotype by stably integrated shRNA expression vector *in vivo* [10].

Inhibin has selective inhibition on the synthesis and secretion of FSH, which is crucial for follicular maturation, ovarian development and ovulation. A large number of studies have carried out polymorphism studies on the three subunit genes INHa, INHβA and INHβB and the association analysis of multiple fetal traits [11]. Kristensen et al.[12] studied the local goat inhibin gene, the results showed that the inhibin gene polymorphism significantly affected the number of litters in goats. It is, therefore, necessary to study the relationship between Kazakh sheep inhibin gene polymorphism and reproductive traits.

Ovary granular cell is the maximum cells in follicle. The growth and proliferation of granular cells is one of the most significant sign of the development of follicle [13]. We cultured the granular cell *in vitro* to imitate the growth and development of follicle and the estrous condition. In this experiment, *INHBA* gene which secreted by sheep ovary granular cells was selected as the target gene. The four recombinant vectors was used to interfere the *INHBA* in transfecting the ovarian granular cells. The efficiency of transfection and the interference was tested, then picked out the best interference efficiency recombinant vector. In this study, the method of shRNA was used to interfere the expression in the granular cells of sheep *in vitro* and *in vivo* to explain the mechanism of *INHBA* in the estrous of non-breeding season and discover a way to effect on the *INHBA* expression which could be used in practice to raise the litter size in the future.

MATERIAL and METHODS

Ethics Statement

This study was approved by the Ethical Committee of Animal Experments, Animal Science and Techonology College, Shihezi University (Number: 2015065). All samples were collected in strict accordance with the committee's guidelines. During the experiment, every effort was made to minimize suffering by the animals.

DNA Samples and Lambing Records

Two hundred and thirty genomic DNA samples were obtained from healthy ewes by intravenous blood collection in Xinjiang, China. In this study, a total of 6 Kazakh sheep in good condition were chosen, including 1 sheep deal with saline as one group and 1 sheep deal with empty vectors and 4 sheep deal with interference recombinant vectors. All the Kazakh sheep in the study aged about four years and weighed 49±3.66 kg that were housed individually under the same feeding conditions including *ad libitum* access to alfalfa and water. Genomic DNA was extracted from blood samples using standard phenol-chloroform extraction protocol [14]. Besides that, all lambing records of them were obtained from the production records in the sheep farms.

Primer Design and PCR Amplification

According to the reported sheep *INHBA* gene sequences (NM_001009458.1), two pairs of primers (PD1, and PD2) were designed to amplify the sheep *INHBA* gene. PD1(F: 5'-GGGGAGGAGGCTGAGGAAGT-3' and R:5'-CACA GTAGTTGGCGTGGTAG-3'), PD2(F: 5'-GAGCAGTCGCACAGA CCTTT-3') were separately used to amplify 484 bp, and 196 bp PCR products for 5'-UTR and exon 1 respectively. The primers were synthesized by Xinjiang Kuntailui Co., Ltd. The PCR was performed in a 25 μL reaction mixture containing 0.4 μM of each primer, 200 μM dNTPs, 1× polymerase buffer (including 1.5 mM $MgCl_2$), 1 units of Taq DNA polymerase (Sangon, China) and approximately 100 ng genomic DNA as template. The cycling protocol was 5 min at 95°C followed by 35 cycles of 94°C for 30 s, X°C annealing for 30 s, 72°C for 30 s, with a final extension at 72°C for 10 min (X°C was 58°C and 56°C for PD1, and PD2 primers, respectively)

Single Stranded Conformation Polymorphism (SSCP) and DNA Sequencing Analysis

All PCR products were subjected to SSCP analysis. Aliquots of 2 μL PCR products were mixed with 8 μL loading dye,

denatured by heating at 98°C for 10 min and immediately placed on wet ice. Denatured samples of P1, P2 and P3 were loaded on 10% PAGE gel in 0.5×TBE buffer and constant voltage 140 V for 14-16 h after a pre-run at 220 V for 50 min. The gel was stained by a silver staining method [15].

The 2 PCR products showed different electrophoresis patterns, which were subcloned to pMD19-T vector (Tiangen, China) and sequenced using a commercial service (Huada, Beijing, China). Nucleotide sequence alignments, translations and comparisons were carried out by using DNAMAN software, respectively.

RNAi Vector Construction of INHBA in Sheep

The sequence of RNAi fragment designed in this experiment, depended on the sequence of *INHBA* gene in GenBank (NM_001009458.1) and the principle of RNAi. The construction of the recombination vectors were chosen PLLU2G lentivirus vector. After getting the four RNAi fragments I-1, I-2, I-3 and I-4; the PLLU2G vectors were digested by the *XhoI* and *HpaI* restriction enzymes (Thermo Fisher Scientific, China). The enzyme system: PLLU2G (500 ng/μL) 55 μL, 10×K buffer 5 μL, *XhoI* and *HpaI* 2.0 μL were added to RNase-free water 50 μL at 37°C for 3 h, and tested by 1% agarose gel electrophoresis and collected the vector after digested. PLLU2G linked with the four RNAi fragments (I-1, I-2, I-3 and I-4) over night at 4°C and set the empty control. The recombined vectors transfected the *Escherichia coli* (Thermo Fisher Scientific, China) competent cell overnight. Next day, the monoclonal colony to corresponding resistance LB liquid medium to propagate was picked and tested by PCR, and determined by primers F: AGGCTTAATGTGCGATAAAAGAC, R: GAGCTTATCGATACCGTCGAC, which were sequenced by Invitrogen.

Cell Culture and Transfection Experiment

The ovary was obtained after killing the sheep and the sample was put into the 75% alcohol for 30 s to remove the bacteria from the surface. Later, the samples were washed by PBS (PH=7.2-7.4) for three times and finally the sample was put into the PBS (Invitrogen, Carlsbad, CA, USA) with penicillin and streptomycin. The follicle which is 2-3 mm was pierced through the injection syringe and the liquid from the follicle was taken out into the DMEM (Invitrogen, Carlsbad, CA, USA) with penicillin and streptomycin, which were rinsed repeatedly. The DMEM liquid was filled with the ovary granular cells. The liquid was transferred into the 15 mL centrifuge tube and centrifuge at 1000 rpm for 10 min. The liquid was added in DMEM to suspend the cells and the mixed liquid was filtrated through the 200 meshsieve filtration. After that 5mL 10% fetal bovine serum was added in DMEM culture and the cells were cultured at 37°C and 5% CO_2 incubator in 9 cm petri plate. The cell adherent firmly after 12 h, which were then changed in fresh nutrient solution and cell state was observed. When the primary cells grown to 80%, the nutrient solution was absorbed and digested at 37°C, the process was observed under the microscope until the cell ecptomas vanished. The cell suspension were transferred to new petri plate and cultured at 37°C and 5% CO_2 incubator until the cells grow to 70%. The cells were cryopreserved for later experiment.

The primarily cells in well growth state were used in the transfection experiment. Two controls were setted, one control was added the DMEM and the other control was added the empty vectors equal to the recombinant vectors amount. The four test group were I-1, I-2, I-3 and I-4 interfere vectors *(Table 1)*, and every test group was repeated three times. After the recombinant vectors were transferred the ovary granular cells for 48 h, the transfection efficiency was the best. The fluorescence expression level was observed in granular cells and the number of the cells were recorded under the fluorescence microscope. If the transfection efficiency was up to 20% to 40%, the later experiment could be started.

Cell Total RNA Extraction

The nutrition solution in the 6 well cell culture cluster was discarded and washed and clean by PBS, added the 1 mL Trizol (Invitrogen, Carlsbad, CA, USA) into each well, placed on the ice for 5 min until the cells completely splitting. Added 0.2 mL chloroform to tube, shakes for 15 s, stayed at room temperature for 2 to 3 min, centrifuged with 12000 rpm at 4°C for 15 min. Absorbed the upper clear liquid to a new centrifugal tube, and added the equal volume of isopropyl alcohol after blending, placed at room temperature for 10-30 min, centrifuged with 12000 rpm at 4°C for 15 min. Tested the RNA through the 1% agarose electrophoresis and measured the concentration. The concentration of RNA was measured using a nucleic acid concentration meter (Thermo Fisher, USA), and the ratio of OD260/OD280 was observed to be between 1.8 and 2.0, the later experiment could be started.

Table 1. *INHβA RNAi fragment*

Name	Interference Fragment Sequence	Action Area/bp
I-1	TGCCC TTGCT TTGGC TGAGA GGATT	2-26bp
I-2	CATCG GGACG GAGGG CAGAA ATGAA	317-342bp
I-3	GCTGC ACTTT GAGAT TTCCC AAGAA	404-429bp
I-4	CCATC CGTCT CTTTC AACAG CAGAA	514-519bp

Reverse reaction divided into two steps, the first step system: 10 μL, 5 ×g DNA Eraser Buffer 2 μL, gDNA Eraser 1 μL, total RNA <1 μg, added water to 10 μL, the PCR reaction system: 42°C 3 min, 4°C 10min; the second step system: added the RNase Free dH20, 5 ×PrimeScript Buffer 2 (for Real Time) 4 μL, respectively, RT Primer Mix and PrimeScript RT Enzyme Mix 11 μL, respectively, the PCR reaction system: 37°C 20 min, 85°C 5 s, 4°C 10 min. After those, the gene primer PCR was used for detection.

Transfection of Sheep Ovarian Granulosa Cells with RNAi Plasmid

When the ovarian granulosa cells reached 80%, the dead cells and the medium were washed with sterile PBS (pH=7.2-7.4), and 1 mL of DMEM medium solution (pH=7.2-7.4) was added. Then DMEM solution (pH=7.2-7.4) was added to two 1.5 mL RNase-free tubes, one of which was added with 4 μL of liposome 2000, and the other was added with 1.5 times the mass of RNAi carrier solution equivalent to liposome. Shake the two mixture and let stand for 10 min. The liposome-containing DMEM solution was then added to the DMEM solution of the RNAi carrier, and the mixture was gently mixed and allowed to stand for 30 min. The liposome and RNAi carrier mixed solution was transferred to a cell culture plate and placed in an incubator *(Fig. 1)*.

Sheep INHBA Real-time Fluorescent Quantitative PCR

Using Primer5.0, according to the sheep *INHBA* (GenBank: NM_001009458.1) and *GAPDH* (GenBank: NM_001190390.1) designed real-time fluorescent quantitative PCR primers. Primer sequences were shown in *Table 2*. The PCR reaction system: cDNA 1 μL (100 ng/μL), each of the primer 0.5 μL (0.5 μmol/L), RNAase free H_2O 10.5 μL, PCRmix 12.5 μL, total 25 μL. The PCR procedure: denaturation 94°C 25 s, anneal 57°C 25 s, extend 72°C 25 s, 35 reaction cycles. The PCR electrophoresis test analyzed by gel imaging.

Recombinant Plasmid Transfer by Ovarian Injections

The sheep blood were collected 3 days before the injection of interference vectors and 7 days after injection and the level of INHBA, E_2, FSH and LH in serum related with estrous were measured to explore the relationship between the INHBA and estrous in non-breeding season.

The experimental sheep were fixed in binding frame and removed the abdominal wool. The location with less vessels of abdominal blood were selected to anesthetic and after 5-15 min, the scalpel was used to open the areas of muscle layer and cortex, explored the ovary and fixed it, the 1 mL medical syringe with 400 μL interference vectors were used to inject ovarian surface for a few points. After the injection, the wound were closed by a suture, and applied penicillin and streptomycin powder to the wound. The eating and exercise of sheep were observed over night and collected 3 mL blood from the sheep in good condition, kept 3 h at 38°C, centrifuged with 3000 rpm for 2 min and collected the upper clean liquid. Enzyme linked immunosorbent assay (ELISA) were used in the determination of serum INHBA, FSH, LH and E_2 levels. The antibody was diluted to a protein content of 1 to 10 μg/mL with 0.05 M PH=9.0 carbonate coating buffer, and 0.1 mL was added to the reaction well of each polystyrene plate. Subsequently, 0.1 mL of a sample to be tested diluted with a certain amount was added to the reaction well, and the mixture was incubated at 37°C for 1 h. After washing, 0.1 mL of freshly diluted enzyme-labeled antibody was added, and the mixture was incubated at 37°C for 0.5 to 1 h and then washed. 0.1 mL of the prepared TMB substrate solution was added, and the mixture was allowed to stand at 37°C for 10 to 30 min. Finally, 0.05 mL of 2 M sulfuric acid was added to each reaction well to terminate the reaction.

Statistical Analysis

Differences in haplotype frequencies were analyzed using a

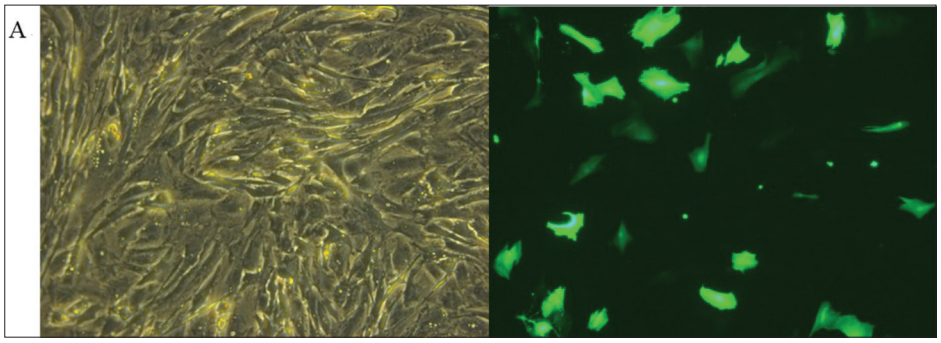

Fig 1. Culture and transfection of ovarian granulosa cells

Table 2. *GAPDH* and *INHBA* RT-PCR primers

Gene Name	Primer Sequence(5′~3′)	Amplification Length/bp	The Annealing Temperature/°C
GAPDH	F:TTCTGCTGACGCTCCCA R:CCTCCACGATGCCAAAG	134	57
INHBA	F:CCTCAAGTCGTGCTGTGTG R:GTCTTCGTGTCACCACTGTCT	187	57

χ2-test. The association between polymorphisms in INHBA gene and the number of lambs in serum and the data of the real-time PCR were evaluated using One-Way ANOVA test. The Data were expressed as the mean±the standard error and all statistical analysis were performed with SPSS for Windows (version 20.0).

RESULTS

SSCP analyses of the PCR-amplified fragments in our study from the 484-bp section of the 5'-UTR of the *INHBA* gene showed three distinct banding patterns in Kazakh sheep (n = 230), including AA, AB and BB *(Table 3)*. However there is no primers 1-1 designed for exon 1 were found to have polymorphic sites of amplified fragments. We used the sequences of the full-length INHBA gene of sheep available in GenBank (accession numbers 443524) as a reference to compare with the sequences we obtained from Kazakh sheep. And subsequent sequence comparison revealed there were two SNPs of the PD1 fragments in Kazakh sheep, including g.79A > G and g.107G > A, which the former was changed to AA- and AB-genotypes and the latter was changed to BB- and AB genotypes. Above all, in our study, it is worth to note that Kazakh sheep is a typical single breed. The genotype frequency and allele frequency of INHBA gene in sheep were shown in *Table 4*, AB-genotype frequency of Kazakh sheep was higher than AA and BB, which showed A-genotype was the predominant allele in all the populations.

Polymorphism information content (PIC), population heterozygosity, effective allele number, and χ2-test results of loci in Kazakh sheep group were shown in *Table 5*. PIC of the populations in Kazakh sheep was separately 0.373 that was ranged within 0.25-0.50, which indicated that the loci was moderately polymorphic in the populations.

According to the statistical results in *Table 3*, which can be considered that there is a significant difference in the number of litters between the Kazakh foreign genotype AA and the AB and BB genotypes (P<0.05). In addition, there was no significant difference in the number of lambs between AB genotype and B genotype (P>0.05).

The colony PCR products including two parts: The length of the positive clone was about 302bp, and the negative clone was about 239bp. Taken the positive clone to sequence to define the base did not mutate, and the results of sequencing are showed in the *Fig. 2*. The *Fig. 2A* was the sequence of the origin RNAi fragments and the *Fig. 2B* was the result of sequencing, both of which showed the two sequencing results were exactly same. According to the results, it indicated that there was no mutated base in the recombinant RNAi vector which could be used in the next ovary granular cell transfection experiment.

The ovarian granular cells were into oval or round just after separation, and the cells were adherent 4-6 h later, and the cells connected with the others by extended filiform pseudopodia, the nucleus was large, the cytoplasm appeared a large number of particles, a single-layer adherent growth and divided fast, and after 96 h of separation the dish was covered. After the petri dish was completely covered, the cells present different shapes such as spindle, irregular triangle, polygon or fan. After 48 h of the transfection of ovarian granular cells with recombinant RNAi vectors, it showed that the transfection effect got the peak and the cells in the 6-well cell culture plate covered about 80% and the transfected fluorescent efficiency was about 40% in the transfected groups and finally reached the basic demands of the cell transfection experiment by observing the fluorescent expression under the fluorescent inverted microscope.

Table 3. *Correlation analysis of INHBA with litter size in sheep*

Group	No.	Genotype	The Number of Lamb
Kazakh sheep	230	AA(82)	1.25±0.06[a]
		AB(114)	1.12±0.03[b]
		BB(34)	1.09±0.08[a]

Table 4. *Data of Heterozygosity (H), Effective number of Alleles (Ne), PIC and χ2-test*

Group	H	Ne	PIC	χ^2-test
Kazakh sheep	0.497	1.987	0.373	2.398 (P<0.05)

Table 5. *The genotype frequency and gene frequency of INHBA gene in Kazakh sheep*

Group	No.	INHBA Primer				
		Genotype Frequency			Allele Frequency	
		AA	AB	BB	A	B
Kazakh sheep	230	0.357(82)	0.496(114)	0.147(34)	0.553	0.447

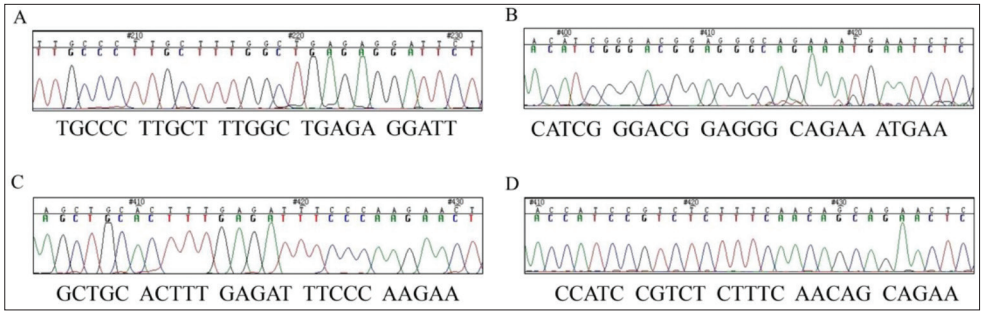

Fig 2. Sequences comparison of recombinant vectors with mRNA of *INHBA*

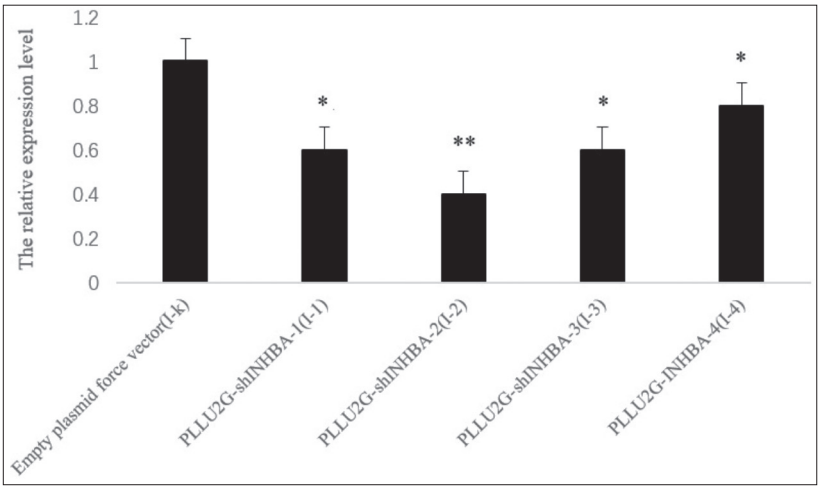

Fig 3. *INHBA* gene mRNA expression in the ovarian granular cells of sheep

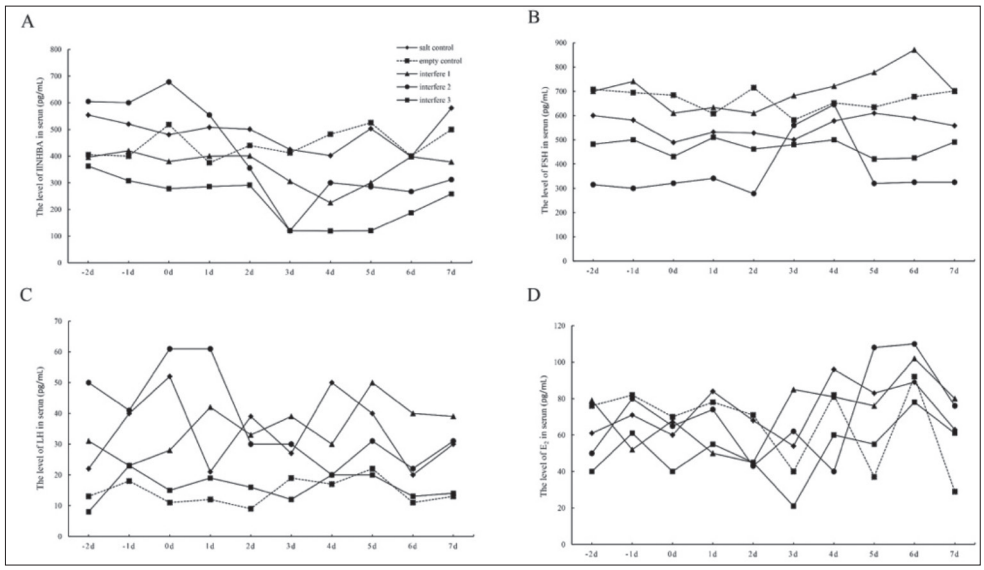

Fig 4. The level of INHBA, FSH, LH and E_2 in serum of sheep

Above all, compared to the empty plasmid force vector (I-K) group, the restructuring plasmid carrier interference on the expression of *INHBA* gene in the ovarian granular cells showed the interference effect, and those relative expression of *INHBA* gene in I-1, I-2, I-3 and I-4 groups after transfection were 0.66, 0.42, 0.62 and 0.66 respectively *(Fig. 3)*, and in turn their interference efficiency were 34%, 58%, 39% and 58%, respectively.

From the *Fig. 4A*, the INHBA level in the salt control and empty control were not changed significantly which keep at the level of 400 pg/mL to 500 pg/mL. However, in the interference group 1, after interference vectors injection, the INHBA level changed at the 2nd day and decreased to the lowest point of 200 pg/mL at the 4th day, then the level began to raise smoothly until the 7th day and the level recovered to 390 pg/mL just as the level before interference. In the interference group 2, the INHBA level dropped sharply at the 1st day after injection until the 3rd day up to the lowest point of 100 pg/mL, then raised gradually. In the interference group 3, the INHBA level decreased to the bottom at the 2nd day and kept at the

bottom of 110 pg/mL for two days and followed shot up. From the *Fig. 4B*, the FSH level in the salt control and empty control was not changed significantly. But in the interference group 1, the level of FSH took off at the 3rd day after injection and reached the peak about 880 pg/mL at the six day, then dropped to the level of 700 pg/mL just as the level before the injection. In the interference 2, the level of FSH raised sharply at the 3rd day of 600 pg/mL and it descended again to the level before injection of about 300 pg/mL. In the interference group 3, the level of FSH did not change significantly. Besides those, more interestingly, in the *Fig. 4C* and *Fig. 4D*, we also noted that there were no significant difference between the level of LH and E_2 before and after the interference recombinant vectors injection, which indicated that the INHBA level was lower in the ovaries and it couldn't lead to the changes of LH and E_2 levels in the ovary following the change of INHBA level. Therefore, those results in the study suggested that the interference recombinant vectors could successfully interfere on the INHBA level in the ovarian granular cells of sheep, and according to those results appeared that the FSH raised following the decreasing level of INHBA and the level of LH fall down as the INHBA raised but the level of E_2 raised along with the INHBA raised.

DISCUSSION

Inhibin is a glycoprotein hormone secreted by the gonads, which can inhibit the synthesis and secretion of FSH in the pituitary [13]. Genetic characteristics of single nucleotide mutation in this study were analyzed in the 5`-UTR and exon 1 of INHBA gene in Kazakh sheep. There were three mutation sites of PD1 site in INHBA, by comparing with the reported ovis INHBA sequences, respectively, among which AA genotype with an A → G mutation in 79bp and BB-genotype with a G → A mutation in 107bp were cause amino acid changes. In this study, we selected *INHBA* gene in Kazakh sheep breeds to study the genotype frequency and gene frequency of INHBA gene, which indicated that A-allele could be the predominant gene in the Kazakh sheep populations and it might provide a choice for selecting the sheep breeds. In the previous studies, there were only few reports about the impact of *INHBA* Gene on animal reproductive traits. In the study of the genetic variation of inhibin A gene and the double lamb trait, Chu et al.[14] found that statin is associated with the double lamb trait. In this study, the inhibin βA gene was studied as a candidate gene associated with lamb litter size in sheep. The result showed that the number of Lambs in AA genotypes of Kazakh sheep was significantly higher than that of AB and BB genotypes ($P<0.05$). There was no significant difference in BB and AB genotypes ($P>0.05$). The number of Lambs in AA genotypes in the 5'-UTR of Kazakh sheep was significantly higher than that in genotypes AB and BB, indicating that allele A is a dominant allele relative to allele B, to a certain extent. It indicates that the homozygous AA mutation in this experiment is positively correlated with the number of lambs in Kazakh sheep in Xinjiang, which is worthy of further study.

Inhibins are produced and secreted by the granulosa cells of the largest follicles during terminal follicular development and is important inhibitors of FSH secretion by pituitary gonadotrophs. A study showed that *INHBA* knock-out mouse exhibited reduced expression of *TGFBR3*, which disrupted *Inhibin-TGFBR3* signaling and therefore increases the secretion of FSH in the mouse anterior pituitary cells [15]. More interestingly, some studies also showed that the lower INHBA levels could lead to the development of a greater number of follicles in lamb, and attenuate INHBA activity could result in higher fertility and ovulation rates in sheep, cattle, and rats using the INHBA vaccines [12].

RNAi possesses post-transcriptional gene silencing mechanism and high specificity, different from traditional DNA level gene knockout technology. In the present study, in order to explore the correlation of INHBA with the reproductive traits in sheep, the RNAi technology was selected and used to construct the recombinant vectors with effective shRNA fragment, and the latter was performed well in the transfection of granular cells *in vitro* and in the ovary of sheep. Han et al.[16] reported that RNAi could inhibit virus production and effectively inhibit pre- and/or post-integration infection events in the HIV-1 life cycle. Si et al.[4] study showed that the H460 cells at 70% confluence was transfected using the optimal small interfering (siRNA) and the *INHBA* knockdown efficiency was 93% by the quantitative RT-PCR and the concentration of *INHBA*-targeting siRNA reduced 6% after 96 h of transfection. However, in the study, we constructed four inducible *INHBA* expression vectors as the experiment groups contain I-1, I-2, I-3, I-4, and an empty vector PLLU2G as the negative control group. The results of the study showed that all the silencing efficiency in the four vectors with inhibiting *INHBA* mRNA expression were 34%, 58%, 39% and 19% respectively, which showed that the better interference efficiency was the I-2 group. Therefore, our results also indicated that the I-2 inducible siRNA expression system could efficiently induce conditional inhibition of *INHBA*, which could conditionally inhibit the expression of *INHBA* in not only established stable clones but also transient transfection cells and finally greatly increase its usefulness and convenience [17]. Beside that, in the study, all the interference recombinant vectors were injected into the follicle of sheep, and then measured the serum INHBA levels in different groups, all of which proved that the INHBA indeed had a closed relationship with the steroid hormones, including FSH, LH and E_2, and further effect on the estrous cycle and reproductive performance in sheep, especially in the non-breeding season.

In conclusion, The PLLU2G vector is based on a well-established and can be used shRNA system, combining the puromycin and eGFP markers on a MSCV-based backbone.

The RNAi vector were successfully constructed and transfected into the follicular granular cells of sheep and then silenced the *INHBA* expression *in vivo*, those results presented in this study clearly indicate that this inducible siRNA expression system could efficiently, conditionally and reversibly inhibit *INHBA* expression in sheep. In a word, this study could be used to set a good theoretical basis of researching the breeding properties and estrus properties of sheep, and the shRNA vector is hopefully used in promoting the fecundity of sheep in practice.

Competing Interests

There are no potential conflicts of interest.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (No. 31460587), and Functional Verification and Development of Seasonal Reproductive Traits in Sheep (No. 2011KLS06). In addition, we are very grateful to Yifan Xie and Mu Jian for their help in this experiment.

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Kafkas Univ Vet Fak Derg
25 (5): 619-626, 2019
DOI: 10.9775/kvfd.2018.21455

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

Research Article

The Effects of Exogenous Fibrolytic Enzymes and a Ferulic Acid Esterase-Producing Inoculant Treatment on Digestibility and Conservation Characteristics of Corn Stover [1]

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[1] *This work was supported by the Research Fund of Ondokuz Mayıs University (Project Number: PYO.VET.1901.17.007)*

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Article ID: KVFD-2018-21455 Received: 03.12.2018 Accepted: 22.04.2019 Published Online: 27.04.2019

How to Cite This Article

Muruz H, Selçuk Z, Salman M, Nuhoğlu Z, Kaya İ, Çetinkaya N: The effects of exogenous fibrolytic enzymes and a ferulic acid esterase-producing inoculant treatment on digestibility and conservation characteristics of corn stover. *Kafkas Univ Vet Fak Derg,* 25 (5): 619-626, 2019.
DOI: 10.9775/kvfd.2018.21455

Abstract

The objective of the current study was to determine the effects of a mixed bacterial inoculant possessing ferulic acid esterase (FAE) activity and exogenous fibrolytic enzyme (EFEs) products on the chemical composition, conservation characteristics and digestibility of corn stover. The moisture level of corn stover was adjusted to 40% with deionized water and then treated with deionized water (control), EFEs (10 U cellulase + 60 U xylanase units g^{-1} of substrate dry matter) or EFEs + FAE inoculant (FAEI) ($1.3x10^5$ cfu g^{-1} fresh forage). The treated stover was then incubated in laboratory mini-silos for 30 days. After the incubation period, the corn stover treated with both EFEs and EFEs + FAEI had a lower pH, ADF and NDF ($P<0.001$), and higher acetic acid and lactic acid ($P<0.001$) than the control stover. In addition, moulds and yeasts were inhibited in stover treated with EFEs + FAEI. The in vitro true dry matter digestibility (IVTDMD) and *in vitro* true neutral detergent digestibility (IVTNDFD) of the stover treated with EFEs and EFEs + FAEI were higher than for the control ($P<0.001$) but there was no significant difference between the EFEs and EFEs + FAEI treatments. From economical point of view, the best treatment was EFEs + FAEI. These results suggest that EFEs played a major role in enhancing the digestibility of corn stover, alone or in combination with FAEI. Moreover the use of EFEs + FAEI promoted a positive response in the conservation characteristics.

Keywords: *Exogenous fibrolytic enzyme, Ferulic acid esterase producing inoculant, Corn stover, In vitro digestibility*

Eksojen Fibrolitik Enzim ve Ferulik Asit Esteraz Üreten Bakteriyal İnokulant Muamelesinin Mısır Samanının Sindirilebilirliği ve Konservasyon Özellikleri Üzerine Etkileri

Öz

Bu çalışmanın amacı, ferulik asit esteraz üreten bakteriyel inokulant (FAEI) ve ekzojen fibrolitik enzimlerin (EFEs) mısır samanının kimyasal kompozisyonu, konservasyon özellikleri ve sindirilebilirliği üzerine olan etkisini araştırmaktır. Mısır samanının nem içeriği deiyonize su ile %40'a ayarlandı ve daha sonra deionize su (kontrol), EFE's (10 U cellulase + 60 U xylanase units g^{-1} of substrate kuru maddesi) ve EFEs + FAEI ($1.3x10^5$ cfu g^{-1} taze kaba yem) ile muamele edildi. Muamele edilen mısır samanı, 30 gün süreyle laboratuvar mini-silolarında inkube edildi. İnkubasyon periyotundan sonra, EFEs ve EFEs + FAEI ile muamele edilen mısır samanının ADF, NDF miktarı ve pH kontrole kıyasla daha düşük ($P<0.001$), asetik asit ve laktik asit içerikleri daha yüksek ($P<0.05$) belirlendi. Buna ek olarak EFEs + FAEI ile muamele edilmiş mısır samanında maya ve küf tespit edilmedi. EFEs ve EFEs + FAEI ile üretilen mısır samanında in vitro gerçek kuru madde sindirilebilirliği (IVTDMD) ve *in vitro* gerçek nötral deterjan sindirilebilirliği (IVTNDFD) kontrolden daha fazla bulundu ($P<0.001$), fakat EFEs ve EFEs + FAEI arasında bir fark saptanmadı. Ekonomik bakımdan en iyi sonuç, EFES + FAEI grubundan elde edildi. Bu sonuçlar, mısır samanının EFEs'nin tek başına veya FAEI ile kombine edilmesinin mısır samanın sindirilebilirliğinin artırılmasında önemli bir rol oynadığını göstermiştir. Dahası, EFEs + FAEI kullanımı konservasyon özelliklerini olumlu etkilemiştir.

Anahtar sözcükler: *Eksojen fibrolitik enzimler, Ferulik asit esteraz üreten bakteriyal inoculant, Mısır samanı, In vitro sindirilebilirlik*

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INTRODUCTION

In Turkey, corn is planted on approximately 680.000 ha, which is approximately 5% of the land used for cereal crops, and corn stover production can reach more than six million tons per year [1]. Despite the abundant production of stover, the low digestion rate of cell wall material by rumen microbes is one of the main problems associated with its use as a feed for ruminants. Therefore, the issue of improvement of its digestibility has received considerable critical attention. Several chemical treatments have been long known to be effective [2]. However, despite these efforts, more than 50% of fiber is still not digested.

Recently, research on the subject has focused on the use of exogenous fibrolytic enzymes (EFEs) such as cellulase and xylanase to improve the nutritive value of low quality forage [3]. Several *in vitro* studies have demonstrated an increase in the fiber digestibility value and complex polysaccharide degradability when corn stover was treated with EFEs [4,5]. Compared with only cellulase or xylanase, the combined application of these enzymes was more effective due to synergism [6]. Moreover, the application of EFEs to forage prior to storage tended to produce a better response via a longer period of interaction between the enzymes and forage than application prior to feeding [7]. However, the pre-storage use of EFEs on forage with a high fiber content has been less well explored [8]. In one study, Mendoza et al.[9] reported that lower forage quality limits the effects of EFEs.

The extent of cell wall digestion is mainly determined by ferulic acid which is cross-linked to polysaccharides by ester bonds and to lignin mainly by ether bonds [10,11]. Other studies have shown that complete or partial hydrolysis of ferulic acid linkages in forage may directly increase the susceptibility of cell walls to ruminal digestion [12,13]. In addition, recent studies have showed that the degradation of ferulic acid linkages by athird-generation ferulic acid esterase producing bacterial inoculant (FAEI), namely *Lactobacillus buchneri*, may have the potential to partially break the ester linkage between ferulic acid and polysaccharides, thereby improving fiber digestibility [14,15]. However, more recent attention has focused on the use of EFEs in combination with FAEI as a potentially effective way to break down the cross linkages in carbohydrates incorporated in plant structural material [16,17]. A considerable amount of literature has been published on EFEs and FAEI [7,16,18]. Most of these studies have only focused on medium to good quality forages [7,16,17]. However, to date, no studies have been published on the effects of EFEs + FAEI as additives on the nutritive value of poor quality forages such as corn stover. Therefore, the principal objective of this study was to investigate the effects of the EFEs, cellulase and xylanase, alone or in combination with a mixed lactic acid bacteria inoculant containing FAE-producing *L. buchneri* on the chemical composition, fermentation and microbiological activity, and *in vitro* digestibility of corn stover.

MATERIAL and METHODS

Crop and Residue Collection

The stover from corn *(Zea mays indurata)* was collected from a farm in the Bafra district of Samsun Province, Turkey in September 2017. After the harvesting of the central rows of the field at 30 cm above ground level, samples of the whole corn stover (leaf, husk, and stalk) were sundried and then chopped into about 30 mm lengths with a straw chopper.

Preparation and Treatment

Deionized water was sprayed on the stover to achieve a final moisture level of approximately 40% of the fresh stover weight. The stover treatments were: 1) control (deionized water, no additives), 2) EFEs and 3) EFEs + FAEI. To ensure a homogeneous distribution, the EFEs and FAEI were dissolved in deionized water and then immediately sprayed on the stover in a 5 min period while it was mixed manually. The treated stover was packed into 1 L laboratory mini-silos (glass jars) which were then sealed, with five replications for each treatment. The silos were stored at room temperature (22ºC) for 30 d.

The EFEs used in the study were a mixture of cellulase and xylanase (Biovet, Bursa, Turkey). The declared enzymatic activities are 7300 IU of cellulose g^{-1} and 45000 IU g^{-1}. The combination of EFEs was applied (10 cellulase units/g of substrate DM + 60 xylanase units/g of substrate DM) according to the methodology of Zhao et al.[19], either alone or in combination with FAEI 11 GFT (Pioneer Hi-Bred, Int., Inc., USA). The FAEI (11GFT: 1.0 x 10^{11} cfu g^{-1} of *Lactobacillus buchneri* LN4017) applied to produce ferulic acid esterase, 2.0 x 10^{10} cfu g^{-1} of *Lactobacillus plantarum* LP7109 and 1.0 x 10^{10} cfu g^{-1} of *Lactobacillus casei* LC3200 were applied at the dosage recommended by the manufacturer, to achieve 1.3 x 10^{5} cfu g^{-1} fresh forage.

Chemical Analysis

After the incubation period, the silos were opened and the topmost spoiled portion of the corn stover was discarded. To determine the pH, 25 g of corn stover from each replicate was blended with 100 mL of distilled water for 10 min [20]. The homogenate was filtered through two layers of cheesecloth and the pH was immediately determined with a digital pH meter (Thermo Orion 710 A+, Thermo Electron Corporation). Two-three drops of toluene were added to approximately 10 mL of sample to prevent fermentation. The samples were stored at -20ºC until they were analysed. The acetic acid and butyric acid contents were determined according to Filipek and Dvarok [21] and the lactic acid content was measured according to the method described by Zhang et al.[22].

The stover samples were oven dried at 60°C for 48 h to achieve constant weight and the dried samples were milled through a 1 mm screen and preserved in labelled plastic containers before chemical analysis and *in vitro* digestion. Pre- and post-storage stovers were analyzed for their ADF, NDF (with alfa-amylase and sodium sulfite) and acid detergent lignin (ADL) contents, according to the methodology of Van Soest et al.[23] in an Ankom$^{200/220}$ Fiber Analyzer. Standard methods were used to determine the organic matter (OM) and ash contents [24].

Microbiological Analysis

When the silos were opened, samples of each treatment were taken for the determination of yeast and mould status. A composite sample (400 g) was taken using sterile gloves and polyethylene bags and sent directly to a laboratory (Samsun Food Control Laboratory Directorate, Turkey). The identities of yeasts and moulds were determined according to ISO 21527-2 criteria [25] and the microbial counts were log10 transformed.

In Vitro Digestibility

In vitro true digestibility (IVTD) was determined with an Ankom Daisy^{II} incubator (Ankom Technology Corp., Fairport, NY, USA) [26], with the unit consisting of a thermostatic chamber (39°C) and 4 rotating jars with a capacity of 2 L each, using an approximately 0.5 g sample of corn stover incubated for 48 h. The reagents, filter bags and samples were prepared according to the procedure specified for the Ankom Daisy^{II} *in vitro* system. The buffer solution consisted of 1.330 mL of buffer A (KH_2PO_4, 10.0 g/L; $MgSO_4.7H_2O$, 0.5 g/L; $CaCl_2{\cdot}2H_2O$, 0.1 g/L and urea, 0.5 g/L; and 266 mL of buffer B (Na_2CO_3, 15.0 g/L and $Na_2S.9H_2O$, 1.0 g/L), mixed in each digestion jar and the pH was adjusted to 6.8. In the analysis, F57 filter bags were used for incubation of the stover. The bags were rinsed with acetone for 3 min and the rinsed bags were then oven dried for 8 h at 60°C. Rumen contents were collected from different sites within the rumen of two donor cows slaughtered in a private slaughterhouse in Samsun Province. The animals were fed with total mixed ration (TMR) as a basal diet. A basal diet (% dry matter [DM]) consisted of corn (38.2%), sun flowe meal (22.5%), barley (23.0%), wheat straw (12.5%), limestone (1.4%), dicalcium phosphate (1.4%), salt (0.8%) and vitamin-mineral premix (0.2%). The rumen contents were transported immediately to the laboratory. They were then strained through four layers of cheesecloth and held at 39°C under a CO_2 atmosphere. The stover samples (0.5±0.01 g) were weighed into the four bags per treatment. The F57 filter bags were placed in the digestion jars filled with 1.596 mL (Buffer A: 1.330 mL + Buffer B: 266 mL) of buffer solution and 400 mL of rumen fluid. After 48 h of incubation, the bags were removed from the jars, and washed four times under running cold water. The bags were then placed in the Ankom$^{2000/220}$ Fiber Analyzer and the manufacturer's procedure for determining the neutral detergent fiber (NDF) amount was followed. The IVTD was calculated as the difference between the DM of the incubated material and the residue after neutral detergent treatment [23]. The IVTNDFD was calculated as the difference between the amount of fiber incubated and the amount recovered after the NDF analysis [26].

Economic Evaluation

According to Oba and Allen [27], 1 unit enhanced in forage NDFD *in vitro* was positively associated with 0.17 kg of dry matter intake (DMI) and 0.25 kg of 4% fat corrected milk yield. Therefore, economical return of the IVTNDFD was calculated for milk production. Price of 1 kg of enzyme and 25 gr of inoculant was 75.00 TRY and 308.00 TRY, respectively. 1 kg of 4% fat corrected milk was 2.10 TRY in year 2019. 1 US Dollar was valued at about 5.82 TRY in April, 2019.

Statistical Analysis

Firstly, the Shapiro-Wilk test was used to determine whether the population was normally distributed. Data management and analysis were carried out by repeated measurements. The difference between means was compared with Tukey's comparison test, with significance assumed for P values <0.05. Analyses were carried out by using IBM SPPSS V21.0 software (IBM Cooperation, Chicago, IL, USA) [28].

RESULTS

The pre-storage stover had high ADF, NDF and ADL contents (75.39, 51.34 and 12.41% respectively) and low IVTD and IVTNDF (55.06 and 37.02%, respectively) *(Table 1)*.

Table 2 displays the results of post-storage chemical composition of corn stover treated with fibrolytic enzymes and inoculant. The ADF and NDF contents were highly significantly (P<0.001) lower in the EFEs and EFEs + FAEI-treated stover than in the controls. However, none of the additives had a significant effect on the ADL content.

The EFEs + FAEI group had a significant (P<0.001) effect on the pH, lactic acid concentration, acetic acid concentration

Table 1. *Nutrient contents and in vitro dry matter digestibility of corn stover in pre-storage*

Item	Pre-storage Corn Stover (±SD)[1]
NDF[2]	75.39±0.34
ADF[2]	51.34±0.52
ADL[2]	12.41±0.10
IVTD[3]	55.06±0.37
IVTNDFD[3]	37.02±0.69

*[1] Means ± SD (n = 3) for corn stover samples collected before storage; [2] **NDF:** neutral detergent fibre; **ADF:** acid detergent fibre; **ADL:** acid detergent lignin (% dry matter); [3] **IVTD:** in vitro true digestibility, **IVTNDFD:** in vitro true neutral detergent digestibility*

and number of yeasts and moulds, compared with the other two groups *(Table 3)*. After the application of EFEs + FAEI, the pH level (4.69) declined and the concentration of lactic acid and acetic acid increased (1.71 and 2.02 g kg^{-1} DM, respectively). Moreover, moulds and yeasts were not detected in stover treated with EFEs + FAEI whereas appreciable numbers of yeasts and moulds were detected in the control and the EFEs.

After 48 h of incubation in the Ankom Daisy^{II} incubator, values of means for *in vitro* IVTD and IVNDFD of corn stover treated with EFEs and FAEI are shown in *Table 4*. The IVTD and IVTNDFD were significantly higher (P<0.001) for the EFEs treatment by 3.83 and 5.16 unit respectively, and EFEs + FAEI by 4.45 and 6.08 unit, respectively, compared to the control (data not shown in the table). However no difference (P>0.05) was found between EFEs and EFEs + FAEI treatments.

Regarding the economic evaluation *(Table 5)*, results indicated that the net profit for EFEs and EFEs + FAEI inclusion in corn stover was higher 2.63 and 3.10 TRY than control, respectively. Best net revenue was recorded for supplemented with EFEs + FAEI.

DISCUSSION

In this study, the NDF, ADF and ADL concentrations before storage of whole corn stover were were lower than the concentrations reported (51.34-55.19, 75.39-89.23 and 12.41-34.04% of DM basis, respectively) by Bhasker et al.[29] and higher than those reported by Cui et al.[30] and Zhao

Table 2. *Post-storage chemical composition of corn stover treated with fibrolytic enzymes and inoculant*

Item	Experimental Groups			SEM[3]	P-value
	Control	EFEs[1]	EFEs + FAEI[1]		
NDF[2]	69.65[a]	64.86[b]	65.04[b]	0.573	<0.001
ADF[2]	46.28[a]	42.92[b]	43.15[b]	0.211	<0.001
ADL[2]	12.10	12.24	12.16	0.222	0.840
Ash	9.20	9.33	9.45	0.167	0.372

*[1] **EFEs:** exogenous fibrolytic enzymes, **FAEI:** ferulic acid esterase-producing inoculant (1.0×10^{11} cfu/g of L. buchneri LN4017, 2.0×10^{10} cfu/g of L. plantarum LP7109, and 1.0×10^{10} cfu/g of L. casei LC3200); [2] **NDF:** neutral detergent fibre; **ADF:** acid detergent fibre; **ADL:** acid detergent lignin (% of dry matter); [3] Standard error of the mean, [a,b] Means (n =5) within a row with different superscripts differ significantly (P<0.001)*

Table 3. *Storage fermentation characteristics and microbial counts of corn stover treated with enzymes and inoculant*

Item	Experimental Groups			SEM[3]	P-value
	Control	EFEs[1]	EFEs + FAEI[1]		
pH	5.43[a]	5.01[b]	4.69[c]	0.043	<0.001
Lactic acid[2]	0.31[c]	0.84[b]	1.70[a]	0.167	<0.001
Acetic acid[2]	0.55[c]	0.84[b]	2.024[a]	0.177	<0.001
Butyric acid[2]	ND	ND	ND		
Microbiology, log_{10} cfu/g of fresh material					
Yeast	5.04[a]	2.3[b]	ND	0.732	<0.001
Mould	3.93[a]	2.90[b]	ND	0.325	<0.001

*[1] **EFEs:** exogenous fibrolytic enzymes, **FAEI:** ferulic acid esterase-producing inoculant (1.0×10^{11} cfu/g of L. buchneri LN4017, 2.0×10^{10} cfu/g of L. plantarum LP7109, and 1.0×10^{10} cfu/g of L. casei LC3200); [2] g kg^{-1} dry matter; [3] Standard error of the mean; [a,b,c] Means (n =5) within a row with different superscripts differ significantly (P<0.001); **ND:** none detected*

Table 4. *In vitro digestibility of corn stover treated with enzymes and inoculant*

Item	Experimental Groups			SEM[3]	P-value
	Control	EFEs[1]	EFEs + FAEI[1]		
IVTD[2]	59.87[b]	63.70[a]	64.32[a]	0.757	<0.001
IVTNDFD[2]	38.54[b]	43.70[a]	44.62[a]	0.968	<0.001

*[1] **EFEs:** exogenous fibrolytic enzymes, FAEI: ferulic acid esterase-producing inoculant (1.0×10^{11} cfu/g of L. buchneri LN4017, 2.0×10^{10} cfu/g of L. plantarum LP7109, and 1.0×10^{10} cfu/g of L. casei LC3200); [2] **IVTD:** in vitro true digestibility, IVTNDFD: in vitro true neutral detergent digestibility (% of dry matter); [3] Standard error of the mean; [a,b] Means (n =5) within a row with different superscripts differ significantly (P<0.001)*

Table 5. *Economic analysis of milk produced in terms of differences in IVTNDFD*

Item	Experimental Groups		
	Control	EFEs[1]	EFEs + FAEI[1]
Enzymes cost[2], TRY	0	0.08	0.08
Inoculant cost[3], TRY	0	0	0.008
Increase in IVTNDFD[4], unit	1 (38.54%)	5.16 (38.54% vs 43.70%)	6.08 (38.54% vs 44.62%)
Milk yield (4% FMC)[5], kg/day	0.25	1.29	1.52
Milk price[6], TRY/kg	2.10	2.10	2.10
Total profit, TRY/day	0.52	2.71	3.19
Net profit, TRY/day	0.0.52	2.63	3.10

*[1] **EFEs:** exogenous fibrolytic enzymes, **FAEI:** ferulic acid esterase-producing inoculant (1.0×10^{11} cfu/g of L. buchneri LN4017, 2.0×10^{10} cfu/g of L. plantarum LP7109, and 1.0×10^{10} cfu/g of L. casei LC3200); [2] Price of 1 kg of enzymes: 75.00 TRY, [3] price of 25 g of inoculant: 308.00 TRY; [4] Differences in in vitro true neutral detergent digestibility (IVTNDFD, % of dry matter) between control and treatments; [5] One-unit of enhanced NDFdigestibility was positively associated with 0.25 kg of 4% fat corrected milk yield [27]; [6] Price of 1 kilogram of 4% fat corrected milk: 2.10 TRY*

et al.[19]. In addition, the IVTD and IVNDFD of corn stover in the present study was higher than that of Zahao et al.[19] These different results are possibly resulted from different physical structures and chemical composition of various corn stover fractions (leaf, husk and stalk) [19].

In the current study, after 30 d of storage EFEs and EFEs + FAEI treatments had lower ADF and NDF contents than control. ADF and ADF concentration of corn stover produced using EFEs primarily reflects the hydrolysis of cell wall carbohydrates, and is in accord with previous studies by Ni et al.[31] and Sun et al.[32] who reported reduced NDF concentration of wheat straw silages produced using a cellulase additive. In contrast, Lync et al.[18] and Coblentz and Hoffman [33] showed that alfalfa hay produced using enzyme products generally had greater NDF concentration than the untreated hay. On the other hand, Lynch et al.[7] showed that the use of fibrolytic enzyme product at ensiling did not affect chemical composition of alfalfa haylage. With respect to chemical composition of corn stover stored in current study, no statistically significant difference was observed between EFEs and EFEs + FAEI. These results are likely to be releated to associated with inadequate breaking of the cross linking bonds in the cell wall by FAEI. In accordance with the present results, Lynch et al.[7] have demonstrated that the use of FAEI with EFEs did not forward the effects of fibrolytic-enzyme treatments.

pH values reflected a good silage conservation [34] and changes in the end pruducts of fermentation [14]. In this study, after incubation for 30 days, even if the pH values of all corn stovers with additive were not below 4.69, they were well preserved. It is attributed from this critical pH value varies with DM content [35]. Liu et al.[36] have found that stylo silage was well preserved at high pH of 5.0 when the DM was 542.9 g kg^{-1}. The DM level of corn strover in this study was approximately 600 g kg^{-1} which resulted in the pH at the level of 4.69 and 5.01. The lower pH (4.69) of corn strover produced using EFEs in combination with FAEI compared with corn stover produced using EFEs alone and control reflects the tendency for increased lactic acid production during storage. This result may be explained by the fact that the *L. plantarum* and *L. casei* populations included in the FAEI inoculant are commonly used as silage additives to increase lactic acid production [37] and/or the breakdown of cell walls by EFEs during incubation. The results also shown that EFEs alone was increased lactic acid and acetic acid concentrations compared to control. It may be that addition of EFEs increased WSC available for LAB from NDF degradation, and that propagation of LAB could be promoted in the stage of storage, which resulted in an increase in lactic acid [38]. The same positive effects of EFEs and EFEs + FAEI have been reported in other research [7,39]. However, Lynch et al.[18] found no effect on the pH and lactic acid content of alfalfa hay treated with EFEs + FAEI at baling compared to fibrolytic enzymes. Another important finding of the present study was that acetic acid production increased significantly in the corn stover treated with EFEs + FAEI compared to the other groups, suggesting that the *L. buchneri* component of FAEI may have converted lactic acid to acetic acid and 1,2-propanediol [40,41]. Similar effects were reported by Kang et al.[12] and Schmidt et al.[42] who treated alfalfa silage with *L. buchneri*. However, this finding contradicts that of Lynch et al.[17] who reported that the acetic acid concentration was unaffected by FAEI in combination with fibrolytic enzymes. These variations could be attributed to the differences in chemical composition of the stored material, seasonal conditions and method of storage.

Yeasts can create conditions favourable for the development of moulds and fungi, as well as other undesirable micro-organisms, which results in losses of organic matter. In the present study, the number of yeasts and moulds was not determined in EFEs + FAEI treatment compared with EFEs treatment and the control. The strain of *L. buchneri* found in the inoculant used in the present study is known to produce acetic acid which has strong antifungal properties [43]. In the present study, an increase in acetic acid production

in the EFEs + FAEI treatment led to the inhibition of yeast and mould multiplication. These findings are in agreement with those of Filya [41] and Muck [44]. Stored corn stover is susceptible to aerobic deterioration, causing loss of dry matter and the development of toxic substances [45]. Therefore, the findings of the present study could be useful in reducing the rate of aerobic deterioration during the storage of corn stover.

In vitro true digestibility and IVTNDFD are an important parameter of evaluating the digestion of forage [46]. Oba and Allen [27] reported that a one-unit of enhanced NDF digestibility *in vitro* was associated with 0.17 of dry matter intake, 0.23 kg of milk yield, and 0.25 kg of 4% fat-corrected milk yield. Therefore, the digestibility of DM and NDF were used to be the first parameter in this study. Results of the present study showed that treatment with EFEs and EFEs + FAEI inreased the amounts of IVTMD and IVNDFD in corn stover compared with the control. These results are likely to be related to fragile and susceptible to ruminal degradation of structural polysaccharides of corn stover during storage with EFEs and EFEs + FAEI groups [7,14]. In accordance with the present result, previous studies have demonstrated that in increase IVTDMD [4,29,47] and IVTNDFD [18,48] with the use of EFEs. In a study investigating *in vitro* DM and NDF digestibility of corn strover, Zhao et al.[19] reported that combination of 10 U/g DM of cellulose with 60 U/g DM of xylanase were screened out based on higher digestibility of DM and NDF, 49.3 and 37.7%, respectively that was lower in present study. In other study, Salem at al.[49] reported that *in vitro* DM digestibility in wheat straw was improved by addition of cellulose and xylanase by 4% in sheep. Similarly in the present study, IVTDM was increased by 6.39% with addition of EFEs compared with control. This outcome is contrary to that of Lynch et al.[17] and Krueger et al.[15] who did not increase DM digestibility or NDFD *in vitro* experiment. This inconsistency may be due to differences of the most digestible fraction of the structural polysaccharides, characteristics of material, enzyme composition and application rate, the period of interaction between forage and enzyme [7,48].

The specific objective of this trail was to examine whether the FAEI could complement EFEs products by increasing access to structural carbohydrates. Contrary to expectations, EFEs + FAEI did not increase the effect of fibrolytic enzymes and thus there was no significant difference between EFEs and EFEs + FAEI. The use of FAEI alone on corn strover was not investigated in this study. However, a possible explanation for this might be that ferulic acid esterase activity initiated by FAEI during storage was insufficient to hydrolyse esterified bonds in the polysaccharides in the cell wall [50]. Similar results, the detailed study of Lynch et al.[18] found that the use of FAEI in combination with EFEs did not improve IVTD and IVNDF of hay after storage compared to EFEs alone. However, the findings of the current study do not support the some previous research [13,14].

The use of IVTNDFD concept has also provided additional information in economic evaluation of forages [27]. The present study showed that the inclusion of EFEs and EFEs + FAEI as a feed additive into the corn stover achieved daily net profit 2.63 and 3.10 TRY respectively over control. These findings agreed with those obtained by Aboul-Fotouh et al.[51] who found that diets supplemented with cellulolytic enzymes economically better than control diet for feeding lactating goats because of higher yields of milk and milk components.

This study on the digestibility of stover showed that the use of EFEs alone and in combination with FAEI improved IVTD and IVTNDFD compared with the control. Another important finding was that FAEI in combination with EFEs did not affect the amounts of IVTDMD and IVTNDFD compared with the application of EFEs alone; however, they improved the fermentation of corn stover by reducing pH and inhibited mould and yeast multiplication compared with EFEs alone and the control. From economical point of view, the best ration was EFEs + FAEI. However, further studies should evaluate these additives under *in vivo* conditions with respect to digestibility, ruminal fermentation and feed intake.

Acknowledgements

This study was supported by the Research Fund of Ondokuz Mayıs University (Project Number: PYO.VET.1901.17.007). The authors thank Gregory. T. Sullivan for helpful comments editing the English in an earlier version of this manuscript.

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Kafkas Univ Vet Fak Derg
25 (5): 627-632, 2019
DOI: 10.9775/kvfd.2018.21472

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

Research Article

Effects of Presence or Absence of a Dominant Follicle Estimated by a Single Ultrasound Examination at the Time of Follicular Aspiration on Superovulatory Responses and Embryo Production in Lactating Simmental Cows [1]

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Article Code: KVFD-2018-21472 Received: 05.12.2018 Accepted: 15.03.2019 Published Online: 15.03.2019

How to Cite This Article

Cirit Ü,A Özmen MF, Köse M, Küçükaslan İ, Çinar EM, Kutsal HG: Effects of presence or absence of a dominant follicle estimated by a single ultrasound examination at the time of follicular aspiration on superovulatory responses and embryo production in lactating Simmental cows. *Kafkas Univ Vet Fak Derg,* 25 (5): 627-632, 2019. DOI: 10.9775/kvfd.2018.21472

Abstract

The aim of the study was to evaluate effects of presence or absence of a dominant follicle (DF) estimated by a single ultrasound examination at the time of follicular aspiration (FA) on superstimulatory and superovulatory responses and embryo production in lactating Simmental cows. At random stages of the estrous cycle, the ovaries of cows (n=42) were examined by transrectal ultrasonography (US) and all follicles ≥3 mm were counted. Donors with <10 follicles 3-8 mm in diameter were considered to have a dominant follicle (group DF+; n=30), while donors ≥10 small follicles 3-8 mm were classified as having no dominant follicle (group DF–; n=12). Just after US examination, all cows were subjected to ultrasound-guided transvaginal aspiration of all follicles ≥5 mm and a progesterone-releasing device was placed in the vagina. Thirty-six h after FA, all cows were superstimulated with FSH, which was given as twice-daily injections over 6 days. Cows were pre-treated with a single dose of 400 IU of eCG 24 h before the start of FSH treatments. It was concluded from this study that the presence of a DF estimated by a single ultrasound examination at the time of FA effects negatively the superstimulatory and superovulatory responses, fertilization rate and embryo quality (P<0.05) but not the number of embryos collected. It was also concluded that estimation of a dominant follicle by a single ultrasound examination at the time of follicular aspiration based on the number of small follicles may be used the selection of potential donor cows and can significantly contribute to improvements in superstimulatory and superovulatory responses and embryo quality.

Keywords: *Superovulation, Cattle, Follicle aspiration, eCG, FSH*

Laktasyodaki Simental İneklerde Follikül Aspirasyonu Zamanında Tek Bir Ultrason Muayenesi İle Tahmin Edilen Dominant Follikül Varlığı veya Yokluğunun Süperovulatör Cevaplar ve Embriyo Üretimi Üzerindeki Etkileri

Öz

Bu çalışmanın amacı, laktasyondaki Simental ineklerde follikül aspirasyonu (FA) zamanında tek bir ultrason muayenesi ile saptanan dominant follikül (DF) varlığı veya yokluğunun süperstimülatör ve süperovulatör cevaplar ve embriyo üretimi üzerindeki etkilerinin değerlendirilmesiydi. Östrus siklusunun tesadüfi aşamalarında, ineklerin (n=42) ovaryumları ultrason (US) ile muayene edilerek ≥3 mm tüm folliküller sayıldı. Üç-8 mm çapında <10 küçük follikülü olan inekler DF'ü var olarak (DF+ grubu; n=30), ≥10 küçük follikülü olan inekler ise DF'ü yok olarak (DF– grubu; n=12) sınıflandırıldı. Ultrason muayenesinden hemen sonra tüm ineklerin ≥5 mm tüm folliküller aspire edildi ve vajina içine bir progesteron salıverici araç yerleştirildi. Tüm ineklere, follikül aspirasyonundan 36 saat sonrasından başlanarak 6 gün boyunca günde 2 kez FSH uygulandı. İneklere FSH uygulamasının başlamasından 24 saat önce 400 IU tek doz eCG ile ön tedavi uygulandı. Çalışmadan, FA günü tek bir ultrason muayenesi ile tahmin edilen DF varlığının süperstimülatör ve superovulatör yanıtları, fertilizasyon oranını ve embriyo kalitesini olumsuz etkilediği (P<0.05), fakat toplanan embriyo sayısını etkilemediği sonucuna varıldı. Çalışmadan ayrıca, FA günü yapılan tek bir ultrason muayenesinde küçük folliküllerin sayısına göre DF varlığının tahmin edilmesi işleminin potansiyel vericilerin seçiminde kullanılabileceği ve süperstimülatör ve superovulatör cevaplar ve embriyo kalitesinin arttırılmasında önemli katkıları olabileceği sonucu çıkarıldı.

Anahtar sözcükler: *Süperovulasyon, Sığır, Follikül aspirasyonu, eCG, FSH*

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INTRODUCTION

Major factors that influence the superstimulatory response in cattle are the status of the follicular wave (FW) at the start of the superovulatory program and the number of follicles ≥3 mm at the beginning of FSH treatments [1]. The ideal time to start FSH treatment in a program is at the emergence of the FW [2]. There are many methods to regulate the time of FW emergence, such as ablation of the dominant follicle (DF), treatment with a combination of estradiol and progesterone (P4) and GnRH treatment [2]. It has been stated that the most reliable method to synchronize wave emergence for superstimulation is follicle aspiration (FA) [2] and that is applied generally at 24, 36 or 48 h before the onset of superstimulation [3]. Aspiration of the DF results in a surge of FSH 1 day after aspiration, with a peak occurring 2 days later, causing the emergence of a new FW [1]. Although it is difficult to utilize in the field, the use of FA technology for *in vivo* embryo production is becoming increasingly common. However, it has been suggested that the synchronization of follicular wave emergence may not be the only requirement for successful superovulation. It was reported that superovulatory response was most dependent on the numbers of follicles entering the wave and a simple ultrasound examination at the start of superstimulatory treatments was highly predictive of the subsequent superovulatory response [4,5]. However, to our knowledge, there is no study investigating the effects of ovarian status at the time of FA on superstimulatory response and in vivo embryo production in cattle.

The goal of superstimulatory treatment is to induce the growth of multiple follicles to produce multiple competent oocytes capable of developing into transferable embryos [5,6]. However, the most significant limiting factor in the success of superovulation has been and continues to be the unpredictability, due to high between-individual variability, in the ovarian response to gonadotropin stimulation [7]. It has been stated that one-third of the donors treated do not answer to superovulation, another third yields an average of one to three embryos and only one-third actually superovulates giving a large number of embryos [8]. Therefore, selection of donors is very important for the success of superovulation. The presence of a DF before superstimulatory treatment decreases superovulatory response [4,9]. However, in order to predict superovulatory response by means of this relation, it is necessary to first monitor the ovaries of donor cattle for at least 4 consecutive days before the initiation of superstimulatory treatment, and confirm whether a follicle is functionally dominant or not. This is impractical under field conditions [9]. However, Bungartz and Niemann [4] clearly demonstrated that the presence or absence of a DF can be determined by a single ultrasound examination using the number of small follicles (3-8 mm in diameter). The researchers also reported that a cow with a DF and more than 10 small follicles have never been observed [4]. Therefore, it has been suggested that potentially good and poor responders to superovulation and the exclusion of poor responders from superovulatory treatment can be diagnosed by a single ultrasound examination at the start of superstimulatory treatments [4].

Knowing the effects of ovarian status at the time of FA on in vivo embryo production may allow eliminating of poor responders from superovulation program and contribute to increase superovulation success. Therefore the aim of the study was to evaluate effects of presence of a DF detected by a single ultrasound examination at the time of FA performed at 36 h before the start of FSH treatments on superstimulatory and superovulatory responses and embryo production in lactating Simmental cows.

MATERIAL and METHODS

All procedures were approved by the Animal Experiments Local Ethics Committee of Dicle University (Approval No: 92406). The study was completed in four replicates. This study was conducted at Ceylanpinar Directorate of Agricultural Enterprise under General Directorate of Agricultural Enterprises (TIGEM) (Şanlıurfa, Türkiye) from March to June 2018. Forty-two lactating Simmental cows housed in a free-stall facility, with a body condition score (BCS) of 3 to 4.5 (1-5 scale), 500 to 650 kg of body weight, milk production of 28.2±0.79 kg/day (mean±SEM) and >60 days in milk were used. The donor cows were maintained under the same nutritional, management and environmental conditions. All the cows underwent a gynecological examination before the commencement of the study.

Follicle Aspiration (FA) and Superovulation

At random stages of the estrous cycle, the ovaries of each cow were examined by transrectal ultrasonography (US, 8-MHz linear-array transducer; Esaote Pie Medical Aqulia, Türkiye) and all follicles ≥3 mm were counted. The criterion for the presence or absence of a dominant follicle (DF) on the day of FA was the number of small follicles 3-8 mm in diameter. Donors with <10 follicles 3-8 mm in diameter (on both ovaries) were considered to have a DF (group DF+; n= 30), while donors ≥10 small follicles 3-8 mm were classified as having no DF (group DF–; n= 12) [4]. Just after the US, cows were subjected to FA to synchronize the emergence of a new follicular wave [10,11]. Prior to FA, each cow received epidural anesthesia (5 to 7 mL of 2% lidocaine; Vilcain, Vilsan, Türkiye) to decrease peristalsis and discomfort [12]. All ovarian follicles ≥5 mm were aspirated *(Fig. 1)* with a 18-gauge disposable needle by using the ultrasound-guided transvaginal approach with a 7.5-MHz convex-array transducer (Nutricell/Esaote-Pie Medical, Campinas, Brazil) by an experienced researcher (ÜC). A progesterone-releasing device (1.38 g progesterone, CIDR; Zoetis Animal

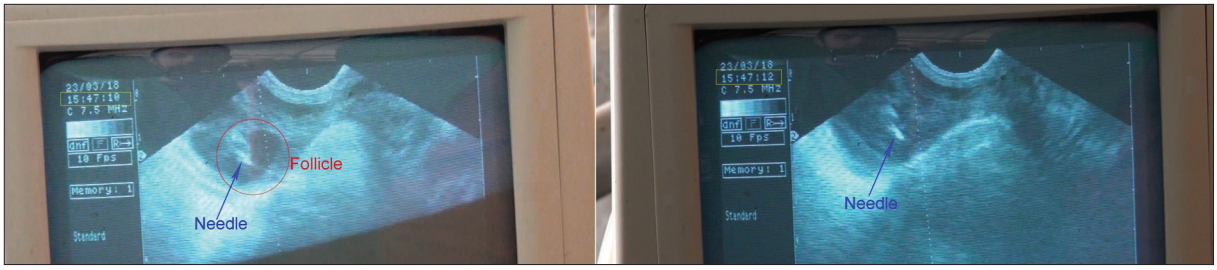

Fig 1. Ultrasound images of an ovary just before *(left image)* and after follicle aspiration *(right image)*

Table 1. *Schematic illustration of the superovulation protocol*

D-1.5	D-1	D0	D1	D4	D5	D6	D7	D8	D14
← CIDR →									
		← FSH →							
US	eCG			PGF (am)		hCG (pm)	TAI+PGF (am)	TAI (am)	US
FA				PGF (pm)		US (pm)	TAI (pm)		Flushing

D: *days;* ***FA:*** *follicle aspiration;* ***TAI:*** *timed artificial insemination;* ***PGF:*** *prostaglandin F2α;* ***US:*** *ultrasound*

Health, Türkiye) was placed in the vagina immediately after FA *(Table 1)*. Approximately 36 h (24 to 48 h) after FA, all cows were superstimulated with a total of 500 µg porcine FSH (pFSH, Stimufol; Reprobiol SPRL, Belgique), which was given as twice-daily injections over 6 days on a decreasing dose schedule (75, 65, 50, 50, 40, 40, 35, 35, 30, 30, 25 and 25 µg) [13]. Cows were pre-treated with a single dose of 400 IU of equine chorionic gonadotropin (eCG, Folligon, im; MSD Animal Health, Türkiye) 24 h before the start of FSH treatments. Cows received prostaglandin F2α (PGF, 25 mg, Dinolytic, im; Zoetis Animal Health, Türkiye) concomitant with the ninth and tenth FSH treatments. The CIDR was removed at the time of the last FSH treatment (36 h after the first PGF) and 24 h after CIDR removal, ovulations were induced with 1500 IU of human chorionic gonadotropin (hCG, Chorulon, im; MSD Animal Health, Türkiye). Timed artificial inseminations (TAI) were performed thrice at 12, 24 and 36 h after hCG treatment using previously tested frozen semen from two different bulls [13]. All cows were treated with PGF concurrent with the first TAI. The ovaries of each cow were examined by transrectal ultrasonography concomitant with hCG treatments to determine the number and size of follicles and positive response to superstimulatory treatment was considered when three or more ovulatory follicles ≥9 mm were found.

Ova/embryo Collection

Prior to flushing, each cow received epidural anesthesia (5 to 7 mL of 2% lidocaine; Vilcain, Vilsan, Türkiye). Ova/embryos were collected non-surgically 6.5 to 7 days after the first TAI using lactated Ringer's solution containing 1% calf serum (CS, N4762; Sigma-Aldrich, USA) and 125 mg/L of kanamycin (Kanovet, Vetaş, Türkiye) [14,15]. Each uterine horn was flushed with 800 to 1000 mL of the solution. A two-way Foley catheter and the interrupted-syringe technique were used for flushing. The aspirates were poured into embryo collection filters (EZ, 017726, IMV, Şark Kemikal, Türkiye). Recovered ova and/or embryos were evaluated for developmental stage and quality at x 50 magnification (Leica S8 APO) using the criteria of the International Embryo Technology Society (IETS) [16]. Embryos were defined as transferable (Grades 1, 2, and 3) and freezable (Grades 1 and 2) [17]. Ovaries were examined with transrectal ultrasonography (8-MHz linear-array transducer; Esaote Pie Medical Aqulia, Türkiye) to determine the presence and number of CL after embryo collection. Animals with 3 or more CL at the time of embryo collection were considered to have responded to the superovulatory treatment [18].

Statistical Analyses

Data were analyzed using a general linear model (GLM) procedure of SPSS. The initial statistical model included study groups as fixed effect and the time of FA (24 to 48 h) BCS, days in milk and milk yield as covariates. The time of FA, BCS, days in milk and milk yield were found insignificant effect as covariates. The non-significant covariates were not included in the final statistical model. The numbers of follicles, total ova/embryos, degenerate, transferable and freezable embryos, fertilized and unfertilized ova and CL were compared using independent samples t-test. Proportional data were compared using Chi-square test [19]. Results were expressed as mean±SEM and differences were accepted as statistically significant when $P<0.05$. Statistical analyses were performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). In group DF+, abnormal vaginal discharge was determined in 4 cows during the period between the first TAI and embryo collection and these cows were excluded from the statistical analyses where superovulatory responses and embryo production were compared.

Table 2. *Mean (±SEM) number of follicles ≥ 9 mm and < 9 mm on the day of hCG treatment in DF+ and DF– groups*

Examined traits	Group DF+ (n= 30)	Group DF– (n= 12)	P value
Number of follicles ≥9 mm	15.1±1.30	23.1±3.20	<0.01
Number of follicles <9 mm	3.0±0.51	3.0±0.39	>0.05
Total number of follicles	18.1±1.50	26.1±3.00	<0.05
Percentage of superstimulatory response (%)	100	100	>0.05

Table 3. *Superovulatory responses and embryo production (mean±SEM) in DF+ and DF– groups*

Examined Traits	Group DF+ (n= 26)	Group DF– (n= 12)	P value
Number of CL on the day of embryo collection	19.4±1.60	26.8±3.31	<0.05
Percentage of superovulatory response (%)	100	100	>0.05
Recovery rate (%)*	57.7	49.4	<0.05
Total ova/embryos recovered	11.2±1.70	13.3±1.47	>0.05
Unfertilized ova	2.1±1.01	0.7±0.36	>0.05
Fertilized ova	9.0±1.27	12.6±1.29	=0.95
Percentage of fertilized ova (%)	81.0	95.0	<0.0001
Degenerate embryos	2.2±0.69	3.2±0.77	>0.05
Transferable embryos (Grades 1-3)	6.7±0.94	9.3±1.18	>0.05
Percentage of transferable embryos (%)	60.3	70.4	<0.05
Freezable embryos (Grades 1 and 2)	5.7±0.91	7.7±1.10	>0.05
Percentage of freezable embryos (%)	51.4	57.9	>0.05

** Total ova and embryos recovered/number of CL detected*

RESULTS

The mean (±SEM) number of ≥9 mm follicles on the day of hCG treatment ranged from 4 to 27 in group DF+ and from 12 to 50 in group DF–. The mean number of ≥9 mm follicles (P<0.01) and total numbers of follicles (P<0.05) on the day of hCG treatment were significantly higher in DF– group than in DF+ group *(Table 2)*.

The mean number of CL at the time of embryo collection were significantly higher in DF– group (26.8±3.31, ranged from 9 to 50) than in DF+ group (19.4±1.60, ranged from 6 to 39) (P<0.05, *Table 3)*.

However, the cows in DF– group had a decreased percentage of ova/embryo recovery than cows in DF+ group (P<0.05). The mean numbers of cows with ≥3 CL (superovulatory response), ova/embryos recovered, unfertilized ova, degenerate embryos, transferable embryos and freezable embryos did not differ between groups (P>0.05, *Table 3)*. However, the cows in DF– group had more increased percentages of fertilized ova (P<0.0001) and transferable embryo (P<0.05) than cows in DF+ group.

DICUSSION

One of the most important advantages of using embryo transfer is acceleration of the dissemination of desirable genetics by increasing the number of offspring obtained from donors with high genetic value [20-23]. It has been well known that the presence of a DF at the start of superstimulatory treatments represses the growing of subordinate follicles and affects the superovulatory response and embryo quality negatively [4]. Ultrasound-guided FA to eliminate the DF is usually performed at random stages of estrus, and the number of collected viable embryos increases when superstimulatory treatments are initiated 24-48 h after FA [3]. However, the effects of estrus cycle and/or follicular status during FA on superovulatory responses and embryo production are unknown. To the best of our knowledge, this is the first study investigating the effects of ovarian status at the time of FA on superstimulatory response and embryo production in cows. Since FA eliminates the DF, it may be expected that the superstimulatory and superovulatory response of cows with or without a DF will be similar. However, the cows estimated to have a DF at the time of FA had lower superstimulatory (mean number of ≥9 mm follicles on the day of hCG treatment; P<0.01) and superovulatory (number of CL at the time of embryo collection; P<0.05) responses than the cows without a DF. A possible explanation of these results may be related to the DF evaluation method. Animals with more than ten small follicles (3-8 mm) were evaluated as being without a DF [4]. This evaluation method allows us to have information about

both the presence of a DF and the number of small follicles in the ovaries. It has been reported that the main factor that affects the superovulatory response is the number of small follicles at the beginning of ovarian superstimulation with gonadotropins [5,24,25]. It was shown that cows with high antral follicle counts (≥3 mm) had significantly more ovulations and produced a greater number of transferable embryos than those with low antral follicle counts [5,24]. Another possible explanation of these results could be that although DF were eliminated with FA, the metabolic products that DF had previously given to the blood might have been continued to exert a repressive effect for a while longer on follicle development.

The fact that the cows in DF– group had an increased number of ≥9 mm follicle on the day of hCG treatment and CL at the time of embryo collection suggested that some of these 3-8 mm follicles present at the time of FA continued growing in response to FSH treatments and ovulated. Similarly, it has been suggested that some of these follicles that are 3-6 mm in diameter before superstimulatory treatment continue growing in response to exogenous gonadotropin, and eventually ovulate [9]. Therefore, it may be postulated that the larger the number of small follicles at the time of FA carrying out 36 h before the start of FSH treatments, the larger the number of follicles that will respond to exogenous gonadotropin and the resulting superovulatory response will be better.

Although the mean numbers of ≥9 mm follicles on the day of hCG treatment and CL at the time of embryo collection were significantly higher in DF– group than in DF+ group ($P<0.05$), the mean numbers of ova/embryos recovered, unfertilized ova, degenerate embryos, transferable embryos and freezable embryos did not differ between groups ($P>0.05$). The possible explanation of this can be that the cows in DF– group had a decreased percentage of ova/embryo recovery than cows in DF+ group ($P<0.05$). Similarly, it has been reported a negative correlation between the number of superovulated follicles and recovery rate [26]. One of the most remarkable results of our study was that the cows in DF– group had more increased percentages of fertilized ova ($P<0.0001$) and transferable embryo ($P<0.05$) than cows in DF+ group, while the mean numbers of ova/embryos recovered and transferable embryos were similar in both groups. These results suggest that the presence of a DF at the time of FA effects negatively the superstimulatory and superovulatory responses, fertilization rate and embryo quality but not the number of embryos collected.

Based on the American Embryo Transfer Association (AETA) survey report, the average number of viable embryos recovered from dairy donors was 5.7 (58% of the total ova/embryos recovered) and from beef donors was 6.9 (54%) in 2017 [27]. Previous studies, in which conventional 4 or 5-day programs were applied, reported that mean number of ova/embryos ranged from 10.2 to 20.5, number of transferable embryos from 4.0 to 12.8 and percent transferable from 39.2% to 77% in Simmental cows [28-34]. In a study analyzing the records of 1596 embryo collections from cows from 13 breeds, the mean number of total and transferable embryos and the rate of transferable embryos were reported as 13.1, 6.6 and 51%, respectively in Simmental cows [28]. However, except for one [29] of these studies, the lactation status (lactating or dry) of the Simmental cows is unclear. The mean numbers of transferable embryos in the present study (6.7 and 9.3 in DF+ and DF– groups, respectively) were comparable to those reported in the literature for Simmental cows [13,28-34]. Besides, the percentage of transferable embryos in DF– group (70.4%) were higher than those of most of the studies [29-33] and than general average reported for Simmental and beef cows [27,28].

One of the most striking results of our study was that fertilization rates were found to be high, especially in DF– group (81.0% and 95.0% in DF+ and DF– groups, respectively). Based on the AETA survey report, the average number of unfertilized ova recovered was 3.1 (29% of total ova/embryos) from dairy donors and 3.5 (27%) from beef donors [35]. Roussel et al.[36] examining nonsurgical embryo recoveries from 1116 beef and dairy cows from 15 different breeds over a 7-yr period reported that combining all embryo collections resulted in 31% unfertilized ova, 11% degenerate embryos and 58% transferable embryos. Similarly, previous studies reported that mean fertilization rates ranged from 65.6% to 79.4% in Simmental cows [29,30,34]. In the present study, in which a superovulation program lengthened to 7 days with eCG pre-treatment was used, cows were timely inseminated three times 12, 24 and 36 h after hCG injection and treated with PGF concurrently with the first TAI. It has been showed that treatment with PGF during the late growing phase of the DF of a wave can result in ovulation by a luteolysis-independent mechanism in prepubertal heifers [37]. Similarly, Ambrose et al.[38] reported that PGF treatment concurrent with AI significantly increased conception rate in dairy cows. The result of the study indicated that FA, lengthened superstimulation program, timings of P4 withdrawal and hCG treatment (P36LH60 strategy), three times AI and/or PGF treatment concurrent with the first TAI might have contributed to high fertilization rates either alone or combined. Besides these factors, it seems that the presence of a DF estimated by a single ultrasound examination at the time of FA also affects fertilization rates and, decreases fertilization rates significantly ($P<0.0001$).

It was concluded from this study that the presence of a DF estimated by a single ultrasound examination at the time of FA effects negatively the superstimulatory and superovulatory responses, fertilization rate and embryo quality but not the number of embryos collected. It was also concluded that estimation of a DF by a single ultrasound examination at the time of FA based on the

number of small follicles may be used the selection of potential donor cows and can significantly contribute to improvements in superstimulatory and superovulatory responses and embryo quality in Simmental cows. Further studies including different breeds are needed to determine the effect of ovarian status at the time of FA.

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Kafkas Univ Vet Fak Derg
25 (5): 633-638, 2019
DOI: 10.9775/kvfd.2018.21505

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

Research Article

The Effect of Intrauterine Infusion of Carvacrol After Insemination on Conception Rate in Repeat Breeder Cows Subjected to Progesteron Based Estrus Synchronization Protocol

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Article ID: KVFD-2018-21505 Received: 07.12.2018 Accepted: 06.05.2019 Published Online: 08.05.2019

How to Cite This Article

Lehimcioğlu NC, Öztürkler Y, Yıldız S, Arı UÇ: The effect of intrauterine infusion of carvacrol after insemination on conception rate in repeat breeder cows subjected to progesteron based estrus synchronization protocol. *Kafkas Univ Vet Fak Derg,* 25 (5): 633-638, 2019. DOI: 10.9775/kvfd.2018.21505

Abstract

Repeat breeder (RB) is one of the crucial causes of economic loss in dairy cows. The aim of this study was to determine the effects of intrauterine carvacrol administration after timed artificial insemination (TAI) on the conception rate (CR) in RB cows. The study involved 155 RB cows returned to service for more than three times and without any significant pathologic defects in genital tract. All cows were subjected to following estrus synchronization method: An intra-vaginal apparatus (IVA) containing 1.55 g progesteron were inserted for 9 days and administered one dose of $PGF_{2\alpha}$ one day before removing IVA, then injected one dose of GnRH 6 h before TAI. All the cows were inseminated in 56th h after removing IVA. All RB cows were randomly divided to three groups. Group I (GI; n=52) and Group II (GII; n=51), treatments groups were infused with 0.1% diluted Carvacrol and 0.1% Lugol's (GII) solution. Group III (GIII; n=51) the control group, was received normal saline only. All infusions were administered one dose of 30-50 mL (according to uterine size) into uterus half hour after insemination. In the observations performed in 21 to 30 days after insemination, non-return rates (NRR) were 71.15%, 69.23% and 68.62% in groups of GI, GII and GIII, respectively. CR detected by rectal palpation at 60 days following artificial insemination were 67.30%, 63.46% and 46.15% in GI, GII and GIII, respectively. However, the differences between groups were not statistically significant. As a result: In the treatment of RB cows, it may be suggested that carvacrol infusion to the uterus may be preferred as it has improved pregnancy rates to some extent. Nevertheless, further investigations are needed to confirm these results.

Keywords: *Cow, Carvacrol, Artificial insemination, Repeat Breeder, Estrus synchronisation, Progestron, PRID, $PGF_{2\alpha}$, GnRH*

Progesteron Temelli Östrus Senkronizasyonu Protokolüne Tabi Tutulan Repeat Breeder İneklerde Suni Tohumlama Sonrası İntrauterin Carvacrol İnfüzyonunun Gebelik Oranları Üzerine Etkisi

Öz

Repeat Breeder (RB), süt ineklerinde ekonomik kaybın önemli nedenlerinden biridir. Bu çalışmanın amacı, RB ineklerinde zamanlanmış suni tohumlama sonrası intrauterin carvacrol uygulamasının gebelik oranlarına (GO) etkilerini belirlemektir. Çalışma, 3 defadan fazla geri dönen, genital sisteminde önemli patolojik bozukluk saptanmayan 155 adet RB inekte yürütüldü. Tüm inekler aşağıdaki östrus senkronizasyonu yöntemine tabi tutuldu: 1.55 g progesteron içeren bir intra-vajinal aparat (IVA), 9 gün boyunca yerleştirildi ve IVA çıkarılmasından bir gün önce bir doz $PGF_{2\alpha}$ uygulandı ve suni tohumlamadan 6 saat önce bir doz GnRH enjekte edildi. Tüm inekler, IVA çıkarıldıktan 56 saat sonra tohumlandı. Tüm hayvanlar rastgele üç gruba ayrıldı. Uygulama grupları olan grup I (GI; n=52) ve grup II (GII; n=51)'ye sırasıyla %0.1 seyreltilmiş Carvacrol ve %0.1 Lugol (GII) çözeltileri verildi. Kontrol grubu grup III'e (GIII; n=51) sadece normal serum fizyolojik infüze edildi. Tüm infüzyonlar, tohumlamadan yarım saat sonra uterus içine bir doz 30-50 mL (uterus boyutuna göre) olacak şekilde uygulandı. Tohumlamadan 21 ila 30 gün sonra yapılan gözlemlerde, geri dönmeyenlerin oranı (NRR), GI, GII ve GIII gruplarında sırasıyla %71.15, %69.23 ve %68.62 olarak belirlendi. Suni tohumlamayı takiben 60. günde yapılan rektal palpasyonla teşhis edilen GO ise GI, GII ve GIII'de sırasıyla %67.30, %63.46 ve %46.15 olarak saptandı. Ancak, gruplar arasındaki farklar istatistiksel olarak anlamlı değildi. Sonuç olarak: RB ineklerin tedavisinde, uterusa carvacrol infüzyonunun gebelik oranlarını bir dereceye kadar arttırdığı için tercih edilebileceği kanaatine varıldı. Fakat, bu sonuçları pekiştirmek için başka araştırmalara ihtiyaç olduğu düşünülmektedir.

Anahtar sözcükler: *İnek, Carvacrol, Suni tohumlama, Repeat breeder, Östrus senkronizasyonu, Progesteron, PRID, $PGF_{2\alpha}$, GnRH*

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INTRODUCTION

Reproductive performance plays a crticial role in productivity of the dairy cattle industry. An ideal reproductive health can be described as "interval of calving-repregnancy in a cow herd is kept in an optimum range of economic scale". The main goal in herd management in dairy cows is considered to be as "getting a calf per year [1]. Repeat breeder (RB) is one of the main problems in cows that leads to reproductive waste and significant decline in profitability in dairy industry due to decreasing of reproductive performance [2]. According to results of one study on RB syndrome which were carried out in Michigan, RB was observed in 24% of 3.309 lactations in twenty-two dairy herds, the cost of delayed conception, extra inseminations, extra veterinary service, and losses due to wastage of livestock were calculated as $385 [3]. In Indian, the incidence of clinical endometritis and sub-clinical endometritis were estimated as 54.15% and 1.40%, respectively in RB cows [4]. Besides, it has been also reported that RB is seen in 5-32% frequency in cattle [5] and alone contributed to reproductive problems in the rate of 2.29 to 42.7% [6-8].

According to the most common description, the cows that are cyclic and nearly showing normal heat, have no clinically detectable pathological lesion of their genital tract, failure of conceiving and returning back to estrus after three mating with a fertil bull has been definited as "RB" [9,10]. RB is caused by an abnormal uterine media established by harmful bacteria, histological lesions and pathological defects such as endometritis, subclinical endometritis, nutritional deficiency, thereby embriyo survival is terminated. RB syndrome depend on many multifactorial situations and causative factors such as uterine inflamation, improper oestrus detection and also endocrine imbalance and uterine infection [11,12]. Although many creative factors are existed under the RB, among the various etiological factors, subclinical endometritis is regarded as the most effective etiological factor which decreases reproductive performance and increases the insidence of RB (52.7%) [13]. Subclinical endometritis is defined as endometrium inflammation without systemic findings and is correlated with delayed uterine involution which depends on various factors such as age, race, nutrition [11].

Many methods have been implemented in the treatment of RB syndrome in cows. For therapeutic process of RB, the effects of GnRH injections [14] intrauterine infusions of various antiseptic and antibiotic solutions [15] had been investigated. However, cattle with metritis are commonly treated by using various antibiotics (gentamicin, oxytetracycline, ampicillin, cloxacillin, penicillin, ceptiofur), antiseptics (2% Lugol's solution, povidine iodine) and hormones [16-18]. Whereas, undesirable resistance effects of antibiotics in animal and humans are well known. Widely use of antibiotic resistant strains of microorganisms requires the discovery of alternative therapeutic compounds. For this purpose, recently, instead of antibiotics, some alternative therapy options are sought and new methods are tried to develop in order to treat the RB [19,20]. At his point, nowadays, the use of medicinal herbs obtained from natural sources has become an option and alternative for therapeutic purposes [21].

From past to present, uterine effective herbal drugs has been used for treatment of endometritis in animals. Thus, it has been reported that polyherbal intrauterine infusion has yielded succesful conception results (70%) compared with control group (40%) in treating in repeat breeder cattle, and it showed that polyherbal drugs have worked safety and also not seen any any irritation effect to mucous membrane of uterus [20]. From this point on, some *in vitro* studies have been performed on carvacrol which has wide spectrum antimicrobial power and is safe in use of animal and human conditions [22]. Carvacrol has also a strong inhibition effect on growing of bacterial isolates except *Pseudomonas aeroginosa* [23]. Moreover, it has been demonstrated that the carvacrol has a inhibitive effect against antibiotic resistant bacteria in vitro and in food, and use of herbal preparations with organic origin in the treatment of cows with toxic puerperal metritis have provided significant benefits in terms of food safety [21,24]. Additionally, it has been demonstrated that the residual of carvacrol or its derivatives in urine was found in very small amounts after one day only in rats [25]. On the other hand, it has been declared that herbal derived oils such as carvacrol and thymol have also been accepted as generally recognized as safe (GRAS) natural antimicrobial and non-antibiotic effective that are traditionally employed to conserve food and enrich flavor [26,27]. Further, it is known that the oils obtained from thyme contain thymol, carvacrol, p-cymene and α-pinene. Many investigators have declared that carvacrol has antioxidant, antibacterial, antiviral, anti-obesity, hepatoprotective, antifungal, antiseptic, anticarcinogenic, anti-inflammatory, spasmolytic, vasoconstrictive, immuno-modulatory effect and biological and pharmacological effects [21,28-30]. In this regard, it has been suggested that Optimum UterFlush, containing carvacrol (Van Beek® Natural Science, Orange City, IA) is an organic certified product used in the treatment of toxic puerperal metritis cows, and it can be used is in the treatment by transvaginally and IU infusion [30]. Although this product has been applied to cure the metritis, but up to now, not tested just after insemination in RB cows in order to treat the probable subclinical endometritis. However, some researchers have showed that application of various drugs as IU after artificial or natural insemination to the RB cows in the field conditions increases the pregnancy rates [18,31-34]. In the past, a similar method had applied by Riedel and Astrom was realised by using dilute iodine tincture and Lugol's solution for the first time in 1935. Since then, this method has been considered as an option for treatment after insemination in the cows carrying light subclinic chronic endometritis and this application has

been called as "Astrom Theraphy after that time" [32]. This intrauterine treatment has been applied either shortly after insemination or 15th minute as well as after 12th or 24th h [18,24,31,33]. However, in the literature search, we did not find any study which has investigated the effect of intrauterine infusion of carvacrol (uteroflash) after artificial insemination combined with oestrus synchronisation protocol. Therefore, under the light of above knowledges, in the present study, it was aimed to investigate the effects of intrauterin carvacrol infusion following artificial insemination in cows exposed to estrus synchronization protocol including PRID plus $PGF_{2\alpha}$ and GnRH.

MATERIAL and METHODS

This study was received approval by the Animal Experiments Local Ethics Committee of Kafkas University (KAU HADYEK; 2017-102).

Upon farmers' demand, a total of 300 cows were examined in accordance with the anamnesis and reviewed records of the owners in 6 intensive barns in three villages, near to Kars Province (40° 25′ 0″ Nort and 43° 4′ 59″ East), Turkey. Among them, repeat Breeder cows were selected as described by Levine [10] and Taşal [35].

In the selection of RB cows the following criteria were applied:

a- Not to be pregnant at least three times breeding

b- Being cyclic

c- Clinically not show any detectable disease

d- No detect any pathological findings in rectal palpation

e- No pathological discharge from the vagina

One hundred fifty five of the cows at various ages (3-7), races (Simental, Swiss Brown and crossbred) and 90-120 days range of postpartum period were considered as "RB cows" based on the above criteria, then included in the study. The remaining cows were disqualified because they were pregnant or have given birth. For feeding of animals, appropriate ration (coarse/concentrate) was given as twice daily with ad-libitum water.

All the cows were subjected to following synchronization method: An intra-vaginal apparatus (IVA; PRID DELTA® containing 1.55 g progesteron, CEVA-DIF) was inserted for 9 days and administered one dose of $PGF_{2\alpha}$ (5 mL/IM Enzaprost®-T, CEVA-DIF) one day before removing IVA, injected one dose (2 mL of GnRH (Ovarelin®, CEVA-DIF, containing 0.1 mg Gonadorelin diasetat/mL 6 h before TAI and then inseminated in 56th h after removing IVA. All animals were randomly divided to three groups. Cows in Group I (GI; n=52) and Group II (GII; n=51) were administered intrauterine with 0.1% diluted Carvacrol (GI) (Optimum UterFlush, Van Beek® Natural Science, Orange City, IA; containing 1 oz fl 13.9 g carvacrol, cinnamaldehydes, tymol, 440 mg yucca extract, stock containing 2.13 mL+ 997.7 mL distilled water) and 0.1% (w/v) Lugol's (GII) solution (1g iodine (I), 2 g Potassium iodide (KI) 1000 mL distilled water), respectively. Cows in Group III (GIII; n=51), control were received intrauterine normal saline only. All infusions were performed as one dose of 30-50 mL (according to uterine size) into uterus half hour after insemination.

Days 21 to 30 following inseminations, the percentage of cows returning to service were determined according to the estrus observations, but, pregnancy rates were confirmed by rectal palpation on the 60th day following insemination. All data were statistically analysed with SPSS (20.0) chi-square test program.

RESULTS

The rates of non-returned cows (NRR) at 21-30 days after the insemination were 71.1% (37/52), 69.23% (36/52) and 68.62% (35/51), in groups GI, GII and GIII, respectively *(Table 1)*. There was no statistically significant difference among groups in view of non-return rates (P=0.958).

Table 1. *The proportion of non-returned cows 21 to 30 days after insemination (NRR%)*

Groups	n	Number of Non-Returned Cows	NRR (%)
Carvacrol (GI)	52	37	71.15
Lugol's (GII)	52	36	69.23
Control (GIII)	51	35	68.62
There was no significant difference among groups (P=0.958)			

Table 2. *Pregnancy rates at the day 60th after insemination*

Groups	n	Number of Pregnant Cows	Pregnancy Rate (%)
Carvacrol (GI)	52	35	67.30
Lugol's (GII)	52	33	63.46
Control (GIII)	51	24	46.15
There was no statistically significant difference among groups (P=0.085)			

Pregnancy rates determined by rectal palpation on day 60 following AI were 67.30% (35/52) 63.46% (33/52) and 46.15% (24/51) in GI, GII and GIII groups, respecrtively *(Table 2)*. There was no statistically significant difference among groups (P=0.085).

DISCUSSION

Although Intrauterine infusions with a variety of antiseptic and antibiotic solutions have provided successful results for therapy in repeat breeder cows for a long time [15] we encountered a limited number of articles directly related to intrauterine infusion of carvacrol or uteroflash after insemination with estrus synchronisation in the literature review documented here.

In the present study, despite no statistical difference was found between percentages of NNR (71.15%, 69.23% and 68.62%) in GI, GII and GIII groups (P=0.490), it was noticed that carvacrol and Lugol's solution groups were numerically higher than the control group.

Pinedeo et al.[30] were treated with Uterflush (Stock Uterflush 3.75 mL/117 distilled water) and povidin iodine (200 mL povidin iodine/2lt distilled water) in natural estrus in the treatment of toxic puerperal metritic cows. They achieved the preganacy rates in 61.7% and 56.6%, respectively. In our study, the pregnancy rates obtained from carvacrol and Lugol's treatment groups (67.30%, 63.46%) were found higher than those of the study mentioned. In the present study, the results of Lugol's treament were also near to those of the values of Öztürkler et al.[31] (71.43%), Çolak and Öztürkler [33] (70%) and Vandeplasche [36] (80%). However, Öztürkler et al.[31] (60%)'s Lugol's results were found to be lower than the control group (69.23%) in those of our study. Additionally, Ahmed et al.[37] provided passable recovery rates (63.64, 61.54 and 60.00%) in the treatments with mineral mixture, GnRH and Lugol's solution in repeat breeder buffalo-cows. This study demonstrates that also the mineral support and special care to animals are positively effected on management of RB animals. Despite any supplementation and specical care did not enforce to animals in the current study, the conception rates were successful. Moreover, Sharma and Singh [38] found that 0.1% Lugol's iodine was successful for the management of suspected fungal endometritis, also they considered it as inexpensive therapeutic choose. At the same time, they have declared that the administration of irritant solutions to healthy uterus may not have an negative effect on conception but infusion of them in to sick uterus may adversely affect fertility in cows [38]. In this point, it is understood that all these research results mentioned above including our study's findindgs show that Lugol infusion also give successful results for treatment of endometritis. Several investigators claimed that it is not possible to determine the subclinical endometritis during routine examinations in RB cows. So, in RB cases, also, it is not exactly possible to diagnose metritis with the rectal and vaginoscopic examination of cows [1,39]. Subclinical endometritis can only be diagnosed by cytological examination, biopsy and the other laboratory methods [40,41]. In our study, any laboratory diagnostic method was not performed in cows except for rectal palpation and clinical inspection in the cows in order to determine only RB cows. Because, as known well, RB cows are mostly suffered from subclinical endometritis [42-45]. Actually, It is necessary to point here that, when the present study was designated, it was speculated that potential slight subclinic endometritis might be major cause of the RB. As it is known, RB might be caused by mainly subclinical endometritis and delayed ovulation or hormonal imbalance. Since the subclinical endometritis has been a major causative factor (in incidence of 52.7%) of RB in cows, the present study was basically established to elimination by assuming presence of such a problem [13]. However, it has been commented that despite both treatments (Uteroflash and Lugol's solution) did not show statistically significant positive effect, but also it can be said that any negative effect was not seen. Nonetheless, it was noteworthy that results of group I (Uterflash) had numerically higher compared to other groups (Lugol's and control group). In this context, in our study, the numerical increase in pregnancy rates of treatment groups compared to the control group can be interpreted that Carvacrol and Lugol's therapies may be effective on subclinical endometritis, so, these results confirm our speculation mentioned above.

On the other hand, it is seen that there are many studies related to intrauterine antibiotic infusion for treatment of endometritis. Such that, Shams-Esfandabadi et al.[45] reported that intrauterin infusion of oxytetracycline and procain penicillin G sodium gave 49.2 and 47.7% pregnancy rates, respectively following first service in dairy cows. Also they did not see any advantage of antibiotic treatment comparing with control group. Besides, Gümen et al.[46] and Mosaferi et al.[47] obtained the lower pregnancy rates (40-44% with cephapirin; 32 and 22% with cephapirin and oxytetracycline) than those of our study's results. It is understood that the results obtained from the present study are superior than those of several studies mentioned above which used the intrauterine antibiotic infusion twenty-four hours after inseminaton. Nevertheless, it is seen that the pregnancy rates of the present study are lower than those of some intrauterine antibiotic infusion's studies such as with cephapirin (70%) and with combination ciprofloxacin and tinidazole (70 to 78%) [48,49] and cephalexin and enrofloxacin (83 and 75%) or similar to an gentamicin treatment (67%) [50]. These studies indicate that impact of uteroflash may be considered as competitive comparing those of other studies that focused on effects of intrauterine antibiotic infusion for treatment of RB. In another study, Oral et al.[19] found that pregnancy rate was 66.6% following the intrauterine application of 5% oregano oil (contains carvacrol) in cows

with chronic endometritis. In the present study, the pregnancy rate obtained from carvacrol group was similar (67.30%) to those of them. Also, Carvacrol group in the present study (67%) was found to be numerically higher than other groups (63.4%, 47%), although there was no statistically significant difference among the all groups (P=0.252). With reference to theese results, it can be commented that carvacrol may have a improving effect on pregnancy rate in RB cows.

In the present study, it was observed that pregnancy rates obtained by rectal palpation in all groups were lower than NRR%. This may be due to the incidence of early and late embryonic deaths during the gestation or fertilisation failure [51]. On the other hand, Inskeep and Dailey [52] reported that conception failure in cows were due to various causes such as embriyonic mortality (57%), late placentation (10%), early placentation (4%), fetal (3%), lethal gene (5%), re-bred (1%) and ovum transport (8%). Differences between the our work and others may be depended on research protocols, care and nutrition conditions, age, breed, environmental factors, different yield characteristics and many unknown attributed factors [53-55]. Thus, in RB Cows, repeat breeding is not only depend on endometrial inflamations and infections, but also accociated with hormonal, mineral and antioxidant imbalance [37].

In accordance with the above litaretures and discussions, it can be argued that carvacrol (uteroflash) increased preganacy rates compared to Lugol's and control group, but it was not also seen negative effect on pregnancy rates. In this respect, it is said that carvacrol has also some advantages such as inhibition effect on antibiotic resistant bacteria and no side effect on uterus as well as improving the fertility.

In conclusion, for treatment of RB cows, it can be suggested that carvacrol infusion to the uterus may be preferred as it has improved pregnancy rates to some extent. But, to confirm these results, further are needed.

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Kafkas Univ Vet Fak Derg
25 (5): 639-643, 2019
DOI: 10.9775/kvfd.2018.21506

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

Research Article

Comparison of Internal Transcribed Spacer Region Sequencing and Conventional Methods Used in the Identification of Fungi Isolated from Domestic Animals

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Article Code: KVFD-2018-21506 Received: 10.12.2018 Accepted: 07.04.2019 Published Online: 07.04.2019

How to Cite This Article

Müştak İB, Sarıçam S, Müştak HK: Comparison of internal transcribed spacer region sequencing and conventional methods used in the identification of fungi isolated from domestic animals. *Kafkas Univ Vet Fak Derg,* 25 (5): 639-643, 2019. DOI: 10.9775/kvfd.2018.21506

Abstract

The aim of this study was to compare gold standard conventional culture method and internal transcribed spacer (ITS) sequence-based analysis in the identification of fungi isolated from domestic animals. A total of 35 animals (15 cats, 11 dogs, 4 horses and 5 chickens) suspected for fungal infection were examined. Of the 35 samples, 20 were found to be positive for fungal culture. Among positive samples 8 (40%) were predominantly found to be dermatophyte species by conventional methods. The ITS regions (ITS1, ITS2 and complete ITS) of fungal isolates were also amplified, sequenced and the results were compared with conventional culture method. The identification results of 18 (90%) fungal species were found to be compatible with both conventional culture and sequencing methods. Comparison of the results demonstrated that, complete ITS regions gene sequencing could be used for the identification of medically important fungi rapidly. Since the results of complete ITS regions gene sequencing were found to be compatible with the results of phenotypic identification, it can be concluded that ITS regions gene sequencing of fungal isolates can be also used as a confirmative tool of conventional culture methods.

Keywords: *Dermatophyte, Fungi, ITS sequencing*

Evcil Hayvanlardan İzole Edilen Mantarların Teşhisinde Kullanılan Konvansiyonel ve ITS Dizi Analizi Metotlarının Karşılaştırılması

Öz

Bu çalışmanın amacı, evcil hayvanlardan izole edilen mantar türlerinin identifikasyonunda altın standart olan konvansiyonel kültür yöntemi ve Internal Ara Bölgeler (ITS) dizi analizi yönteminin karşılaştırılmasıdır. Çalışmada, mantar enfeksiyonlarından şüphelenilen toplam 35 hayvan (15 kedi, 11 köpek, 4 at ve 5 tavuk) incelendi. 35 örnek içinde 20 tanesinin mantar kültürleri pozitif bulundu. Pozitif örnekler arasında 8 (%40) tanesi konvansiyonel yöntemlerle ağırlıklı olarak dermatofit türleri olarak bulundu. İzolatların ITS bölgeleri (ITS1, ITS2 ve ITS bölgesinin tamamı) amplifiye edildi, sekanslandı ve elde edilen sonuçlar kültür yöntemiyle karşılaştırıldı. Hem konvansiyonel hem de dizi analizi yöntemiyle elde edilen identifikasyon sonuçları 18 (%90) örnekte birbiri ile uyumlu bulundu. Bu sonuçlar doğrultusunda, ITS bölgesinin tamamının dizi analizi, mantarların hızlı teşhisi için kullanılabileceğini ortaya koydu. ITS gen bölgesinin tamamına ait dizi analizi sonuçlarının fenotipik identifikasyon sonuçları ile uyumlu olması, ITS gen bölgesi dizi analizinin konvansiyonel yöntemlerin doğrulanmasında bir araç olarak kullanılabileceğini ortaya koymuştur.

Anahtar sözcükler: *Dermatofit, Mantar, ITS dizileme*

INTRODUCTION

The fungi are saprophytic eugenic organisms that can easily spread in the environment [1]. Fungal infections, which have increased over the last three decades, cause severe health problems, from superficial infections to nail and skin, mucocutaneous candidiasis to invasive infections, sometimes with high mortality in humans and animals. High mortality is not only associated with impaired organ function, chronic lung diseases, neurological disorders, blindness, or impaired vision but also associated with late diagnosis of the causative agents [2].

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It is known that only 300 of 100.000 fungal species cause infection in humans or animals. Some fungal species (e.g. *Candida* sp.) can survive both in humans and animals; some types of fungi, such as dermatophytes are both zoonotic and highly contagious [3].

For the treatment and control of fungal diseases, faster and more accurate identification is very important [2]. Accurate identification of fungi is also important for the evaluation of biodiversity, taxonomy and species identification. Conventional identification by morphology and biochemical tests is time consuming and also requires experienced laboratory personnel. Therefore, DNA barcoding, RNA polymerase I and II, translational elongation factor 1-α, β-tubulin and internal transcribed region (ITS) are frequently used for the molecular identification and phylogenetic analysis of fungi [2].

In fungi, the ribosomal RNA operon covers the 5.8S, 18S, 28S ribosomal subunit genes and ITS1, ITS2 un-transcribed regions [1]. For various species of fungi, ITS regions are accepted as standard barcoding site since there is genetic diversity among fungal species [2]. Polymerase chain reaction based methods used to amplify the ITS regions by universal primers and sequence analysis of these regions are currently used for the identification of fungi [4].

The aim of this study is to compare the ITS region (ITS1, ITS2 and complete ITS) sequencing analysis and conventional fungal culture method in the identification of fungi obtained from clinical samples of domestic animals.

MATERIAL and METHODS

Material and Media Used

Skin scraping, swap and hair samples obtained from clinically fungal infection suspected domestic animals (15 cats, 11 dogs, 4 horses and 5 chickens) at different ages and sexes were used as material. After direct microscopic examination with 15% KOH, isolation of fungi was performed by conventional culture method using Saboraud Dextrose Agar (SDA) (Oxoid, USA). All samples were inoculated on SDA and incubated aerobically at 25°C for 7-14 days [5,6].

Identification of Fungi

Slides were prepared from fungal colonies and stained with lacto phenol cotton blue (LFPM) (Merck, Germany). Fungal colonies were identified macroscopically according to the colony characteristics (colony diameter, color, surface appearance, pigment formation etc.) and microscopically according to the conidium, hyphae and spore structures [5,6]. The isolates identified as dermatophytes were also cultured on dermatophyte agar (Becton Dickinson, USA) and Trichophyton agar (Thermo Scientific, USA) to confirm the identification. Tests were performed within two weeks of the initial isolation. Twenty fungal strains identified were cultured onto SDA to be used in molecular analysis.

Standard Strains

Microsporum canis AMF-12, *Trichophyton rubrum* AMF-19 and *Aspergillus niger* AMF-8 strains obtained from the Ankara University Faculty of Veterinary Medicine, Department of Microbiology culture collection were used as reference positive controls in the study.

DNA Extraction

Rapid DNA extraction from colonies on SDA was performed according to the method of Liu et al.[7]. The concentration of the DNA extracts was measured by spectrophotometer (Thermo Scientific NanoDrop 1000). The obtained DNA samples were stored at -20°C until molecular analysis.

Amplification of ITS Regions

The PCR assay was performed in a total 25 mL reaction volume containing 2 mL of template DNA, 0.2 mM dNTPs (10 mM dNTP mix, Thermo Scientific USA), 3 mM of $MgCl_2$ (Thermo Scientific, USA), 2.5 mL PCR reaction buffer, 2U Taq DNA Polymerase (Thermo Scientific, USA) and 0.2 mM of each primer. Thermal cycle conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 60°C, 58°C and 58°C for 30 s (ITS1, ITS2 and complete ITS region respectively) and 72°C for 1 min. Finally, there was 7 min at 72°C for final extension [8].

Sequencing of ITS Regions

The ITS1, ITS2 and complete ITS region were sequenced with the ABI 3500 Genetic Analyzer device using the BigDye Terminator v3.1 Cycle Sequencing Kit with primers that were shown in *Table 1* [8]. Analysis of the sequences was performed using the CLC Main Workbench 7.7.3 (Qiagen) and the obtained data were compared with fungal sequences in NCBI GenBank. The decisions about the fungal genus and species name were given according to the similarity scores obtained from the NCBI GenBank by BLAST analysis [9]. The ITS regions of rRNA gene sequences were also deposited in GenBank as shown in *Table 2*.

RESULTS

A total of 20 samples among 35 samples were found to be positive for the existence of fungi by direct examination and mycological culture. Sequence-based identification of 18 strains among 20 positive isolates were found to be compatible with conventional phenotypic methods.

Based on the structure of conidium, fungal hyphae, spore, mycelium and mycological cultures on media of the fungal colonies, twelve non-dermatophytes species *[A. niger* (n=2), *A. flavus* (n=1), *A. tubingensis* (n=1), *Penicillium expansum* (n=1), *P. chrysogenum* (n=1), *P. dipodomyicola* (n=1),

Table 1. *Primers used for ITS1, ITS1-2 and ITS1-5.8S-ITS2 gene regions amplification*

Name	Primer Sequences (5'-3')	Amplification Region	Amplicon Size
1F 1R	TCCGTAGGTGAACCTGCGG GCTGCGTTCTTCATCGATGC	ITS1	150-250 bp
2F 2R	GCATCGATGAAGAACGCAC TCCTCCGCTTATTGATATGC	ITS2	300-350 bp
1F 2R	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	Complete ITS (ITS1-5.8S-ITS2)	380-650 bp

Table 2. *Conventional culture and ITS region sequence-based identification results of fungi*

Strain	Accession	Culture Results	ITS1 Region	ITS2 Region	Complete ITS
AVMF1	MK461906	*A. flavus*	*Aspergillus* sp.	*Aspergillus* sp.	*A. flavus*
AVMF2	MK461907	*A. niger*	*A. niger*	*Aspergillus* sp.	*A. niger*
AVMF3	MK461908	*A. alternata*	*Alternaria* sp.	*Alternaria* sp.	*Alternaria* sp.
AVMF4	MK461909	*A. alternata*	*Alternaria* sp.	*Alternaria* sp.	*Alternaria* sp.
AVMF5	MK461910	*A. niger*	*A. niger*	*Aspergillus* sp.	*A. niger*
AVMF6	MK461911	*A. tubingensis*	*Aspergillus* sp.	*Aspergillus* sp.	*A. tubingensis*
AVMF7	MK461912	*M. nanum*	*M. nanum*	*M. nanum*	*M. nanum*
AVMF8	MK461913	*T. verrucosum*	*T. verrucosum*	*T. verrucosum*	*T. verrucosum*
AVMF9	MK461914	*S. schenckii*	*S. schenckii*	*Sporothrix* sp.	*S.schenckii*
AVMF10	MK461915	*T. tonsurans*	*T. tonsurans*	*Trichophyton* sp.	*T. tonsurans*
AVMF11	MK461916	*T. rubrum*	*T. rubrum*	*T. rubrum*	*T. rubrum*
AVMF12	MK461917	*M. canis*	*Microporum* sp. *A. otae*	*Microporum* sp. *A. otae*	*M. canis* *A. otae*
AVMF13	MK461918	*T. rubrum*	*Trichophyton* sp.	*T. rubrum*	*T. rubrum*
AVMF14	MK461919	*T. schoenleinii*	*T. schoenleinii*	*T. schoenleinii*	*T. schoenleinii*
AVMF15	MK461920	*T. mentagrophytes*	*Trichophyton* sp.	*Trichophyton* sp.	*T. mentagrophytes* *A. vanbreuseghemii*
AVMF16	MK461921	*P. dipodomyicola*	*Penicillium* sp.	*Penicillium* sp.	*P. dipodomyicola*
AVMF17	MK461922	*M. hiemalis*	*Mucor* sp.	*M. hiemalis*	*M. hiemalis*
AVMF18	MK461923	*G. candidum*	*Galactomyces candidum*	*Galactomyces candidum*	*G. candidum* *Galactomyces candidum*
AVMF19	MK461924	*P. expansum*	*Penicillium* sp.	*Penicillium* sp.	*P. expansum*
AVMF20	MK461925	*P. chrysogenum*	*Penicillium* sp.	*Penicillium* sp.	*P. chrysogenum*

Sporothrix schenckii (n=1), *Geotrichum candidum* (n=1) and *Alternaria alternata* (n=2)] and eight dermatophytes species *[M. nanum* (n=1), *T. tonsurans* (n=1), *T. rubrum* (n=2), *T. schoenleinii* (n=1), *T. mentagrophytes* (n=1), *T. verrucosum* (n=1) and *M. canis* (n=2)] were identified.

Four *Aspergillus* species identified phenotypically as *A. niger* (n=2), *A. flavus* (n=1) and *A. tubingensis* (n=1) were also identified to the species level by complete ITS regions sequencing.

Eight dermatophytes conventionally identified as *M. nanum* (n=1), *T. tonsurans* (n=1), *T. rubrum* (n=2), *T. schoenleinii* (n=1), *T. mentagrophytes* (n=1), *T. verrucosum* (n=1) were also identified by complete ITS sequencing except for *M. canis* (n=1) which was identified as *Arthroderma otae* by sequencing.

The non-dermatophyte fungi phenotypically identified as *P. expansum* (n=1), *P. chrysogenum* (n=1), *P. dipodomyicola* (n=1), *S. schenckii* (n=1) and *G. candidum* (n=1) were also identified by complete ITS sequencing. Two *A. alternata* were identified to the species level by conventional methods. However, their sequence-based identification could not be determined at the species level. *Mucor hiemalis* (n=1) which was identified to the species level phenotypically, had sequence-based identification of *Mucor hiemalis* with 99% homology. The results of conventional culture and sequence analysis results of the ITS regions were shown in *Table 2*.

DISCUSSION

Frequently used conventional methods in fungal identification are based on the colony morphology, microscopic examination and several biochemical tests which are also time consuming and subjective. In some cases, there is also

a need for experienced personnel to enable identification to the species level [10].

In order to obtain reliable and faster results, methods based on sequence analysis of ITS regions have been developed and used in routine laboratories [11]. ITS region is accepted and frequently used as a genetic marker for the identification of fungi [12,13]. It is known that ITS regions 1 and 2 are more variable than the other subunit genes among fungi [14,15]. Thus, sequencing of ITS gene regions was chosen for the molecular identification of fungal strains in this study. In regard to this information in this study ITS1, ITS2 and complete ITS region sequencing was used to differentiate the fungi identified by conventional culture methods. Fungal identification results of 6 (30%) samples by ITS1 and ITS2 gene sequencing were found to be compatible with each other and the conventional culture results to the species level. On the other hand, fungal identification results of all samples (100%) by complete ITS region gene sequencing were found to be compatible with conventional culture results.

Molecular techniques were used in several studies for the identification of *Aspergillus* species obtained from environmental and clinical samples [16,17]. In a study, Henry et al.[18] investigated *Aspergillus* species by ITS region sequence analysis and compared the obtained sequences with GenBank sequences using BLAST. They concluded that both ITS1 and ITS2 regions were necessary for the identification of *Aspergillus* isolates to the species level [18]. In another study, Pryce et al.[11] showed that clinical *Aspergillus* isolates had compatible ITS1 and ITS2 sequence results with conventional culture methods. The results of our study found to be compatible with the study of Henry et al.[18] since *A. tubingensis* (n=1), *A. flavus* (n=1) and *A. niger* (n=2) strains were correctly identified to the species level by complete ITS region sequence analysis in comparison to the conventional culture results. It can be said that both ITS regions have to be used for the appropriate identification of the *Aspergillus* sp. because the sequences obtained from both regions exhibited different results. Further studies by increasing the number of samples can demonstrate the discriminative power of ITS1 and ITS2 gene regions for *Aspergillus* species.

CLSI recommends the ITS region sequencing in the identification of *Alternaria* sp. and also several reports supported this statement by using ITS region in the phylogenetic analyses of *Alternaria* species [19-21]. However, it is also stated in the CLSI guideline MM18-A, some *Alternaria* species have nearly identical ITS sequences. In compatible with the CLSI, the results of *Alternaria* ITS sequences exhibited similar BLAST scores with more than one *Alternaria* sp. Thus, in this study ITS sequence-based identification of *Alternaria* strains (n=2) which were identified phenotypically to the species level, was evaluated to the genus level.

Generally common dermatophytes can be identified especially by ITS1 region sequencing [9]. CLSI stated that for the differentiation of *T. rubrum* complex, the sequence analysis of the ITS regions is sufficient to identify only *T. rubrum* and *T. violaceum* species. However, the sequence analysis of rDNA 28S D2 region is required for the other species in the genus [9]. In this study, all dermatophyte isolates except for *M. canis* had compatible conventional and sequence-based identification result. *M. canis* which was identified conventionally, was found as *A. otae* by sequencing. It is known that dermatophytes may have two species names according to their sexual (teleomorph state) and asexual (anamorph state) forms. When *M. canis* find a compatible environment, it changes into sexual form which is called *A. otae* [22]. Similarly, the teleomorph state of *T. mentagrophytes* organism is called *A. vanbreuseghemii*. These results did not change the decision about the results of both complete ITS sequence-based and conventional culture for their compatibility in the identification of common dermatophytes.

Pryce et al.[11] identified a *G. candidum* strain (n=1) to the species level by sequencing of complete ITS region. However, in this study, *G. candidum* strain was identified with 100% homology as *Galactomyces candidum* to the species level by complete ITS, ITS1 and ITS2 regions. As in dermatophytes, *G. candidum* has a synonym name as *Galactomyces candidum* which is the anamorph state of this organism.

Kwiatkowski et al.[23] identified five *Penicillium* species except for *P. marneffei* to the species level by both complete ITS and D1/D2 region sequencing. It is also stated in CLSI guidelines only for the identification of *P. marneffei* alternative DNA targets must be used [9]. In this study for the identification of *Penicillium* genus, while the sequence analysis of ITS1 and ITS2 gene regions were not sufficient, complete ITS region sequencing was found to be discriminative for this genus.

It can be concluded that, the results of conventional culture and complete ITS region sequencing of clinical fungal isolates were found to be compatible. Also in this study it was shown that sequence-based identification is a rapid, accurate and reliable method and may be used for the confirmation of the results obtained from the conventional culture methods which is based on subjective observation in fungal identification.

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Kafkas Univ Vet Fak Derg
25 (5): 645-650, 2019
DOI: 10.9775/kvfd.2018.21521

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

Research Article

The Effects of Oxytocin and $PGF_{2\alpha}$ Injections on Semen Quality and Libido in Buck

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Article ID: KVFD-2018-21521 Received: 10.12.2018 Accepted: 10.05.2019 Published Online: 16.05.2019

How to Cite This Article

Çebi Şen Ç, Tekin K, Çil B, Akçay E: The effects of oxytocin and $PGF_{2\alpha}$ injections on semen quality and libido in buck. *Kafkas Univ Vet Fak Derg,* 25 (5): 645-650, 2019. DOI: 10.9775/kvfd.2018.21521

Abstract

The aim of the present study was to evaluate the effects of exogenous oxytocin and $PGF_{2\alpha}$ on seminal quality and libido sexualis in bucks. To investigate the role of these hormones on male fertility, semen samples from 20 Norduz bucks (3-4 years of age) were collected with an artificial vagina twice a week with five replications in breeding season. Bucks were randomly assigned to five groups, control group was administered with 2 mL of sodium chloride, 0.9% (w/v)) i.m., whilst the experimental groups were administered with oxytocin 10 IU, i.v. (Group 1, n = 5), oxytocin 20 IU, i.v. (Group 2, n = 5), $PGF_{2\alpha}$ 5 mg, i.m. (Group 3, n = 5) or $PGF_{2\alpha}$ 10 mg, i.m. (Group 4, n = 5) 20 min before each collection. There was no statistical difference between the treatment groups in terms of motility (P>0.05). However, semen volume, concentration, abnormal spermatozoa rate, intact membrane rate and libido results were statistically significant among the different groups (P<0.05). As a result, administration of 20 IU oxytocin twenty minutes prior to semen collection in bucks did not improve overall semen quality, however, libido, semen volume, and sperm concentrations were increased. In contrast to oxytocin, $PGF_{2\alpha}$ administration has led to a slight decrease in libido and has shown moderate effects on semen quality.

Keywords: *Buck, Libido, Oxytocin, $PGF_{2\alpha}$, Semen quality*

Tekelerde Oksitosin ve $PGF_{2\alpha}$ Enjeksiyonlarının Sperma Kalitesi ve Libido Üzerine Etkisi

Öz

Bu çalışmanın amacı, ekzojen yoldan uygulanan oksitosin ve $PGF_{2\alpha}$'nın seminal plasma ve libido seksüalis üzerine olan etkilerini değerlendirmekti. Bu hormonların sezon içi erkek reprodüksiyonu üzerindeki rolünü araştırmak için, 20 Norduz tekesinden (3-4 yaş) haftada iki kez suni vajina ile alınan sperma numuneleri, beş replikasyon ile gerçekleştirildi. Tekeler rastgele beş gruba ayrılarak, sperm alma işleminden 20 dk önce, kontrol grubu 2 mL sodyum klorür, %0.9 (w/v)) ile i.m., deney grupları ise oksitosin 10 IU, i.v. (Grup 1, n = 5), oksitosin 20 IU, i.v. (Grup 2, n = 5), $PGF_{2\alpha}$ 5 mg, i.m. (Grup 3, n = 5) ve $PGF_{2\alpha}$ 10 mg, i.m. (Grup 4, n = 5) olarak uygulandı. Deney grupları arasında motilite açısından istatistiksel fark tespit edilmedi (P>0.05). Ancak sperma hacmi, konsantrasyon, anormal spermatozoa oranı, intakt membran oranı ve libido sonuçları gruplar arasında istatistiksel olarak anlamlı bulundu (P<0.05). Sonuç olarak, sperma alımından yirmi dakika önce 20 IU oksitosin enjeksiyonu sperma kalitesini değiştirmezken, libido, sperma hacmi ve sperm konsantrasyonunu artırdı. Oksitosinin aksine, $PGF_{2\alpha}$ uygulaması libidoda hafif bir azalmaya neden olurken ve sperma kalitesinde orta derecede etkiler gösterdi.

Anahtar sözcükler: *Libido, Oksitosin, $PGF_{2\alpha}$, Semen kalitesi, Teke*

INTRODUCTION

The use of assisted reproductive techniques in goat breeding provides additional advantages for cryopreservation and artificial insemination [1]. In some cases, certain animals constantly have a high libido and good semen quality for evaluation, freezing or insemination, while others are reluctant for collection and have low quality ejaculates with decreased volume and concentration or other inadequate spermatological characteristics [2-4]. During the

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last decades, specific hormones (oxytocin, prostaglandins, testosterone and GnRH) were introduced to increase the sperm output, quality of male-related reproductive deficiencies and to regulate the breeding activity [5-9].

Oxytocin is a peptide structure hormone and has numerous peripheral actions such as lactation, smooth muscle contraction, wound healing, natriuresis, sexual arousal and mostly known as social behaviour hormone which increases trust and reduces fear, monogamous pair and maternal bonding [10,11]. Prostaglandin (PG) is mainly used for synchronisation of the females [12,13]. It is a physiologically active lipid compound which is secreted from several tissues and is derived from arachidonic acid by the action of cyclooxygenase (COX) isoenzymes COX1 and COX2 [14]. The development of mice deficient in COX1 and/or COX2 has shown that COX2–null female mice are infertile. PG enhances progressive motility of spermatozoa by stimulating the contraction of the vaginal smooth muscle [15]. Administration of $PGF_{2\alpha}$ prior to semen collection has led to an increase in sperm output in buffalos [16], dogs [17], and stallions [18,19]. Oxytocin has been known as a female hormone but the role of oxytocin associated with reproductive physiology in the male animal needs to be elucidated. Increasing the contractility in the male reproductive tract by modulating steroidogenesis is the specific role of this hormone [20]. Hypothalamic nonpeptide oxytocin is one of the most potent mediators of drug-induced penile erections in laboratory animals. Moreover, oxytocin treatment prior to ejaculation has improved the ejaculate quality by increasing the concentration of sperm in the ejaculate of the bull [21], ram [22], rabbit [23] and man [24]. Oxytocin receptors have been determined from testes, epididymis, prostate, penis and the epididymal smooth muscle of several species [19,25]. There is a growing evidence that $PGF_{2\alpha}$ and oxytocin are important factors in determining sperm transport throughout the entire epididymis of bulls, buffalos, rams, rabbits and stallions [17]. In parallel with above mentioned studies, we aim to evaluate the effects of exogenous oxytocin and $PGF_{2\alpha}$ on seminal quality and libido in Norduz bucks.

MATERIAL and METHODS

Animals and Semen Collection

This study was conducted according to ethical laws and regulation of Ankara university animal experiments local ethics committee. 20 Norduz bucks (3-4 years of age) were barned at Research Farm of Ankara University, Faculty of Veterinary Medicine (40°05'53.5"N 32°37'19.6"E). The bucks were maintained under the constant nutritional regime and with water ad libitum.

At the beginning of the study, twenty min prior to semen collection all animals were administered with 2 mL physiological saline (Sodium Chloride, 0.9% (w/v)) i.m. and ejaculations were collected with artificial vagina from each buck as a control group (Control Group, n = 20). Afterwards, bucks were randomly assigned to four groups and each group was administered with oxytocin (Hormonipra, HIPRA) 10 IU, i.v. (Group-1, n = 5), oxytocin 20 IU, i.v. (Group-2, n = 5), $PGF_{2\alpha}$ (Gestavet-Prost, HIPRA) 5 mg, i.m. (Group-3, n = 5) or $PGF_2\alpha$ 10 mg, i.m. (Group-4, n = 5) twenty min before each collection, with a total of 5 replications. A total of 120 ejaculates (20 for control and 100 for experimental design) were collected with an artificial vagina, twice a week from 20 mature bucks during the breeding season. After collection, ejaculates were placed in a water bath (33°C) for further evaluation of spermatological parameters [26].

Libido Assessment

The behavioural signs of libido (leg kicking, sniffing, vocalization, flehmen reaction, mounting without thrust and mounting with ejaculation) were recorded as the total score for libido. Reaction time was assessed as the interval between the buck's entrance into the collection room and the initiation of ejaculation. Libido was evaluated at a scale of 0 to 4, with 0 being the total absence of sexual desire and 4 being the highest level of sexual desire giving minimal time to mount the teaser animal [27].

Semen Evaluation

Semen volume, total sperm motility, sperm concentration, sperm morphology, membrane integrity and pH were recorded [18].

Sperm Motility

Motility assessment was performed using a phase-contrast light microscopy (x100) (Olympus BH-2, Olympus Optical Co. Ltd., Japan) on a pre-heated stage (37°C). Five microscopic fields from separate 5 µL aliquots of the same sample were evaluated by two trained technicians. The mean percentage of the three successful evaluations was determined as total motility [18].

Spermatozoa Concentration

Spermatozoa concentration was identified according to the haemocytometry method. Semen was diluted with Hayem solution (1 g NaCl, 5 g Na_2SO_4, 0.5 g $HgCl_2$ and 200 mL bidistilled water) at a ratio of 1:100. Mean spermatozoa count was calculated from three replicate of each sample at a magnification of 400x and recorded as $x10^6$ mL [18].

Sperm Morphology

For morphological assessment of the sperm, a drop of a mixture containing 150 µL semen mixed with 1 mL of Hancock's solution *(Sodium saline solution:* 9.01 g NaCl and 500 mL of double-distilled water. *Buffer solution*: (1) 21.682 g $Na_2HPO_4 \times 2H_2O$ and 500 mL of double-distilled water; (2) 22.254 g KH_2PO_4 and 500 mL of double-distilled water. Amounts of 200 mL of (1) and 80 mL of (2) were mixed to

obtain 280 mL of buffer solution. The final Hancock solution was mixed as follows:150 mL sodium saline solution, 62.5 mL formalin, 150 mL buffer solution and 500 mL double distilled water) was evaluated using a bright field microscopy (x 400) (Olympus CX21FS1, Olympos Optical Co. Ltd., Japan) under immersion oil. At least 200 spermatozoa were counted to determine the percentage of abnormal spermatozoa [18].

The Hypo-Osmotic Swelling Test (HOST)

Spermatozoa membrane integrity was assessed with the hypoosmotic swelling test (HOST), based on swollen and curled tails. 20 µL of semen added into 200 mOsm hypo-osmotic solution (9 g fructose, 4.9 g trisodium citrate and 100 mL distilled water) and the mixture was incubated for 30 min at 37ºC. Subsequent to incubation, 0.1 mL of the mixture was evaluated using a bright-field light microscopy (Olympus CX21FS1, Olympus Optical Co. Ltd., Japan). At least two hundred spermatozoa were evaluated and sperm cells that have coiled or swollen tails were defined as spermatozoa with intact membrane integrity [28].

Statistical Analyses

Semen parameters were statistically analyzed using one-way ANOVA for (GLM procedure of SigmaStat 4.0 Statistical Software) while libido scores were analyzed by Kruskal Wallis Test. Significant differences were considered with $P<0.05$.

RESULTS

According to obtained results, there was not any statistical difference between the dosage groups regarding motility. However, in terms of semen volume, concentration, abnormal spermatozoa rate, intact membrane rate, and libido test, results were statistically significant among the different dose groups ($P<0.05$).

Average semen volume of Group two (1.34±0.16 mL) was found significantly higher than other groups, whereas mean values of $PGF_{2\alpha}$ groups (Group-3 and Group-4) were found lower than control value ($P<0.05$). When the concentration was taken into account, Group two has statistically higher mean value than $PGF_{2\alpha}$ groups. For abnormal spermatozoa rate, a significant increase in Group one was observed regarding other groups. A significant decrease of mean intact membrane percentages was evident at Group four comparing to group one *(Table 1)*.

A significant increase in libido evaluation scores for oxytocin groups (3.04±0.64; 3.2±0.5) was observed *(Table 2)*. The bucks, which were administered $PGF_{2\alpha}$, were reluctant for mating and showed a decrease in libido. Thus, it might have enhanced hyperthermic and psychological stress in animals. Therefore, the duration of ejaculation was longer in $PGF_{2\alpha}$ groups than the other groups.

DISCUSSION

Numerous pharmacological substances have been proven to improve male reproductive performance in many species [29]. It is a known fact that hormones play the major role in the regulation of male reproductive functions as in sexual arousal, control of sexual behaviour, the onset of erection and ejaculation, and the post-ejaculatory detumescence [30].

Libido is an important factor in male reproduction and oxytocin plays a major physiological role in sexual behaviours. Libido is one of the most important factor in male reproduction and regulated by primarily testosterone, dopamine respectively. However, a study [31] showed that electrical stimulation of the glans penis elicits a specific activation of 40-50% of oxytocinergic neurons in the paranuclear nucleus of the hypothalamus. During ejaculation, oxytocin probably associated with ejaculation through hypothalamus. This is also must be in relation with systemic pulsation of oxytocin on sexual behaviour [32].

Table 1. *Effects of different doses of oxytocin and $PGF_{2\alpha}$ administration on ejaculate characteristics in bucks*

Treatment	n	Volume (mL)	Motility (%)	Concentration (x10⁹ sperm/mL)	Abnormal Spermatozoa Rate (%)	Intact Membrane (%)
2 mL PWS (Control)	20	1.14±0.17^{b}	56.50±0.82	3.18±0.006^{ab}	24.55±0.87^{b}	62.00±0.95^{ab}
10 IU Oxytocin (Group 1)	25	1.20±0.18^{b}	56±0.71	3.24±0.06^{ab}	27.76±0.78^{a}	64.52±0.86^{a}
20 IU Oxytocin (Group 2)	25	1.40±0.13^{a}	57±0.82	3.32±0.03^{a}	23.16±0.45^{b}	63.28±0.99^{ab}
5 mg $PGF_{2\alpha}$ (Group 3)	25	0.96±0.22^{c}	56.4±0.74	3.07±0.05^{b}	25.40±0.63^{ab}	61.40±0.60^{ab}
10 mg $PGF_{2\alpha}$ (Group 4)	25	0.89±0.15^{c}	54.60±0.64	3.05±0.05^{b}	23.16±0.47^{b}	60.40±0.65^{b}

^{a,b,c} Different letters within the same column indicate a significant difference ($P<0.05$) One Way ANOVA

Table 2. *Effects of different doses of oxytocin and $PGF_{2\alpha}$ administration on the libido of bucks*

Treatment	n	Libido Scores
2 mL PWS (Control)	20	2.55±0.51^{b}
10 IU Oxytocin (Group 1)	25	3.04±0.73^{a}
20 IU Oxytocin (Group 2)	25	3.2±0.5^{a}
5 mg $PGF_{2\alpha}$ (Group 3)	25	2.24±0.44^{bc}
10 mg $PGF_{2\alpha}$ (Group 4)	25	2.12±0.33^{c}

^{a,b,c} Different letters within the same column indicate a significant difference ($P<0.05$) Kruskal Wallis Test

During the sexual arousal, oxytocin has shown a slight increase while during the ejaculatory phase a considerable increase has been presented in rams, bulls, rabbits and humans as well. Peripherally released oxytocin participates in sexual satiety and assists the sperm transport by contracting the reproductive tract [33].

Although the administration of oxytocin stimulates sexual behaviour and performance in many mammalian species, our data indicate that oxytocin administration 20 min prior to semen collection did not improve the semen quality of bucks, however detectable effects on the ejaculation time and sperm output was observed. In the present study, the role of oxytocin on libido was clearly displayed through an increase in libido test score. In addition to that, the overall duration of semen collection was shortened.

Oxytocin hormone can be effective on mating behaviour and erectile function and it can modulate the androgen regulation. It acts on smooth muscle cells of the epididymis. In addition to that, oxytocin can stimulate the release of endothelin-1 from the caput epididymis [32]. There is a growing evidence that oxytocin is one of the most potent mediators of drug-induced penile erections in laboratory animals, most likely by increasing NO-synthase activity in the paraventricular nucleus of the hypothalamus. Intra-cerebroventricular injection of synthetic oxytocin was followed by yawning and penile erection within 5 min in rats, whereas application of a potent non-peptide oxytocin receptor antagonist, as well as a competitive inhibitor of NOsynthase, reduced penile erections and copulatory behaviour in a dose-dependent manner [34]. The role of central oxytocin in the control of ejaculation has been demonstrated in rabbits and rodents as well. After the oxytocin treatment, the number of intromission before ejaculation was reduced and oxytocin promoted ejaculatory behaviour by shortening ejaculation latency and post-ejaculatory refractory period [35-37]. However, attempts to elicit sexual behaviour in previously nonresponsive male rats were not successful. Consistently with previous research, in the present study, the administration of 10 or 20 IU oxytocin hormone has positively affected the libido and shortened the collection time as well.

Although 10 IU i.v. oxytocin administration did not lead to any statistically significant increase in semen volume or sperm concentration, doubling the dose of oxytocin led to a significant increase in both parameters. Even though parallel studies with our results exist, there is also a contradiction with previous studies. In addition, there is a negative correlation between the volume of semen and DNA fragmentation [38]. The implementation of the exogenous oxytocin hormone just before the ejaculation, bull [21], buffalo [16], ram [22,34], rabbit [23] and rats caused to an increase in the number of sperm in the ejaculate. While a study [39] reported that, administration of 7 IU oxytocin 5 min before ejaculation has been found to increase the number of spermatozoa in rams, Berndtson and Igboeli [21], suggested that, 50 IU iv oxytocin injection has no positive effect on spermatological characteristics in the bull. In another study, after administration of 10 IU of oxytocin in the male do had no effects on ejaculate characteristics [29]. Knight and Lindsay [36] reported that exogenous administration of oxytocin hormone 10 min. prior to semen collection had resulted with an increase of sperm concentration. Moreover, in vitro addition of oxytocin did not improve motility or abnormal spermatozoa rate [40]. This conflict may be due to oxytocin dose and the specific time of hormone administration.

The neuropeptide oxytocin can be found in the mammalian testis and cauda epididymis and it enhances sperm transport by improving seminiferous tubular and testicular capsule contractile activity. It has been shown in vitro that in the absence of oxytocin contractile activity of seminiferous tubules is reduced [41]. However, it can be restored by addition of exogenous oxytocin [42]. The increase of spermatozoa number in the ejaculate after oxytocin administration is assumed that can be related to the forceful contraction of the efferent tubules and testes. Oxytocin increases the seminiferous tubules rhythmic contractions which taken forward the spermatozoa from lumen towards the rete testicles [17,43]. Effect of oxytocin is partly mediated via stimulation of an increase in the synthesis of prostaglandins by the seminal vesicles. It influences the secretion rate of the male accessory glands, which, may account for the increased volume seen in the ejaculate of rams and buffalo following administration. Within the prostate, both directly and via interactions with androgen metabolism, it has been shown to affect gland growth [32].

In the present study, the concentration of spermatozoa number did not increase statistically after with 10 IU oxytocin administration however with 20 IU a significant increase was observed. In other words, this can be related to epididymal contraction dose as well. Motility and semen pH was not affected by the treatment and showed similar results with the control group [29,40]. With 10 IU oxytocin group, there was a slight increase in abnormal spermatozoa rate.

It is a well-known fact that $PGF_{2\alpha}$ is a component of the seminal fluid in many species. This hormone is related to the increase of contractility in both the male and female genital tracts. It is also believed that prostaglandins are involved in the ejaculation process and may have some effect on libido [44]. Exogenous $PGF_{2\alpha}$ has been shown to cause masturbation, spontaneous erection and ejaculation in the stallion. It has also been used for ex-copula ejaculation in stallions [18]. In the rabbit corpus cavernosum smooth muscle, endogenous prostaglandins have a local effect on reinforcement of neurally mediated and spontaneous contractions and enhancing the detumescence of the penis [45]. In contrast to our study, in the ejaculate of bulls, rabbits, rams and stallions $PGF_{2\alpha}$ has been reported to

increase both the volume and spermatozoa concentration [46]. $PGF_{2\alpha}$ is also believed to cause contractions on the male reproductive system. In the boar, spermatozoa concentration did not affect by $PGF_{2\alpha}$ administration, although an increase in the volume of the semen has been reported [47]. The mechanism behind this increase has not been fully understood yet. Nevertheless, in the present study, $PGF_{2\alpha}$ has shown detrimental effects on semen quality, especially with a high dose on intact membrane rate. In addition to that, libido score was decreased following the $PGF_{2\alpha}$ administration. Among the livestock species, goats are more vulnerable due to their sensitive behavioural pattern. In this study, we also observed that administration of $PGF_{2\alpha}$ has increased the reaction time before ejaculation, comparing to oxytocin group. Our observation is highly associated with the libido results, which suggest that prostaglandins are involved in the development of hyperthermia and the ACTH response induced by psychological stress.

In conclusion, administration of 20 IU oxytocin twenty min before the semen collection have increased both the semen volume and the concentration of spermatozoa in bucks. Administration of oxytocin has increased the libido although there were not any improvements in semen quality. In contrast to oxytocin, $PGF_{2\alpha}$ administration has led to a decrease of libido and has detrimental effects on semen quality. We concluded that administration of oxytocin stimulates sexual behaviour and performance in bucks.

Conflict of Interest

The authors declare no conflict of interest.

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Kafkas Univ Vet Fak Derg
25 (5): 651-658, 2019
DOI: 10.9775/kvfd.2018.21543

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

Research Article

Economic Feasibility of Package Beekeeping Application in Turkey: A Case Study of Edirne Province

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[1] *This study was supported by the Ministry of Agriculture and Forestry, General Directorate of Agricultural Research and Policies, Turkey (Project number: TAGEM/HAYSÜD 14/06/01/10)*

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Article ID: KVFD-2018-21543 Received: 12.12.2018 Accepted: 21.04.2019 Published Online: 28.04.2019

How to Cite This Article

Adanacıoglu H, Kosoglu M, Saner G, Topal E, Yucel B: Economic feasibility of package beekeeping application in Turkey: A case study of Edirne province. *Kafkas Univ Vet Fak Derg,* 25 (5): 651-658, 2019. DOI: 10.9775/kvfd.2018.21543

Abstract

In this study, it was discussed whether the package beekeeping was an economical alternative to traditional beekeeping in Turkey. An experiment involving two different applications was carried out in the province of Edirne, which has a short production season due to the cold climate conditions. The control groups representing the colonies formed by the artificial swarms and the colonies formed by the package bees were compared in terms of economic feasibility in this experiment. The results of this study showed that package beekeeping was more advantageous for beekeeping enterprises when the artificial swarms' application was taken into consideration. The purchase price of package bees for beekeeping enterprises in Edirne Province should be below US$39.52. In other words, package beekeeping for Edirne province is profitable for enterprises at prices below US$ 39.52. The package beekeeping can provide savings for beekeepers whose bees overwinter in cold climates. It was determined that if the bees were not wintered, the beekeeping enterprises could save US$31.63 per hive in this study. According to the results, it is expected that the dissemination of package beekeeping will have a hugely positive impact on beekeeping enterprises for North, Northwest and Eastern Anatolia Regions of Turkey.

Keywords: *Package bees, Beekeeping, Economic feasibility, Profitability*

Türkiye'de Paket Arıcılık Uygulamasının Ekonomik Olarak Uygulanabilirliği: Edirne İli Örneği

Öz

Bu çalışmada, paket arıcılığın Türkiye'de geleneksel arıcılık faaliyetine ekonomik bir alternatif olup olmadığı tartışılmıştır. Bu amaçla, soğuk iklim koşulları nedeniyle kısa bir üretim sezonuna sahip olan Türkiye'nin Edirne ilinde iki farklı uygulamanın yer aldığı bir deneme gerçekleştirilmiştir. Bu denemede yapay oğuldan oluşan kolonileri temsil eden kontrol grupları ile paket arılardan meydana gelen koloniler ekonomik yapılabilirliği açısından karşılaştırılmıştır. Bu çalışmanın sonuçları, arıcılık işletmelerinde yapay oğul uygulamasına göre paket arıcılığın daha avantajlı olduğunu göstermiştir. Edirne ilinde arıcılık işletmeleri için paket arı satın alma fiyatının 39.52 ABD Dolarının altında olması gerekmektedir. Diğer bir ifade ile, Edirne ili için paket arıcılık, 39.52 ABD Doları'nın altındaki fiyatlarda işletmeler için karlı olmaktadır. Paket arıcılık, arılarını soğuk iklimlerde kışlatan arıcılar için tasarruf sağlayabilmektedir. Arılar kışlatılmadığı takdirde arıcılık işletmelerinin kovan başına 31.63 ABD doları tasarruf edebileceği belirlenmiştir. Paket arıcılığın yaygınlaşmasının Türkiye'nin Kuzey, Kuzeybatı ve Doğu Anadolu bölgelerindeki arıcılık işletmeleri üzerinde son derece olumlu bir etki yaratması beklenmektedir.

Anahtar sözcükler: *Paket arıcılık, Arıcılık, Ekonomik uygulanabilirlik, Karlılık*

INTRODUCTION

New honey bee colonies can be acquired in different ways, such as established colonies, nucleus colonies, package bees and swarms. The main advantages of package bees are that they cost cheaper than established colonies or nucs, easy for beginners to handle, and there is little possibility of the bees having a severe brood disease [1].

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Especially, package bees are preferred by beekeepers due to the colony losses caused by wintering. Cengiz and Erdoğan [2] pointed out that the vast majority of colony losses occurred in the winter months. Ucak Koc [3] put forward that significant colony losses during wintering had been reported in the USA (30%), Europe (1.8-53%) and the Middle East (10-85%) since 2006. Maucourt et al.[4] cited that severe winter losses had pushed beekeepers to multiply colony numbers by producing more nuclei during the productive season. According to them, multiplying colonies were achieved by creating a new colony with a young mated queen and either just bees (package bees) or brood and bees (nucleus bees). Withrow et al.[5] emphasized that beekeepers often relied on purchasing 'packages' of bees, consisting of ~10.000-12.000 workers and a young mated queen in order to offset these annual colony losses.

Beekeepers in Turkey also produce a relatively small amount of pollen besides honey. The sales of live bees, i.e. package bees, is very important to obtain alternative income in beekeeping. However, due to long wintering and hard climate conditions, significant colony losses are experienced. The fact that both the cold zone beekeeper and the hot zone beekeeper have the potential to generate additional income with the apiculture application reveals the importance of the issue. This can be seen as an opportunity to increase production alternatives in beekeeping, to improve beekeeping and the economic situation of beekeepers.

Beekeepers in the Mediterranean and Aegean regions of Turkey, which are suitable for the production of package bees, will be able to obtain additional income by this method and produce healthy and clean honey to be presented to consumers. From package beekeepers to entrepreneurs, beekeepers who aim to increase the capacity and to strengthen their colonies will have the opportunity to find a healthy and cheap colony [6].

The colony production in spring season is also done in the Aegean and Mediterranean regions of Turkey which have a suitable climate for bee production in spring season. These bee colonies can be sent to North and West Anatolia regions that have the negative weather conditions and short-term source of nectar, and also to North West Anatolia, that experiences adverse weather conditions after early spring. Thus, while the beekeeper in the Aegean and the Mediterranean will obtain safe and high income with colony production, the bee producers in North, Northwest and Eastern Anatolia will be less affected by the colony losses caused by wintering, and will be able to benefit from the nectar stream in the spring season [7].

Package beekeeping practices are carried out in countries with different climates. Therefore, the economic conditions of the country directly affect the applicability. The risk of winter loss increases the beekeeping enterprises in the regions where the climate conditions are severe and prolonged, and harsh winters are experienced. Package beekeeping began to develop in the US in the late 19th century. In order to reinforce the bee colonies in the northern region of North America, the beekeepers in the more tempered Southern region tried to meet the bee demand of the Northern beekeepers, where heavy winter conditions and bee colonies yield significant winter losses. According to the increasing demands, Southern beekeepers have turned to bee and queen bee production, which is more secure than honey production and they have created a model called *Package Beekeeping* by transporting bees in small packages in order to minimize transport inputs [8].

Package beekeeping is widespread in North America, Australia, New Zealand and Russia [6]. The winter losses in the beekeeping sector in Europe caused the ¼ of all colonies to collapse and due to the weakness of honey bee colonies at harvest time, packaged bee transfer from Austria had to be done and success was obtained from the package bees transferred to the hives in early spring season [9].

According to a study carried out in Saudi Arabia by A.A. Al-Ghamdi et al.[10], the country imports 200,000 exotic package bees annually due to the shortage of local bees. However, the imported colonies are only surviving for one honey harvest or season [10]. There is a literature on how to make more applications of package beekeeping. Package sizes vary according to the producers of the region. The most common dimensions are 15x25x35 and 15x22.5x40 cm. The packages are sold to the extent that they are suitable to the desired bee quantity.

The weight of the packages is usually 1.5 kg but can vary between 1 kg and 2.5 kg. For instance, in Canada, packaged bees usually exist in 1 or 1.5 kg packages, containing 8.000 and 12.000 bees, respectively [11]. Package bees are usually purchased in the spring season because they are used to replace winter losses or to obtain a new colony. Approximately 1 kg worker bee refers to a population of 7.000 worker bees. Packages may be with queen bees and/or except queen bees. A queen-package contains a fertilized queen bee, a young worker bee and a feeder with the desired weight in the cage [8,12-15].

Punnett and Winston [16] compared various combinations of package and nucleus production in April. In their study, conducted at Southwestern British Columbia, colonies were monitored through the season following the removal of packages and nuclei to determine the biological and economic impact of the package and/or nucleus production. They found that all colonies used for bee production yielded greater economic returns than the control colonies, from which no packages or nuclei were removed. According to the authors, the results indicate

that both package and nucleus production is feasible in the Lower Fraser Valley area of BC, and would provide local beekeepers with additional income [16].

Tahirov et al.[17] stated that population density and honey yield have increased depending on the convenient transport of bee colonies to favorable regions during the season. According to the results of another research, it was reported that the wax production was higher in the colonies supported by the package bees [18]. In a study in which an economic analysis of feeding periods and varieties of bees were made, it was determined that in the autumn season, high protein content (21%) was found to be more profitable and economical than pollen feeding in spring season [19].

It has been seen that the production and trade of package bees have been done in different countries of the world for many years. On the other hand, the option to establish new colonies with package bees in Turkey has been neglected until now. In recent years, there has been considerable debate on whether package beekeeping is feasible to the beekeeping enterprises in Turkey. However, no study has been carried out to determine whether package beekeeping is feasible or not regarding the beekeeping enterprises in the cold climate regions of Turkey.

In this study, it was discussed whether the package beekeeping was an economical alternative to traditional beekeeping in Turkey. For this purpose, an experiment involving two different applications was carried out in Edirne province of Turkey, a region having a short production period due to the cold climate conditions. In this experiment, the control groups representing the colonies formed by the artificial swarms and the colonies formed by the package bees were compared economically.

MATERIAL and METHODS

Data

This study was performed on the data from experiments conducted in Edirne province, Turkey [20] *(Fig. 1)*. One of the reasons for the selection of this province in the study is that the region has cold climate conditions. Therefore, the wintering period in the beekeeping activity in the region is longer. Besides, Edirne Province also represents the region with high colony losses due to the climate changes observed in early spring. Edirne also has wide sunflower planting areas.

Thrace is the region where wintering loss is especially the highest. In addition, this study was also carried out in Yozgat province with heavy winter conditions. But, the production in the same year did not occur due to adverse climate conditions and the data was not used in this research.

The genotype of the Anatolian bee *(Apis mellifera* L.) adapted West Aegean conditions was used in the experiments. This genotype has been obtained from the treatment material by the Aegean Agricultural Research Institute about 10 years. The control group of Edirne is local genotype which is adapted to the conditions of Thrace. In all groups, the queen bees are sisters of the same queen bee.

The experiments were established on May 2, 2016. Two groups were formed as package bee and artificial swarm in the experiments. The experiments were carried out according to the Completely Randomized Design, which will cover 12 beehives in each group *(Table 1)*. Considering the main nectar flows in the region, package bees and artificial swarms were sent eight weeks before the beginning of the main nectar flow. After the package bees were

Fig 1. The geographic location of the study region

Table 1. *The design of experiments by groups*

Groups	Design of Experiments	The Number of Colonies
Group 1	1.5 kilogram packages of bees	12
Group 2	Artificial swarms (3 to 5 frames)	12

transferred to the hives, the colonies were fed intensively up to the nectar flow. The honey was harvested in July 2016.

Economic Analysis

In this study, the economic feasibility of package beekeeping in terms of the enterprises dealing with beekeeping was examined with some economic evaluations.

In the course of the economic analyses carried out in the study, production costs, unit cost, absolute profitability, relative profitability, gross margin and net return were calculated. Besides, a break-even analysis was used in order to determine which price levels for package bees will be profitable for the beekeeping enterprises.

The cost items of honey production were classified into variable and fixed [21,22]. In honey production, total variable costs include subsequently, feed costs (sugar), medication (parasite and disease control), wax foundation, transportation of hives, hired labor, location rental fees, colony replacement costs, packaging of honey (jar), and repairs and maintenance. After taking the total variable costs of honey production, an interest of the total variable costs was calculated by charging a rate of 7% (annual average nominal credit interest rate for Turkish Lira) on total variable costs and added to total variable costs [23,24].

Fixed costs involve the interest on the hive, machinery and equipment investment, depreciation and administrative costs. Interest on the hive, machinery and equipment investment was calculated by charging a rate of 8% [24]. Interest amounts added to the variable and fixed costs were calculated by taking the production periods into consideration in Edirne Province. The production period covers 5 months for the package bee application and 7 months for the artificial swarm application. Administrative costs were estimated at 3% of total variable costs [19]. Depreciation was estimated using the straight-line method and the depreciation rate for hive, machinery and equipment was accepted 10% [21].

The total production cost equals fixed costs plus variable costs. The net return was calculated by subtracting total production costs from total gross revenues. The unit cost of honey (per kg) was identified by dividing the total production costs that were incurred by the total of variable and fixed costs of the honey production.

Gross margin analysis was carried out in this study in order to compare the profitability of the colonies formed by the artificial swarms and the colonies formed by the package bees. Gross margin analysis is one of the oldest and simplest analytical tools used in farm management. It has been used in some economic studies for analyzing the profitability of farm production practice [25].

Kay et al.[26] defined gross margin as a difference between gross income and variable costs. They also cite that income above variable costs is sometimes called the gross margin of an enterprise.

One of the most important objectives of this study is to determine the purchase price of the package bee in terms of the beekeeping enterprises that buy package bees. The break-even analysis was used to determine the price level which beekeeping enterprises can buy package bees.

One of the most common tools used in evaluating the economic feasibility of a new enterprise or product is the break-even analysis. The break-even point is the point at which revenue is precisely equal to costs. There is no profit and no loss occurs at this point [27]. In the break-even analysis, the package bee price was found to same net return to zero since the price of the package bee, which was one of the variable cost items, was not known.

The economic analysis values calculated in Turkish Lira were converted to US Dollar with the exchange rates released by the Central Bank of the Republic of Turkey. The average exchange rate between Turkish Lira (TRY) and the US dollar (USD) for May, June, and July 2016 was taken as 2.94 USD/TRY [28].

RESULTS

The results of production costs and profitability analysis were shown by artificial swarm and package bee applications in this section. According to both applications, production costs were given in *Table 2* and it is seen that the production costs vary according to the applications.

Variable and fixed costs associated with honey production per hive are given in *Table 2* by the artificial swarm and package bee applications and it is seen that these costs do not differ much from the applications. While the total variable costs per hive in artificial swarm application was US$94.25, this cost was US$94.91 in the package bee application. In variable costs, hired labor and colony replacement costs were identified as significant cost items.

It was determined that the colony replacement cost was slightly lower in the package bee application than the artificial swarm application. The colony replacement cost per hive was US$39.52 for package beekeeping, while it was US$42.52 in artificial swarm application. In the package bee application, the price level equalizing the net income

Table 2. *Production costs by applications (US$ per hive)*

Items	Artificial Swarm	Package Bees
Feed costs (sugar)	10.88	13.06
Medication (Parasite and disease control)	1.02	0.68
Wax foundation	2.81	2.81
Transportation of hives	6.29	6.29
Hired labor	21.41	21.41
Location rental fees	0.68	0.68
Colony replacement costs	42.52	39.52
Packaging of honey (jar)	5.69	7.49
Repairs and maintenance	0.28	0.28
Interest on variable costs (7%) (2)	2.67	2.69
Total Variable Costs (1+2)=3	**94.25**	**94.91**
The interest on the hive investment** (8%)	1.48	1.48
The interest on the machinery and equipment investment ** (8%)	0.50	0.50
Depreciation for hives	4.46	4.46
Depreciation for machinery and equipment	1.41	1.41
Administrative costs (3%)	2.83	2.85
Total Fixed Costs (4)	**10.68**	**10.70**
Total Production Costs (3+4)	**104.93**	**105.61**

** The average exchange rates between Turkish Lira (TRY) and the US dollar (USD) for May, June, and July 2016 is $1 = 2.94 TL [28]*
*** Represents potential interest income if funds were placed elsewhere*

Table 3. *Honey cost, absolute and relative profit in artificial swarms and package bee applications*

Items	Artificial Swarm	Package Bees
Production cost per hive (US$)	104.93	105.61
Honey yield per beehive (kg)	14.20	18.70
Honey cost per kg (US$)	7.39	5.65
Average price paid to beekeepers for honey (US$ per kg)	5.10	5.10
Absolute profit (US$ per kg)	-2.29	-0.54
Relative profit	0.69	0.90

Table 4. *Gross margin and net return in artificial swarms and package bee applications (US$ per hive)*

Gross Revenue (1)	Artificial Swarm	Package Bees
	82.65	105.61
Honey	72.45	95.41
Beeswax	10.20	10.20
Total Variable costs (2)	94.25	94.91
Gross Margin (1-2) (3)	-11.60	10.70
Total Fixed Costs (4)	10.68	10.70
Net return (3-4)	-22.28	-

Table 5. *Break-even price above total expenses and net returns for purchase price combinations of package bees, per hive*

Purchase Price of Package Bees (US$ per hive)	Net Returns (US$ per hive)	Extra Income Earned Due to Absence of Winter Losses (US$ per hive)	Total Net Returns (US$ per hive)
17.01	23.86	31.63	55.50
25.51	14.85	31.63	46.48
34.01	5.83	31.63	37.47
39.52 (Break-even price)	**0.00**	**31.63**	**31.63**
42.52	-3.18	31.63	28.45
51.02	-12.19	31.63	19.44
59.52	-21.21	31.63	10.43
61.22	-23.01	31.63	8.62
68.03	-30.22	31.63	1.41

to zero at the break-even point was taken as the basis as the colony replacement cost. When considering the fixed costs, the total production cost per hive was US$104.93 in artificial swarm application and US$105.61 in package bee application.

The highest honey yield per colony/beehive was obtained from the package bee application. In the artificial swarm and package bee applications, the average honey yields per colony were 14.20 kg and 18.70 kg respectively *(Table 3)*.

While the honey cost per kg in package bee application was US$5.65, this value was calculated as US$7.39 in the artificial swarm application *(Table 3)*. The relative profit per hive was observed that package bee application was more profitable than artificial swarm application.

Although the relative profit value was less than 1 in both applications, this value was found to be closer to 1 (0.90) in the package bee application. It is important to note that the relative profit obtained for the package bee application

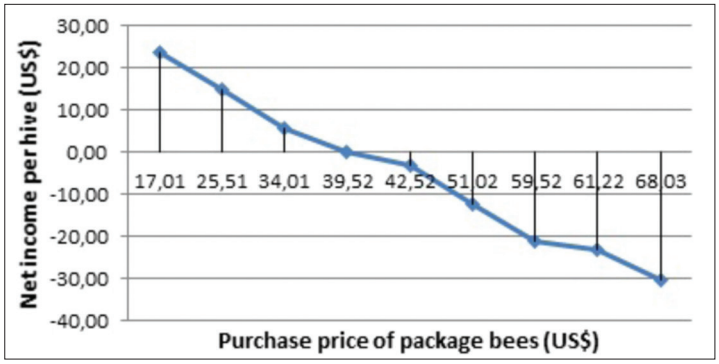

Fig 2. Breakdown of net returns for purchase price combinations of package bees by the break-even point

is calculated according to the price at the breakeven point. It is predicted that the relative profit will be higher in package bee prices below the break-even point.

While the gross margin per hive in package bee application was US$10.70, this value was calculated as -US$11.60 in the artificial swarm application *(Table 4)*. When considering only variable costs, this result shows that package bee application is a profitable activity regarding beekeeping enterprises.

In order to determine which price levels will be profitable for the beekeeping enterprises, the net return to be obtained by the beekeeping enterprises was calculated for purchase price combinations of package bees. As mentioned above, the break-even price which can be accepted as the point of transition to the profitability of the enterprises was determined as US$39.52. According to this result, the purchase price of package bees for beekeeping enterprises in Edirne Province should be below US$39.52 *(Table 5; Fig. 2)*. In other words, package beekeeping for Edirne province is profitable for enterprises at prices below US$39.52. There was a cost saving due to absence of winter losses in case of packet beekeeping in Edirne. This value was US$31.63.

DISCUSSION

Package beekeeping becomes profitable for the beekeeping enterprises at prices below US$39.52 for Edirne province. It is not yet known whether the beekeeping enterprises can supply package bees below this price level because the package beekeeping sector shows new developments in Turkey. A private company in Turkey started sales of frameless package bees with queen in 2019. Price of 1.5 kg bees and young queen bee is totally 370 TRY (about US$64) in the spring of 2019 for this private company.

However, in the case of high package bee prices in the domestic market, "importation of package bees may be an option for beekeeping enterprises. According to a study carried out in Saudi Arabia by Al-Ghamdi and Nuru [29], the price of a local *Apis mellifera yemenitica* colony is relatively high at USD 100-120 per colony in Saudi Arabia. The authors point out that the country annually imported around 100.000 *Apis mellifera carnica* and *Apis mellifera ligustica* bee colonies from Egypt and Australia. In this study, the price was shown to be one of the reasons for importation of package bees. The authors emphasized that the average price of imported package bees was US$30-40 per colony [29].

Other countries in the world meet the need for package bees by import. In Canada, almost 100% of bee packages are imported. New Zealand accounted for about 100% of bee packages imported into Canada in 2016 [30].

Live bee exports from New Zealand were 15.139 one kg packages in 2016-17. A package of bees exported from New Zealand generally consists of 1 kg of bees housed within a ventilated cardboard tube or a cardboard and wire screen box about the size of a shoe box. The package may hold a supply of sugar syrup and queen bee in a cage. All packages and the majority of the queen bees go to Canada. The exporting season is from late January to May. The price for bulk bees, which was the price paid to the beekeepers for export in New Zealand, ranged from $31 (US$21.46) up to $35 (US$24.23) in 2016-17 [31].

On the other hand, package bees prices paid to the beekeepers in 2017 are higher in United States. For operations with five or more colonies in United States, the average price paid in 2017 for packages was US$76 per package. The average price paid in 2017 for operations with less than five colonies was US$117 per package [32].

The results of this study showed that package beekeeping was more advantageous for beekeeping enterprises according to the artificial swarm application. The package beekeeping can provide savings for beekeepers whose bees overwinter in cold climates. In this study, it was determined that if the bees were not wintered, the beekeeping enterprises could save US$31.63 per hive.

In line with the break-even point analysis, the price level in which the package beekeeping will be profitable was determined as US$39.52 per hive for beekeeping enterprises in Edirne. In other words, package beekeeping will be an activity that provides profit for the enterprises in the region at every price that occurs below this price level.

Many beekeepers in Turkey do not have enough information about the package beekeeping. According to this study's preliminary results, it is expected that the dissemination of package beekeeping will have a hugely positive impact on beekeeping enterprises in Turkey. However, further studies are needed to determine the economic impact of package beekeeping on beekeeping enterprises. Preliminary findings strengthen the perception that the effectiveness of beekeeping enterprises can be increased with the adoption of this system in Turkey.

The Ministry of Agriculture and Forestry only provides hive and queen bee supports. There is no support for package beekeeping. Package beekeeping should be included to the scope of beekeeping supports by The Ministry of Agriculture and Forestry for dissemination of package beekeeping in Turkey.

It has been seen that package beekeeping system has been applied in the world for many years. It is also known that packaged honey bees are exported to different countries. As a result, with the expansion of package bee production in Turkey, it is expected that the marketing initiatives for exportation of package bees will increase.

Acknowledgements

Funding for this research was provided by Ministry of Food, Agriculture and Livestock, General Directorate of Agricultural Research and Policies, Turkey under grant number TAGEM/HAYSÜD 14/06/01/10. The authors would like to thank the Ministry of Agriculture and Forestry, General Directorate of Agricultural Research and Policies, Turkey for its financial support.

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Kafkas Univ Vet Fak Derg
25 (5): 659-664, 2019
DOI: 10.9775/kvfd.2018.21545

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

Research Article

SNPs Detected in the *SIRT1* and *H-FABP* Genes and Their Association with Growth Traits in Yak

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Article Code: KVFD-2018-21545 Received: 12.12.2018 Accepted: 10.04.2019 Published Online: 10.04.2019

How to Cite This Article

Gui L, Sun Y, Han Y: SNPs detected in the *SIRT1* and *H-FABP* genes and their association with growth traits in Yak. *Kafkas Univ Vet Fak Derg,* 25 (5): 659-664, 2019. DOI: 10.9775/kvfd.2018.21545

Abstract

The aims of this study were to investigate whether the Sirtuin 1 *(SIRT1)* and Hear fatty acid binding protein *(H-FABP)* genes could be used as candidate genes in the breeding of yak. In 409 indigenous Chinese yaks, two single nucleotide polymorphisms (SNPs) were identified through DNA sequencing technology, including a SNP within the 5'UTR of *SIRT1* (g.1906A>G), and a SNP in the exon 1 of *H-FABP* (g.6643C>T). The chi-square test suggested that all the variations were in Hardy-Weinberg equilibrium (P>0.05). An association analysis suggested that g.1906A>G of *SIRT1* gene and g.6643C>T of *H-FABP* gene exhibited significant correlation with body weight and body length (P<0.01). These results indicated that these SNPs could be used as meritorious and available genetic markers in yak growth traits breeding.

Keywords: *SIRT1 gene, H-FABP gene, Growth traits, Yak, Single nucleotide polymorphism*

Yak Sığırında SIRT1 ve H-FABP Genlerinde Belirlenen Tek Nükleotid Polimorfizmleri ve Büyüme Özellikleri İle İlişkisi

Öz

Bu çalışmanın amacı Yak sığırı yetiştiriciliğinde Sirtuin 1 *(SIRT1)* ve Kalp tipi yağ asidi bağlayıcı protein *(H-FABP)* genlerinin kullanılabilirliğinin araştırılmasıdır. Yerel 409 Çin yak sığırında *SIRT1* geninin (g.1906A>G) 5'UTR'sinde ve *H-FABP* geninin (g.6643C>T) ekzon 1'inde olmak üzere iki adet tek nükleotid polimorfizmi (SNP) DNA sekanslama teknolojisi ile tespit edildi. Ki kare testi tüm varyasyonların Hardy-Weinberg denklemi içerisinde olduğunu gösterdi (P>0.05). İlişki analizi, *SIRT1* geninin g.1906A>G ve *H-FABP* geninin g.6643C>T'sinin vücut ağırlığı ve uzunluğu ile anlamlı derecede ilişkili olduğunu gösterdi (P<0.01). Bu sonuçlar, SNP'lerin Yak sığırı büyüme özelliklerine bağlı yetiştiricilikte kullanılabilecek önemli genetik belirteçler olabileceğini göstermiştir.

Anahtar sözcükler: *SIRT1 geni, H-FABP geni, Büyüme özellikleri, Yak, Tek nükleotid polimorfizmi*

INTRODUCTION

Yak *(Bos grunniens)* was distributed mainly in the Qinghai-Tibetan Plateau at altitudes from 3000 m to 5000 m above sea level, which could survive in conditions of extreme harshness with extreme cold, poor oxygen concentrations, and low air pressure [1,2]. At present, the total population of yak in China were estimated to be 15 million and accounted for over 90% of those distributed all over the world [3]. This species provided hides, meat, and milk for local Tibetans needs and played a crucial role in contribution to the animal husbandry economy in Qinghai-Tibetan Plateau [4].

Mammalian Sirtuin 1 *(SIRT1)* was localized in the nucleus, wherein it influenced the activity of transcription factors via deacetylate histones [5]. In vivo and vitro, *SIRT1* regulated body growth through modulated insulin resistance and body glucose equilibrium [6,7]. In response to fasting, the secretion of insulin in *SIRT1*$^{-/-}$ mice was significant restrained [8], when compared with wild-type littermates in pancreatic β-cells. In turn, β-cells-specific *SIRT1*-overexpression transgenic

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mice influenced adenosine triphosphate (ATP) production by repressing uncoupling protein 2 *(UCP2)*, consequently contributing to enhanced glucose-stimulated insulin secretion [9]. *SIRT1* deacetylated peroxisome proliferator-activated receptor gamma coactivator 1-alpha *(PGC-1α)* at multiple lysine sites, resulting in alteration of genetic programs for gluconeogenesis and glycolysis in the liver [10]. In addition, *SIRT2* deacetylated forkhead box O 1 *(FoxO1)*, in parallel with influences the activation of NeuroD and MafA, thereby inhibiting the secretion of insulin in β-cells [11].

Hear type fatty acid binding protein *(H-FABP)* was expressed predominantly in hear and skeletal muscle [12], wherein played a crucial role in signal transduction pathways such as the uptake or utilization of long chain fatty acids [13]. The *H-FABP*-nullt mice exhibited improved insulin sensitivity, which was perhaps related to the increased reliance on glucose [14]. The expressions of *UCP2*, *UCP3*, *ACOX1*, and *PGC-1α* involving in lipid and glycolysis metabolism were modified by the knockdown of *H-FABP* gene in brown adipocytes [15]. As a transcriptional factor, *KLF15* gene regulated diverse arrays of biological processes including cell proliferation, differentiation and apoptosis [16]. Previous studies demonstrated that the *KLF15* gene might modify the core promoter of *H-FABP* gene, thereby influencing the growth-related traits in mammal [17].

Based on the functional role in metabolism, polymorphism of *SIRT1* and *H-FABP* genes had been previously demonstrated in pig [18], cattle [19], and human [20]. However, there were no reports on associations between the variations of these two genes and growth traits in yak. The main purpose of this study was to evaluate the genetic association between polymorphism of the *SIRT1* and *H-FABP* genes and growth traits in Chinses domestic yak.

MATERIAL and METHODS

Experimental Animals

In total, 409 female yak aged from 12 to 24 months were randomly collected, which belonged to five farm reared in Qilian county, Qinghai Province of China. They were reared under the same environmental dry-lot nutrition standard conditions. Blood samples were collected from the jugular vein, and stored at -20°C. Genomic DNA was extracted using a DNA extraction Kit (OMGAM Bio-Tek, Doraville, USA) following instructions provided in the attached protocol. The DNA concentration was estimated spectrophotometrically, and then the DNA was diluted to 50 ng/uL. Meanwhile, the phenotypes traits including body weight, body length, withers height and chest circumference were measured according to Gilbert's method [21]. For the accuracy of the results, all individuals were measured once by the same person.

SNP Screening and Genotyping

Available sequence information from yak *SIRT1* gene (Genbank accession no NW_005395486.1) and *H-FABP* (Genbank accession no NW_005395183.1) were used to design PCR primers *(Table 1)*. 5 primer pairs were synthesized by Sangon Biotech (Shanghai, Chian) Co., Ltd. The PCR was carried out in 30 uL mixture containing 50 ng DNA, 10 pM of each primer, 0.2 mM dNTP, 2.5 mM $MgCl_2$, and 0.5 U Taq DNA polymerase. The cycling protocol was as follows: initial denaturation at 94°C for 5 min, with 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at optimum temperature, and elongation at 72°C for 30 s. The final extension was performed at 72°C for 10 min. All PCR products were sequenced in forward direction by using the ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA), and the results were analyzed by DNAMAN software version 5.2.2 (Madison, WI, USA). In this study, all 409 samples were genotyped by directly sequencing method of PCR products respectively.

Statistical Analysis

Genotyping and allele frequencies, Hardy-Weinberg equilibrium (HWE) and polymorphism information content (PIC) were calculated by online website *(http://www.msrcall.com/Gdicall.aspx)*. The effect of genotypes of SNPs on the

Table 1. *Primers used for PCR amplification*

Primer Name	Primer Sequence (5' to 3')	Annealing Temperature	Product Length	Amplified Region
SIRT1-L1	CCTGATTTCATTGGGATA	62.5°C	777 bp	5'UTR
	AAGGCTGAGCAAATAACC			
SIRT1-L2	CTTGGACTTGGCATTCTC	60.0°C	351 bp	Eexon 4
	TGGGCTCTTTACCACTCT			
SIRT1-L3	TTTTGGCTTACAGGAACT	58.3°C	749 bp	3'UTR
	AGGCGTTTACTAATCTGC			
H-FABP-L1	CTATGTAACGTCTTTGAAGG	61.0°C	509 bp	Eexon 1
	ACAGGCAACAGGTAGATGCT			
H-FABP-L2	GGCTGGCTGAGCTCTGGCTC	60.5°C	548 bp	Eexon 2
	AGTGAGGCTTTGTGCTCTGC			

growth traits was analyzed by SPSS software (Version 18.0). The model applied was: $Y_{ijk} = \mu + G_i + A_j + F_k + E_{ijk}$, where Y_{ijk} is the trait value for each individual, μ is the overall population mean, G_i is the fixed effect associated with the i^{th} genotype, A_j is the fixed effect of the i^{th} age, F_k is the fixed effect of the i^{th} farm and E_i is the random error. In this model, P-values less than 0.05 were considered to be statistically significant.

RESULTS

SNP Detection and Genetic Diversity Analyses

As is presented in *Fig. 1*, two variations were identified through DNA sequencing analysis, including one SNP within 5'UTR of *SIRT1* gene (g.1906A>G), and one SNP located in exon 1 of *H-FABP* gene (g.6643C>T). The g.1906A>G locus had 3 genotypes (AA, AG and GG, respectively). The g.6643C>T locus had 2 genotypes, the TT genotype was not observed in the sampled animals.

Table 2 illustrates the genotypic and allelic frequencies at locus of *SIRT1* and *H-FABP* genes. The "A" allele of g.1906A>G (*SIRT1*) and the "C" allele of g.6643C>T (H-FABP) were found to be predominant (72.98% and 90.10%, respectively). In addition, the g.1906A>G had moderate polymorphic status (0.25 <PIC<0.05).

Association Analysis

In the present study, one SNP (g.1906A>G) in the 5'UTR of *SIRT1* gene was identified. Statistical results showed that the animals with genotype AA exhibited significantly higher body weight and body length compared to genotype GG (P=0.000, P=0.004, respectively).

For the *H-FABP* gene, statistical analyses indicated animals with genotype CC of g.6643C>T had significantly higher body weight and body length than those with genotype CT (P=0.000 and P=0.031, respectively), demonstrating that allele "C" might be associated with an increase in growth traits in yak *(Table 3)*.

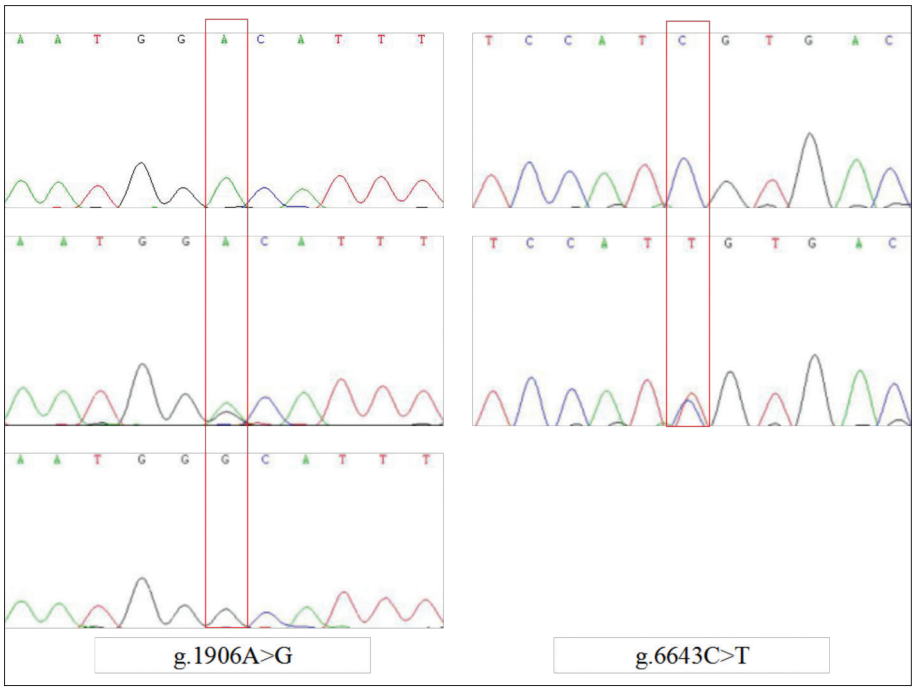

Fig 1. The sequencing map of the SNPs

Table 2. *Distribution of genotype and allele frequencies in yak*

Site	Genotype (N)	Genotypic Frequency (%)	Alleles	Allele Frequency (%)	χ^2 (HWE)	PIC
g.1906A>G (*SIRT1*)	AA (225)	55.01	G	72.98	P>0.05	0.32
	AG (147)	35.94	T	27.02		
	GG (37)	9.05				
g.6643C>T (*H-FABP*)	CC (328)	80.20	C	90.10	*P*>0.05	0.16
	CT (81)	19.80	T	9.90		
	TT (0)	0.00				

χ^2 (HWE): Hardy-Weinberg equilibrium χ^2 value, Hard-Weinberg equilibrium (P>0.05), Hardy-Weinberg disequilibrium (P<0.05)

Table 3. *Association of growth traits with marker genotypes within SIRT1 and H-FABP genes in yak*

Site	Traits	Genotype (Mean±SE)			P Value
		AA	AG	GG	
g.1906A>G (*SIRT1*)	BW (kg)	175.09±0.87^{A}	165.58±1.10^{B}	156.90±2.15^{C}	0.000
	BH (cm)	106.98±0.63	104.64±0.78	105.84±1.55	0.313
	BL (cm)	120.89±0.37^{A}	116.89±0.46	113.27±0.91^{B}	0.004
	CC (cm)	144.91±0.39	142.48±0.49	140.16±0.94	0.147
g.6643C>T (*H-FABP*)		CC	CT		
	BW (kg)	172.41±0.75^{A}	160.38±1.24^{B}		0.000
	BH (cm)	107.62±0.28	106.30±0.56		0.464
	BL (cm)	119.99±0.30^{a}	113.79±0.63^{b}		0.031
	CC (cm)	143.87±0.33	142.52±0.67		0.232

^{A,B,C} *Means with different superscripts are significantly different (P<0.01)*

DISCUSSION

Through deacetylating specific transcription factors (i.e., *PPARγ* and *FOXO1)* [22], the *SIRT1* gene modulated mitochondrial capacity [23], insulin secretion [9] and plasma glucose levels [24]. Previously, a polymorphism (g.-274A>G) within the promoter region of *SIRT1* gene in a Nanyang cattle breed was significantly correlated with body height and body weight [25]. Three SNPs (g.25764G>A, g.25846A>G and g.25868T>C) of *SIRT1* gene were identified in Qinchuan cattle, and were observed to be associated with body length and withers height [26]. Additionally, the novel 12-bp indel of *SIRT1* gene were associated with body weight, chest circumference and height at hip cross Chinese beef cattle [27]. In the present study, the statistical results showed that the individuals with genotype AA of g.4845C>T of *SIRT1* gene were significantly associated with body weight and body length than the other genotype. Those results were consistent with the previous findings of other species [18-20].

The g.1906A>G was located in region of 5'UTR of *SIRT1* gene, without changing the structure of amino acid. Recently, there were some reports about the effects of variants located in region of 5'UTR on the gene expression pattern [28]. A SNP within 5'UTR region of zinc finger, BED-type containing 6 *(ZBED6)* gene resulted in transcription factor change, and then altered the transcription activity and mRNA expression in beef cattle [29]. A 5'UTR-region SNP of growth hormone-releasing hormone receptor *(GHRHR)* gene locus had been found to be associated with body weight and average daily gain in Chinese cattle [30]. Thus, it was an interesting work to find out the mechanism for the association between these 5'UTR-region SNPs and the growth traits in yak.

The *H-FABP* gene contained four exons, and was expressed predominantly in skeletal muscle and subcutaneous fat [12]. Because of its important role in metabolism, association between *H-FABP* polymorphisms and economic characters in livestock was extensively studied. In Berkshire pig breed, one SNP *(H-FABP/Hinf*1) was found to be associated with the live weight and fatty acid composition [31]. Similarly, this SNP had a significant effect on moisture, tenderness and flavor score of Korean native pig [32]. For the g.6643C>T of *H-FABP* locus, only 2 genotypes were detected. The result was due to the lack of genotype in the population or the small size of experimental population. The genotypic frequencies of g.6643C>T of *H-FABP* gene conformed to Hardy-Weinberg equilibrium might be a result of random mating in yak [33]. Additionally, our experimental populations belonged to intermediate genetic diversity. This reflected that this genetic marker could provide more reasonable and effective genetic information [34].

Sequence alignment showed that the g.6643C>T of *H-FABP* gene was synonymous (Thr→Thr), and thus did not change the structure of the encoded protein. It had been demonstrated previously that synonymous SNPs could modify the stability of mRNA [35,36], thereby influencing the phenotypic traits in mammal. A synonymous mutation (g.25557A>G) in the silent information regulator 6 (*SIRT6*) gene was found to be associated with intramuscular fat in Qinchuan cattle [37]. Additionally, synonymous mutation (c.72G>A) of the *UCP2* gene was related to growth performance, carcass characteristics and meat quality in rabbits [38].

Results of the present study suggested that g.1906A>G of *SIRT1* and g.6643C>T of *H-FABP* were significantly correlated with growth traits in yak. It inferred that these SNPs could modify stability/expression of mRNA, therefore influencing the growth-related phenotypes in yak.

In summary, genotypes of *SIRT1* and *H-FABP* were confirmed to be significantly associated with body weight and body length in yak. Our investigation provided evidence that both *SIRT1* and *H-FABP* genes could be used as molecular markers for better growth traits of yak.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

The research was supported by Science and Technology Department Project of Qinghai province (2019-SF-A3, 2018-ZJ-922Q, 2017-NK-108), the Open Project of Qinghai University (2018-ZZ-08, MKY-2018-01), Weng Hongwu Original Research Fund of Peking University (WHW201509).

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Kafkas Univ Vet Fak Derg
25 (5): 665-672, 2019
DOI: 10.9775/kvfd.2018.21558

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

Research Article

The Effect of Single Amino Acid Substitution in *SecA2* on Protein Translocation and Pathogenicity of *Listeria monocytogenes*

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Article ID: KVFD-2018-21558 Received: 15.12.2018 Accepted: 02.05.2019 Published Online: 02.05.2019

How to Cite This Article

Fang C, Chen X, Liang X, Fang X, Gao K, Chen J, Gu Y, Yang Y: The effect of single amino acid substitution in *SecA2* on protein translocation and pathogenicity of *Listeria monocytogenes*. *Kafkas Univ Vet Fak Derg,* 25 (5): 665-672, 2019. DOI: 10.9775/kvfd.2018.21558

Abstract

Listeria monocytogenes is an important zoonotic pathogen that cause severe listeriosis with high mortality in immunosuppressive humans. Infection and pathogenicity of *L. monocytogenes* was mediated by several surface proteins that translocated by secretion systems. Our previous genomic study showed the secretion systems of the virulent and low-virulent strains were different in *secA2* and two hollin genes. To confirm whether the pathogenicity of the two strains was determined by the difference observed in secretion system. We deleted *secA2* and the two hollin genes to compare the pathogenic phenotypes. Our data showed that *secA2* but not the two hollin genes affected the pathogenic phenotypes. To further confirm whether the single base mutant in *secA2* affected the protein pathogenicity and translocation ability of *SecA2*, we complemented the *secA2* deletion mutant strain with $secA2_{Lm850658}$ and $secA2_{M7}$, which encode *SecA2* with Asn567 and Lys567, respectively. Our data showed that *secA2* mutant complement with $secA2_{Lm850658}$ instead of $secA2_{M7}$ significantly improved the adhesion and invasion ability to epithelial cells Caco-2 and bacterial load in mice liver and spleen at both 24 and 48 h post infection. Cell surface protein analysis indicated that only *SecA2* with Asn567 could restore the protein translocation ability. Taken together, our study demonstrated that single amino acid mutant in *SecA2* affected the protein translocation and pathogenicity of *L. monocytogenes* for the first time.

Keywords: *Listeria monocytogenes, Secretion system, SecA2, Pathogenicity*

SecA2'de Tek Amino Asit Yerdeğiştirmesinin *Listeria monocytogenes*'te Protein Translokasyonu ve Patojenite Üzerine Etkisi

Öz

Listeria monocytogenes önemli bir zoonotik patojen olup immunsupresyone insanlarda yüksek mortaliteye sahip şiddetli listeriozise neden olur. *L. monocytogenes* enfeksiyonu ve patojenitesi sekresyon sistemi tarafından salınan çeşitli yüzey proteinleri tarafından oluşturulur. Yapılan önceki genomik çalışma virülent ve düşük-virülent suşların sekresyon sistemlerinin *secA2* ve iki hollin genlerinde farklı olduğunu gösterdi. İki suşun patojenitelerindeki farkın sekresyon sistemlerinde gözlenen farktan kaynaklanıp kaynaklanmadığı araştırıldı. Çalışmada, patojenik fenotipleri karşılaştırmak amacıyla *secA2* ve iki hollin geni çıkarıldı. Elde edilen sonuçlar iki hollin geninin değil de *secA2* geninin patojenik fenotipi etkilediğini gösterdi. *secA2* geninde tek baz mutantının protein patojenitesini ve *SecA2*'nin translokasyon kabiliyetini etkileyip etkilemediğini test etmek amacıyla *secA2* gen delesyon mutant suşunu sırasıyla Asn567 ve Lys567'i kodlayan $secA2_{Lm850658}$ ve $secA2_{M7}$ ile tamamladık. Elde edilen veriler, $secA2_{Lm850658}$ ile tamamlanan *secA2* mutantının anlamlı derecede Caco-2 epitel hücrelerine adezyon ve invazyon kabiliyeti ile fare karaciğer ve dalak dokularında enfeksiyon sonrası 24 ve 48. saatlerde bakteriyel yığılmayı artırdığını gösterdi. Hücre yüzey protein analizi, sadece Asn567'li *SecA2*'nin protein translokasyon kabiliyetini koruyabildiğini gösterdi. Tüm sonuçlar değerlendirildiğinde, *L. monocytogenes SecA2*'de tek amino asit mutasyonunun protein translokasyonu ve patojeniteyi etkilediği ilk kez gösterilmiştir.

Anahtar sözcükler: *Listeria monocytogenes, Sekresyon sistemi, SecA2, Patojenite*

INTRODUCTION

Listeria monocytogenes is an important foodborne pathogens which can cause severe foodborne disease called listeriosis with an overall 20-30% mortality rate in immunosuppressive patients [1,2]. This bacterium can survive in various environments including water, soil, food, food processing plants, gastrointestinal tracts and even the

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cytosol of eukaryotic cells [3]. To successfully switch from a saprophytic to a pathogenic bacteria, *L. monocytogenes* secreted a variety of proteins involved in colonization and infection [4-6]. These surface proteins were translocated by the six well known secretion systems, including the Sec system, the Tat pathway (Twin-arginine translocation), the FEA (Flagella Export Apparatus), the FPE (Fimbrilin-Protein Exporter), the Hollins and the Wss [WXG100 (proteins with WXG motif of ~100 residues) secretion system]. Among these secretion systems, the Sec system considered to be the main force for protein secretion in *L. monocytogenes* [7]. Sec system was composed of essential SecYEG translocon, peripheral SecA ATPase and other accessory components [8,9]. Among them, SecYEG forms a protein channel through the cytoplasmic membranes, and SecA hydrolyzes ATP to provide energy for protein translocation [10]. SecA2 as one of the accessory components of the Sec system also made a contribution to the translation of several surface proteins [11-13].

SecA2 was firstly identified and demonstrated to contribute to bacterial virulence by Laurel L. Lenz [14]. Unlike SecA, which was essential for *L. monocytogenes* survival, SecA2 was not required for the bacterial viability [10], but SecA2 played important roles in pathogenesis. Soon after the discovery, seventeen SecA2-dependent secreted and surface proteins, which including two autolysins (Iap and NamA) that contributed to cell wall hydrolysis and bacterial virulence, were identified by proteomics approach [11]. More recently, Sandra et al.[12] analyzed the SecA2-dependent exo-proteome and newly found 13 proteins that were associated with cell wall metabolism, bacterial adhesion and biofilm formation. And deleted SecA2 also induces biofilm formation and impacts biofilm architecture, and promotes bacterial aggregation [13]. Moreover, SecA2 primed protective anti-*L. monocytogenes* cell immune response which might be resulted from one or several of the SecA2 secreted proteins released inside the cytosol of infected cells [15]. Taken together, SecA2 played important roles in proteins translocation and pathogenicity of *L. monocytogenes*, but the mechanisms of SecA2 in protein translocation remain unclear. Our previous study showed the secretion systems of the virulent and low-virulent strains were with a little difference [16]. We hypothesized that the difference in secretion systems between the virulent and low-virulent strains affect the virulence associated proteins translocation and finally determine their pathogenicity. To confirm this hypothesis, we deleted the components with difference between the two strains and then studied the roles. Taken together, we demonstrated the single amino acid residue mutant in SecA2 significantly affect its roles in protein translocation and pathogenicity for the first time.

MATERIAL and METHODS

Bacterial Strains, Plasmids and Culture Conditions

L. monocytogenes virulent strain Lm850658 and low-virulent strain M7 were used as the wild-type strains and cultured in brain heart infusion medium (BHI, Oxoid, UK) at 37°C. *Escherichia coli* DH5α was employed as the host strain for the shuttle plasmid pKSV7 and cultured in Luria-Bertani medium (LB, Oxoid) at 37°C. Stock solutions of ampicillin (50 mg/mL), chloramphenicol (10 mg/mL) and gentamycin (100 mg/mL) (Sangong Biotech, China) were added to media with the required concentrations.

Construction of Deletion and Complement Mutants

A homologous recombination strategy was used for the construction of *secA2* and *LMM7_0111* and *LMM7_1303* deletion in Lm850658 and M7 according to previous studies [16,17]. The homologous fragments were amplified with primer pairs listed in *Table 1* and purified with Gel Purification Kit (GK2042, Generay Biotech. Co. Ltd.). Then fragments were digested with indicated restriction enzymes (Takara, Japan) and ligated to the temperature-sensitive shuttle vector pKSV7 and transformed into DH5α. Positive clones were then confirmed by PCR and sequencing. Plasmids containing inserted fragments were subsequently extracted and electroporated into *L. monocytogenes* competent cells. Transformants were grown at a non-permissive temperature (41°C) on BHI containing chloramphenicol (10 μg/mL) to promote chromosomal integration. The recombinants were passaged in BHI without antibiotic at a permissive temperature (30°C) to enable plasmid excision and curing. The deletion mutants were identified by PCR with primers listed in *Table 1*.

For the complemental strains, *secA2* encoding sequence of Lm850658 and M7 were amplified by PCR and then purified with Gel Purification kit. After restriction digestion with appropriate enzymes, the PCR fragment was cloned into pIMK2 following the P_{Help} promoter. The recombinant plasmids were then electroporated into Lm85065-ΔsecA2 competent cells. The transformants were plated on BHI agar containing kanamycin (50 μg/mL), then positive clones were picked up and identified by PCR and designated as $C\Delta secA2_{Lm850658}$ and $C\Delta secA2_{M7}$, respectively.

Adhesion and Invasion Assay

Adhesion and invasion assays in Caco-2 and HeLa cells were conducted as previous research [18]. Briefly, overnight cultures were harvested by centrifugation (5.000g for 10 min), resuspended in 10 mM PBS (pH 7.4) and adjusted to 0.25 at $OD_{600\ nm}$. The Caco-2 or HeLa cells (about 2×10^5 per well) were seeded in 12-well plates (Corning, USA), incubated at 37°C and 5% CO_2 for 18-24 h and infected with *L. monocytogenes* at MOI of 10:1. For adhesion, cells were washed with PBS and then lysed with deionized water at 1 h post infection (hpi) . For estimation of invasion, cells were washed with PBS at 1 hpi and incubated for an additional h in RMPI 1640 medium containing 10% FBS and 100 μg/mL gentamycin. The cells were lysed at indicated times and 10-fold diluted for plating on BHI agar. The agar plates

Table 1. *Primers used for construction deletion and complement mutants*

Primers	Sequences (5′-3′)
M0111-a	CGGGATCCAAATGAGTTTGAATGTTATTACAGG
M0111-b	TTTCTGTCCTTTGCTAAATTTCATCATACCTCACAATCTTTTTATGAAA
M0111-c	ATGAAATTTAGCAAAGGACAGAAA
M0111-d	GGGGTACCGTAACAAATCTCTACACCGATGGT
M0111-F	GGAATTGCAGTGATAGAGTCAGGT
M0111-R	CATCTACTGCAACATGAAAGCTAAC
M1303-a	CGGGATCCTATGCAAGTGCAAGTGCTGAAAGT
M1303-b	TTCTTTCTTCATAATACATAGTCATTTATTATTCCTCCTAATGCCAAATTA
M1303-c	ATGACTATGTATTATGAAGAAAGAAG
M1303-d	GGGGTACCATAGTAGTAAACAGGCACTTTAAAC
M1303-F	AAAGATTTAGTATTACCAATTTCA
M1303-R	TAGTAGTTGGAGTATCTGCTGTAA
secA2-a	CGCGGATCCTAGTCCCTTTACTTTGAGCGATA
secA2-b	GAAAAAATCAGACGTAGGTTGTATTTATATAACATCCTCCATACCTTCT
secA2-c	AATACAACCTACGTCTGATTTTTT
secA2-d	CGGGGTACCATGTTTGTATTCAATTTATCTTTTAT
secA2-F	ACGAATGGTTCTAAAGACATGA
secA2-R	AGCCGGGATTTCTTACTATTTA
secA2-CF	CATGCCATGGGACAGAATTATGATGATCGCAA
secA2-CR	CGGGATCCTTAACCTTGAATTAGACCATCTGG

were incubated overnight at 37°C for colony counting. Adhesion was expressed as the ratio of recovered colonies to the initial inoculated; while invasion was calculated as the ratio of recovered colonies after gentamycin treatment to initial inoculated.

Protein Samples Preparation and Detection

Five milliliter overnight cultures were incubated in 100 mL fresh BHI medium for 6 h at 37°C and harvested by centrifugation (15.000g, 10 min). Surface proteins were extracted from bacterial pellet with 2% SDS (30 mg wet weight per milliliter of 2% SDS) at 37°C for 1 h. Protein samples were analyzed by 12% SDS-PAGE gel and then probed with indicated antibodies as our previous research [19].

Virulence in Mice Model

The assay was conducted as previous research [16]. Four-weeks age female ICR mice, six per group, were acclimatized for three days in a standard class II laboratory animal facility. Overnight cultures were treated as above with adjusted $OD_{600\ nm}$ at 0.6. Mice were incubated by intraperitoneal injection with 2×10^4 CFU bacteria. At 24 and 48 hpi, mice were euthanized, liver and spleen samples were homogenized, and diluted appropriately for plating counting on BHI agar plates. For the LD_{50} assay, five mice per group were inoculated intraperitoneally with serial ten-fold dilutions of indicated bacteria in PBS. Mice in the control group were received PBS only. The LD_{50} values were calculated by using the trimmed Spearman-Karber method on the mortality data during the 10 days observation period. All animal experiments were approved by the Laboratory Animal Management Committee of Yangtze University (Approval No. 20161212).

Statistical Analysis

All data comparisons were analyzed using the two-tailed homoscedastic Student's T-test. In all cases, differences with $P<0.05$ and $P<0.01$ were considered as statistically significant and marked with * and ** respectively. The GraphPad Prism 5 software was used for producing the graphs.

RESULTS

We previously reported the genomes of *L. monocytogenes* lineage III low virulent strain M7 and virulent strain Lm850658 [16]. To explore the mechanisms of that determine the pathogenicity of *L. monocytogenes*, we compared the pathogenic associated phenotypes of strains M7 and Lm850658. Our data showed that M7 exhibited much smaller mobility than strain Lm850658 *(Fig. 1A)*, and the invasion ability of M7 was significantly lower than that of Lm850658 to both Caco-2 cells and Hela cells *(Fig. 1B, 1C)*. As mobility and infection to host cells were mediated by the bacterial surface proteins, we analyzed the compositions of the secretion systems of strain M7 and Lm850658. Our data showed that the

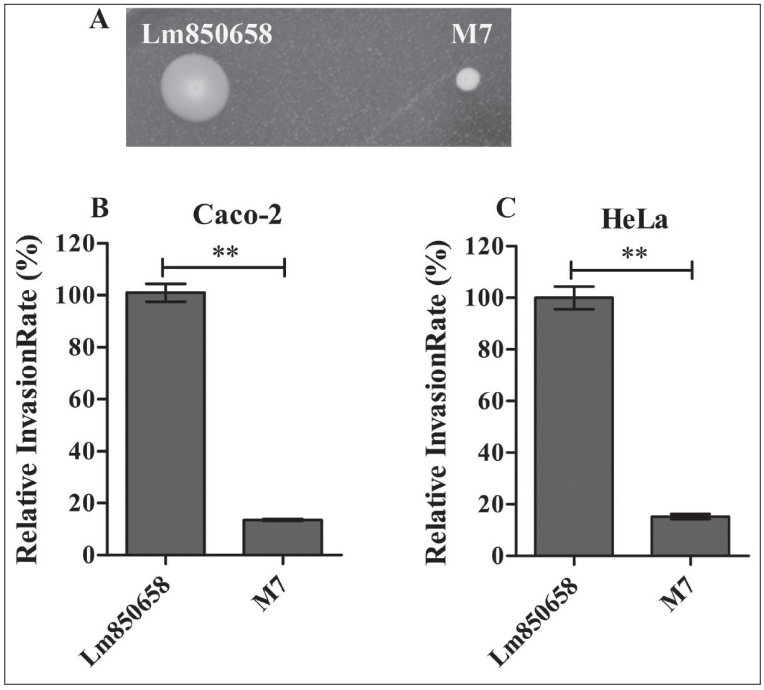


Fig 1. The virulent strain Lm850658 exhibited stronger mobility and invasion ability than that of the low-virulent strain M7. Mobility was tested on the soft agar plate (0.3% agar) under 30°C for 48 h **(A)**, and Invasion ability was conducted on epithelial cell Caco-2 **(B)** and HeLa **(C)**. Data was presented as mean±SD of three independent experiments and ** indicated the statistical significance with P<0.01

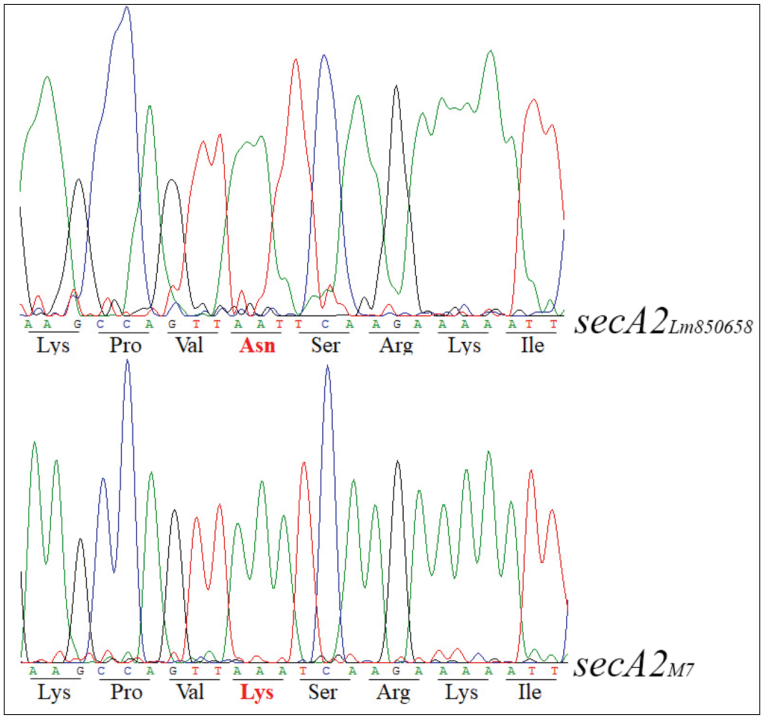


Fig 2. The *secA2*-1701T in Lm850658 was mutant to *secA2*-1701A in M7, which resulted in the Asn567 was mutant to Lys567 of SecA2

encoding genes of the six secretion systems, including the Sec system, the Tat pathway, the FEA, the FPE, the Hollins and the Wss, in strain M7 and Lm850658 were identity except for the *secA2* of Sec system and two hollin genes (*LMM7_0111* and *LMM7_1303*) of Hollin system *(Table 2)*. Sequences analysis showed the *secA2*-1701T in Lm850658 was mutant to *secA2*-1701A in M7 which resulted in SecA2 Asn567 mutant to SecA2 Lys567 *(Fig. 2)*.

To clarify whether the difference in secretion system composition affects the pathogenicity of *L. monocytogenes*, we constructed *secA2* deletion mutant in Lm850658 and *LMM7_0111* and *LMM7_1303* deletion mutant in M7, respectively. We found that deleted *secA2* in Lm850658 significantly defected the adhesion and invasion ability to Caco-2 cell and decreased virulence to mice *(Fig. 3)*. While the two hollin genes deletion made no difference on infection to epithelial cells or virulence to mice *(Fig. 3)*. To confirm the role of the mutant SecA2, the two complemental strains CΔsecA2$_{Lm850658}$ and CΔsecA2$_{M7}$ which encoding SecA2 Asn567 and SecA2 Lys567 respectively. Our data showed that the CΔsecA2$_{Lm850658}$ instead of CΔsecA2$_{M7}$,

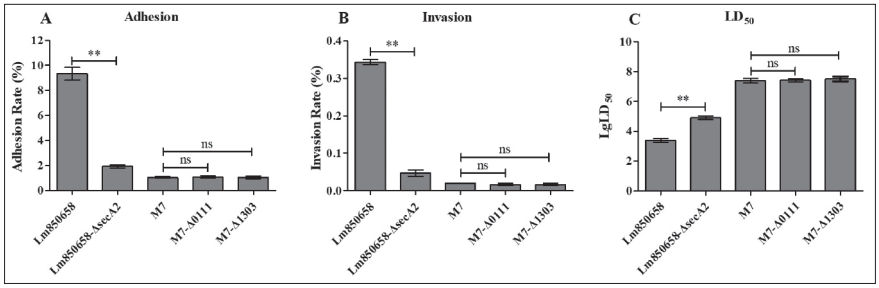
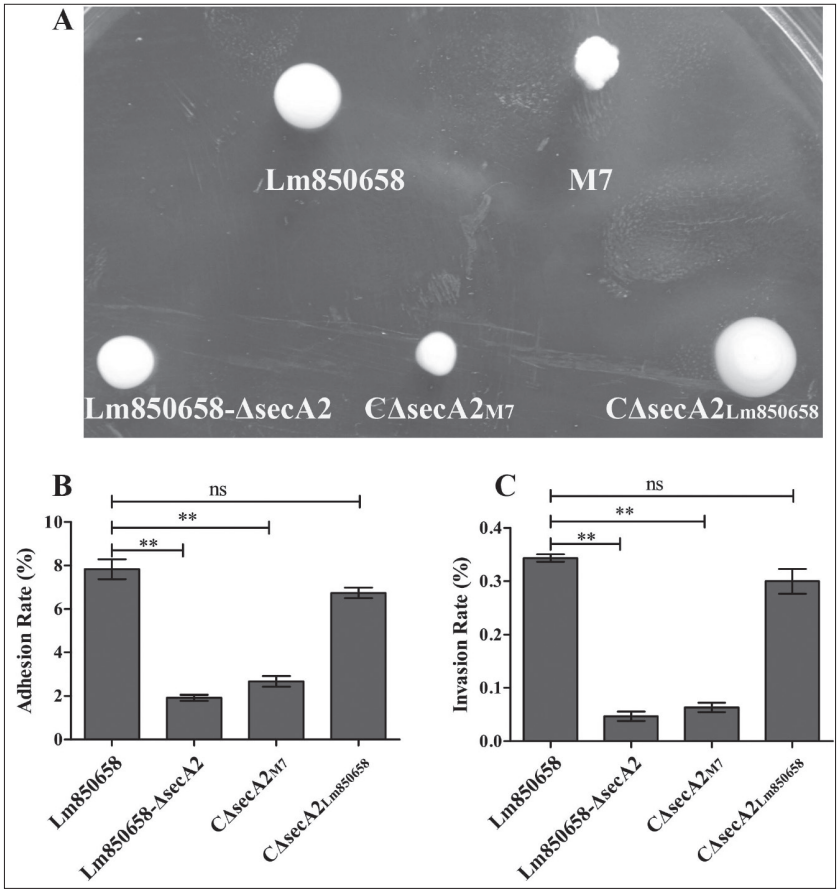


Fig 3. The secA2 deletion instead of the two hollin genes deletion defected the pathogenicity of *L. monocytogenes*. The infection ability **(A** & **B)** and LD50 **(C)** of the wild-type strains and the mutant strains was conducted on Caco-2 cells and ICR mice respectively. Results were presented as mean±SD of three independent experiments and ** indicated the statistical significance with P<0.01

Fig 4. Complement with $secA2_{Lm850658}$ instead of $secA2_{M7}$ restored the mobility and infection ability of *secA2* deletion mutant strain. Mobility was tested on the soft agar plate (0.3% agar) under 30°C for 48 hours **(A)**, adhesion **(B)** and invasion **(C)** ability was conducted on Caco-2 cells. Data was presented as mean±SD of three independent experiments and ** indicated the statistical significance with *P*<0.01



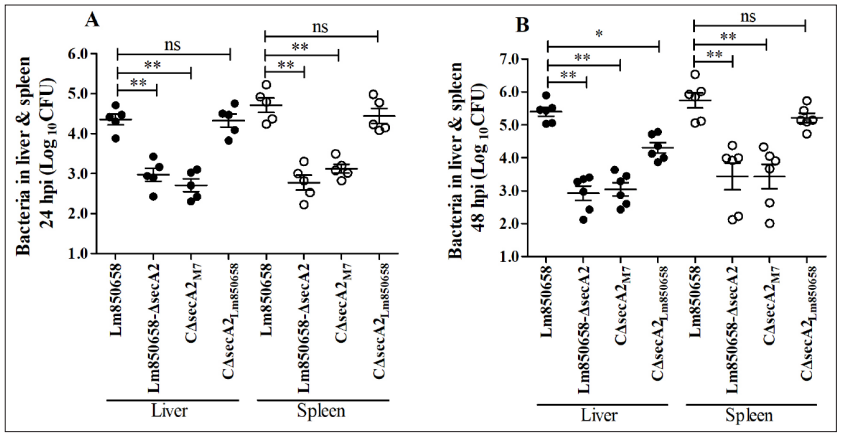


Fig 5. Complement with $secA2_{Lm850658}$ instead of $secA2_{M7}$ significantly increased bacteria load in the liver and spleen of mice at both 24 **(A)** and 48 **(B)** h post infection. Data was presented as mean±SD of five or six mice, * and ** indicated the statistical significance with P<0.05 and P<0.01 respectively

could regain the mobile ability, adhesion and invasion ability and virulence to mice as the wild type Lm850658 *(Fig. 4)*. To further determinate the role of SecA2 Asn567 on the pathogenicity of *L. monocytogenes*, we conducted virulence assay on mice model. Our data showed that the spleen and liver bacteria load of Lm850658-ΔsecA2

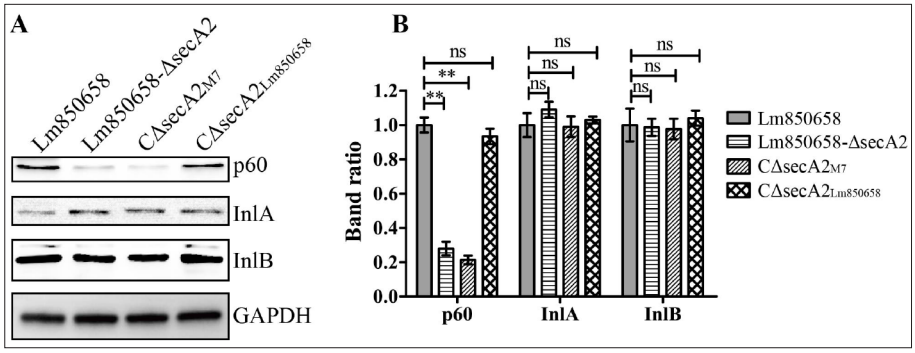


Fig 6. Complement with $secA2_{Lm850658}$ instead of $secA2_{M7}$ significantly increased translocation of p60 but not InlA or InlB. Data was presented as mean±SD of three independent experiments and ** indicated the statistical significance with P<0.01

Table 2. *Secretion systems of Lm850658 and M7*

Systems[a]	Components	EGD-e	Lm850658	M7	Length	Identity[b]
Sec	SecY	Lmo2612	LM850658_2631	LMM7_2724	431	I
	SecE	Lmo0245	LM850658_216	LMM7_0268	59	I
	SecG	Lmo2451	LM850658_2397	LMM7_2493	77	I
	SecDF	Lmo1527	LM850658_1517	LMM7_1613	754	I
	YajC	Lmo1529	LM850658_1519	LMM7_1615	108	I
	YidC	Lmo1379	LM850658_1346	LMM7_1465	275	I
	YidC	Lmo2854	LM850658_2882	LMM7_2975	287	I
	FtsY	Lmo1803	LM850658_1799	LMM7_1895	328	I
	Ffh	Lmo1801	LM850658_1797	LMM7_1893	450	I
	SecA	Lmo2510	LM850658_2456	LMM7_2552	837	I
	SecA2	Lmo0583	LM850658_560	LMM7_0613	776	N567K
	SipX	Lmo1269	LM850658_1229	LMM7_1350	188	I
	SipY	Lmo1270	LM850658_1230	LMM7_1351	189	I
	SipZ	Lmo1271	LM850658_1231	LMM7_1352	180	I
	Lsp	Lmo1844	LM850658_1841	LMM7_1937	154	I
Tat	TatA	Lmo0362				
	TatC	Lmo0361				
FPE	ComGA	Lmo1347	LM850658_1313	LMM7_1432	340	I
	ComGB	Lmo1346	LM850658_1312	LMM7_1431	343	I
	ComGC	Lmo1550	LM850658_1540	LMM7_1636	236	I
FEA	FlhA	Lmo0680	LM850658_662	LMM7_0715	691	I
	FlhB	Lmo0679	LM850658_661	LMM7_0714	348	I
	FliR	Lmo0678	LM850658_660	LMM7_0713	253	I
	FliQ	Lmo0677	LM850658_659	LMM7_0712	90	I
	FliP	Lmo0676	LM850658_658	LMM7_0711	255	I
	FliH	Lmo0715	LM850658_697	LMM7_0750	230	I
	FliI	Lmo0716	LM850658_698	LMM7_0751	433	I
Holins	TcdE	Lmo0128	LM850658_102	LMM7_0155	140	I
	φA118	Lmo2279	LM850658_2548	LMM7_2644	86	I
	Holin			LMM7_0111		
	Holin			LMM7_1303		
Wss	YukAB	Lmo0061	LM850658_56	LMM7_0056	1496	I
	EsaA	Lmo0057	LM850658_52	LMM7_0052	1071	I
	EssA	Lmo0058	LM850658_53	LMM7_0053	171	I
	YukC	Lmo0060	LM850658_55	LMM7_0055	398	I
	YukD	Lmo0059	LM850658_54	LMM7_0054	83	I
	EsaC	Lmo0062				

[a] Tat, Twin-arginine translocation, FPE, Fimbriae Protein Exporter, FEA, Flagella Export Apparatus, Wss, WXG100 secretion system; [b] I, indicated that amino acid sequences between Lm850658 and M7 were identical

infected mice were significantly reduced. Complement strain CΔsecA2$_{Lm850658}$ which expressed SecA2 Asn567 could restore these phenotypes but complement strain CΔsecA2$_{M7}$ which produced SecA2 Lys567 made no difference on the pathogenicity phenotypes *(Fig. 5)*.

Invading into host cells was an important process of *Listeria* infection, which was mediated by the internalin family members and other cell surface proteins, such as InlA, InlB, p60 and Ami [4]. But only p60 and Ami instead of InlA and InlB were reported as the substrates of SecA2 of *L. monocytogenes*. To confirm how the SecA2 mutant affects the pathogenicity of *L. monocytogenes*, we analyzed the content of these surface proteins of wild type strain Lm850658 and indicated mutant strains. Our data showed that surface p60 was significantly reduced in Lm850658-ΔsecA2 and CΔsecA2$_{M7}$ *(Fig 6)*. While the major invasion factor InlA and InlB were exhibited no significant difference in the wild-type, *secA2* mutant and complemental strains *(Fig. 6)*.

DISCUSSION

L. monocytogenes is an important foodborne pathogen because of the severity of its infections in immuno-suppressive humans, like pregnant women, old people and children [20]. *L. monocytogenes* could invade into a variety of host cell types [21], escape from the primary and secondly vacuoles, proliferate in host cytoplasm and recruit actin polymerization to spread to adjacent cells [22]. Each step of the infection process was mediated by specific virulent factors, which were secreted to the bacterial surface by secretion systems [7,23]. It was believed that the Sec secretion systems played important roles in the survival and pathogenicity of *L. monocytogenes* [7]. SecA2 as an accessory component of Sec system was found that had no threat to the bacterial life, but made a contribution to specific proteins translocation and the pathogenicity of *L. monocytogenes* [11,24]. Here we demonstrated that SecA2 Asn567 mediated the surface proteins translocation and pathogenicity of *L. monocytogenes* for the first time.

Comparative genomic analysis showed SecA2 Asn567 were conserved in most of *L. monocytogenes*, and SecA2 Lys567 was only found in strain M7 and the other two representative low-virulent strains HCC23 and L99 [25]. Although previous studies identified the substrates of SecA2 by proteomic methods and demonstrated *secA2* mutant defected the pathogenicity of *L. monoctygoenes* [11,12], little was known about the relationship between structure and function of SecA2 in *L. monocytogenes* or other bacteria. As the essential ATPase SecA and the non-essential SecA2 were conserved in many gram-positive bacteria and they contained similar domains [10], and SecA2 was shared 42% identical and/or 62% similar amino acid residues with the SecA in *L. monocytogenes* [14], SecA2 Asn567 was located in the C terminal domain (CTD). Unlike other domains, the CTD in SecA2 or SecA were various across species [10], so mutation in the CTD might result in different effects on the functions of SecA2.

In this study, we found *secA2* deletion mutant strain complement with *secA2* from the virulent strain Lm850658 and low-virulent strain M7 exhibited the same phenotypes as the original strains respectively *(Fig. 4, Fig. 5)*. These data indicated that the CTD of SecA2 might contribute to the pathogenic phenotypes of *L. monocytogenes*. These phenotypes including mobility in the environments and invading into the host cells *(Fig. 4)* but not the survival ability in macrophage and migration among the cells (data not showed). This phenomenon prompted us to analyze the cell surface proteins of the wild type and the secA2 mutant strains. Our data showed that the p60 (also named as invasion associated protein, Iap) from Lm850658-ΔsecA2 and CΔsecA2$_{M7}$ was significantly less than that of the wild-type strain Lm850658 and CΔsecA2$_{Lm850658}$ *(Fig. 6)*. But the InlA and InlB which were the major factor that contributed to the infection of *L. monocytogenes* into host cells have remained unchanged. This has consisted with previous reported that SecA2 were mediated the translocation the cell wall hydrolases p60 and Ami, and *Listeria* adhesion protein (LAP), an alcohol acetaldehyde dehydrogenase homolog (lmo1634) [11,26].

Taken together, we demonstrated that SecA2 Asn567 determinate the mobility in on agar and pathogenicity of *L. monocytogenes*. Further study might focus on how this amino acid mutant affects the structure and function of SecA2.

Statement of Author Contributions

CF and XYC and conducted experiments, analyzed data and drafted the manuscript. XYL, XWF, KLG, JC and YFG were involved in data collection and analysis. CF and YYY designed the entire experiments, contributed to data analysis and critical reading the manuscript.

Acknowledgments

This study was funded by National Natural Science Foundation of China (grant No. 31802208) and Key Laboratory of Prevention and Control Agents for Animal Bacteriosis (grant No. KLPCAAB-2018-05) to Dr. Chun Fang.

Compliance with Ethical Standards

Disclosure of potential conflicts of interest: All authors declare no conflict of interest.

Research involving Human Participants and/or Animals: This article does not contain any studies with human participants. ICR mice used for virulent assay were approved by the Laboratory Animal Management Committee of Yangtze University (Approval No. 20161212).

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Kafkas Univ Vet Fak Derg
25 (5): 673-682, 2019
DOI: 10.9775/kvfd.2018.21598

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

Research Article

Probiotic Shelf Life, Antioxidant, Sensory, Physical and Chemical Properties of Yogurts Produced with *Lactobacillus acidophilus* and Green Tea Powder [1]

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[1] *A part of this study was presented in "Third International Congress on CoCoa Coffee and Tea 2015, 22-24 June, 2015, Aveiro, Portugal" as a poster presentation*

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Article ID: KVFD-2018-21598 Received: 22.12.2018 Accepted: 23.05.2019 Published Online: 25.05.2019

How to Cite This Article

Çakmakçı S, Öz E, Çakıroğlu K, Polat A, Gülçin İ, Ilgaz Ş, Seyyedcheraghi K, Özhamamcı İ: Probiotic shelf life, antioxidant, sensory, physical and chemical properties of yogurts produced with *Lactobacillus acidophilus* and green tea powder. *Kafkas Univ Vet Fak Derg,* 25 (5): 673-682, 2019. DOI: 10.9775/kvfd.2018.21598

Abstract

The aim of the present study was to determine the effects of the addition of green tea powder (GTP) in the production of yogurt on survival of *Lactobacillus acidophilus* (P) and the yogurt properties. Four yogurt groups (yogurt without P and GTP, Control, C; yogurt with P and without GTP, PC; P + 1% GTP; and P + 2% GTP) were produced. The yogurt samples were stored at 4°C. The addition of GTP into milk did not affect the viability of yogurt bacteria during fermentation. The highest count of *L. acidophilus* was detected in P + 2% GTP. The *L. acidophilus* count was high up to the 7th day (7.54 log cfu/g). Yeast and mold were not counted (<2 log cfu/g) in any yogurt sample during storage. GTP has antioxidant properties that could be attributed to the presence of phenolic and flavonoids compounds. The panelists preferred the PC and P + 1% GTP samples during the storage period. As a result of this research, we can suggest the consumption and production of probiotic yogurt with 1% GTP supplement.

Keywords: *Yogurt, Green tea powder, Lactobacillus acidophilus, Shelf life, Antioxidant activity, Phenolic compounds*

Lactobacillus acidophilus ve Yeşil Çay Pudrası İle Üretilen Yoğurtların Probiyotik Raf Ömrü, Antioksidan, Duyusal, Fiziksel ve Kimyasal Özellikleri

Öz

Bu araştırmanın amacı, yoğurt üretiminde yeşil çay pudrası (GTP) ilavesinin *Lactobacillus acidophilus* (P) canlılığı ve yoğurt özellikleri üzerine etkilerini belirlemekti. Bu amaçla, dört farklı yoğurt çeşidi üretildi [P ve GTP ilavesiz yoğurt (Kontrol, C); sadece P içeren yogurt (PC); P + %1 GTP ilaveli yoğurt ve P + %2 GTP ilaveli yoğurt]. Yoğurt örnekleri 4°C'de muhafaza edildi. Süte GTP ilavesi, fermantasyon sırasında yoğurt bakterilerinin canlılığını etkilemedi. En yüksek *L. acidophilus* sayısı P + %2 GTP ilaveli yoğurtta tespit edildi. *L. acidophilus* sayısı 7. güne kadar yüksekti (7.54 log kob/g). Depolama süresince yoğurt örneklerinin hiçbirinde maya ve küf bulunmadı (<2 log kob/g). GTP, fenolik ve flavonoid bileşiklerinin varlığına bağlanabilecek antioksidan özelliklere sahipti. Panelistler depolama süresince PC ve P + %1 GTP örneklerini daha çok beğendiler. Bu araştırma sonucunda, %1 GTP katkılı probiyotik yoğurt üretimi ve tüketimini önerebiliriz.

Anahtar sözcükler: *Yoğurt, Yeşil çay pudrası, Lactobacillus acidophilus, Raf ömrü, Antioksidan aktivite, Fenolik bileşikler*

INTRODUCTION

Demand for functional foods is growing rapidly due to increased awareness of consumers about the impact of food on health. Yogurt, one of the best known of the foods that contain probiotics, is a popular food [1,2]. Probiotics such as *Lactobacillus acidophilus* are most commonly incorporated into yogurts worldwide. Considering that

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probiotic properties are strain-dependent, possible beneficial effects of consuming yogurt containing *L. acidophilus* are controlling various types of diarrhea and urogenital infections, alleviating lactose intolerance, preventing gastro-intestinal diseases, lowering serum cholesterol levels, anticarcinogenic activity, reducing allergic symptoms, antioxidative properties, stimulation of the immune system, improving resistance to various diseases [2,3]. The number of probiotic bacteria that are required to produce the health benefits is not entirely clear, but to exert the beneficial health effects, the count of probiotic bacteria in the food product should be adequately high 10^6-10^8 cfu/mL or g during shelf life [4,5]. In recent years, there has been increasing interest in the use of natural food additives. Probiotic foods can be supplemented with other active components with the goal of providing additional functional properties.

Green tea and green tea components are known to provide a wide range of benefits to human health [2,6]. Green tea is the least processed form and thus, retains all the healthy ingredients in their natural forms. Many studies have evaluated tea, tea polyphenols and tea extracts as factors for decreasing the risk of cardiovascular diseases and various types of cancer [7,8]. The beneficial effects of green tea have been attributed to the strong antioxidative and other health benefits of the rich green tea phenolic compounds, known as tea catechins [6-9]. Tea polyphenolics such as catechins are known to possess an antimicrobial effect against many microorganisms including pathogens, but these compounds do not inhibit lactic acid bacteria (LAB) [7,10]. Preliminary studies were conducted to see the effects of the addition of tea extract [8] and tea infusions [7,11] to milk on properties of yogurt during its production by fermentation.

Green tea powder (GTP) was selected in this study on the basis of the benefits of green tea to human health and the fact that it is a widely consumed beverage worldwide. The aims of the present study were to study the possibility of manufacturing a new functional probiotic yogurt. We investigated whether the addition of GTP would increase the nutritive value and functionality of yogurt and evaluated the effect of GTP addition on the survival of *L. acidophilus* and other LAB as well as the physical, chemical, sensory characteristics and antioxidant capacities of yogurts. It is possible that the use of GTP in yogurt technology can contribute to improvements in the quality, safety and functionality of probiotic yogurt. Previously, the effects of green tea supplementation on the yogurt were studied [12], but to our knowledge, this is the first study of the use of GTP for the supplementation of probiotic yogurt.

MATERIAL and METHODS

Materials

Fresh tea leaves were harvested and processed at the Atatürk Tea and Horticultural Research Institute (Rize, Turkey). The harvested green tea leaves were steamed, rolled, dried, ground and sieved. The particle size of GTP used as supplement was less than 355 µm. Raw cow's milk was purchased from Atatürk University Pilot Dairy Plant (Erzurum, Turkey). *Lactobacillus acidophilus* DSMZ 20079 was imported from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The direct-to-vat system yogurt culture (YC-350) *(Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus)* (Peyma-Hansen, Istanbul, Turkey) was used as the yogurt starter.

Manufacture of the Yogurt Samples

Yogurt samples were manufactured from cow's milk. The production of yogurt samples is shown in *Fig. 1* for clarity. The YC-350 culture *(Streptococcus thermophilus* and *Lactobacillus bulgaricus)* and *L. acidophilus* (3.2×10^8 cfu/g) was added (except for the Control sample). The yogurt samples were cooled and stored at 4°C for 28 days (for microbiological analysis) and 21 days (for physicochemical analysis). The yogurt samples produced by the inoculation of milk were divided into four groups (C: Control group yogurt; PC: yogurt with *L. acidophilus* and without GTP; P+1% GTP: yogurt with *L. acidophilus* and 1% GTP (w/v); P+2% GTP: yogurt with *L. acidophilus* and 2% GTP (w/v) *(Fig. 1)*.

Microbiological Analysis

The effect of the addition of GTP at various levels (0%, 1% and 2%) on the survival of the *L. acidophilus* strain during the production of yogurt was investigated. In addition, the effects of the use of *L. acidophilus* and GTP at various concentrations on the viability of yogurt cultures in the production of yogurt were examined. For each sample, 10 g of yogurt was diluted in 90 mL of 0.85% (w/v) NaCl solution and homogenized in a sterile polyethylene bag by using a Stomacher (Mayo HG400 Stomacher, Milan, Italy) for 2 min. Serial dilutions were made in 0.1% peptone in 0.85% NaCl, and all determinations were made in duplicate [13]. The numbers of yeast and mold (Dichloran Rose-Bengal Chloramphenicol Agar [DRBC Agar]; Merck) were determined according to Harrigan [13]. MRS, M17 and MRS bile agars (Merck) were used for the enumeration of LAB and determined according to Vinderola and Reinheimer [14]. The agar plates were incubated for 5-7 days at room temperature (yeast and mold), 3 days at 35-37°C in an anaerobic jar *(L. bulgaricus, S. thermophilus* and *L. acidophilus)*. The microorganisms were counted on the 1st, 7th, 14th, 21st and 28th days of the storage.

Physical and Chemical Analysis

The yogurt and milk samples were analyzed for total solids, protein and fat analysis using the method of AOAC [15]. The pH of the samples was measured using a pH meter (Mettler-Toledo AG 8603 Schwerzenbach, Switzerland). Titratable acidity (lactic acid, %) and syneresis were determined according to Atamer and Sezgin [16]. The syneresis, pH,

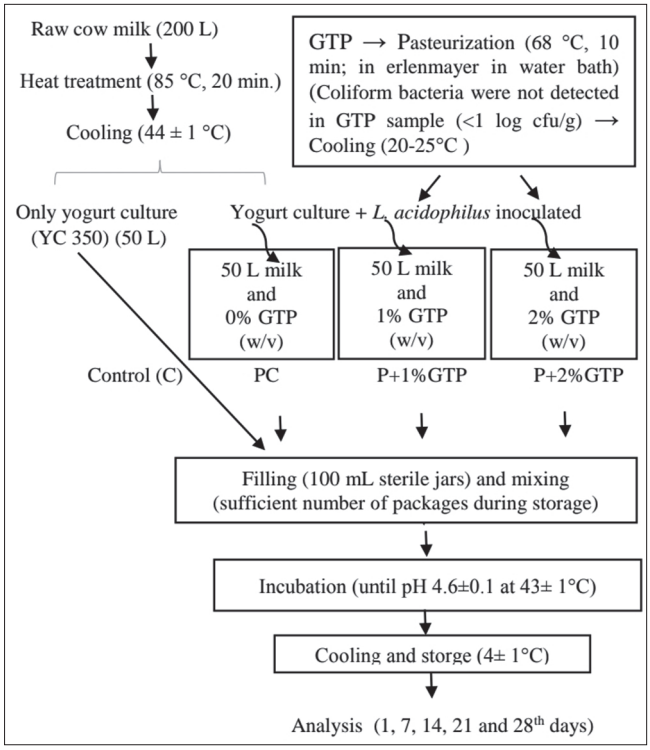

Fig 1. Production of Control and GTP yogurt groups

C: Control: (probiotic and GTP free); **PC:** Probiotic control: (with probiotic and GTP-free), **P+1% GTP:** (probiotic + 1% GTP); **P+2% GTP:** (probiotic + 2% GTP)

titratable acidity and sensory properties were assessed once a week. Dry matter, fat and protein contents of samples were determined at the beginning of the storage.

Antioxidant Analysis

Reducing Power Abilities: The Fe^{3+}-reducing ability of the yogurt samples and GTP was assayed using the $Fe^{3+}(CN)_6$-$Fe^{2+}(CN^-)_6$ reduction method [17]. The cupric ion (Cu^{2+})-reducing power was used as a second method for the analysis of the samples. The Cu^{2+}-reducing capability was analyzed according to the spectroscopic method of Apak et al.[18] with a modification. Another reducing power assay is the ferric-reducing antioxidant power, which is based upon reduction of the Fe^{3+}-TPTZ complex under acidic conditions [19].

Radical-Scavenging Abilities: The DPPH radical-scavenging activity of samples was determined according to the method of Blois [20]. The ABTS radical-scavenging activity of the samples was analyzed using the method described by Re et al.[21]. The DMPD radical-scavenging ability of the samples was analyzed according to the method described by Fogliano et al.[22]. The superoxide radical ($O_2^{\cdot-}$)-scavenging activity of the samples was analyzed in accordance with slight adjustments [23].

Metal-Chelating Ability: The metal-chelating ability of samples was determined according to the method of Gülçin and Daştan [24]. For this purpose, various concentrations of sample were mixed 0.25 mL $FeSO_4$ solution (2 mM), and 2,2′-bipyridine solution (0.2% in 0.2 M HCl) were used. The absorbances of the samples were recorded spectrophotometrically at 562 nm. EDTA is used as a standard ferrous ion (Fe^{2+}) chelator.

Total Phenolic and Flavonoid Contents: The total phenolic contents of samples were assessed using the Folin-Ciocalteu method [25] according to the equation Absorbance (λ_{760}) = 0.0021 × total phenols (gallic acid equivalent [GAE; µg]). The total phenolics in foods were estimated using the Folin-Ciocalteu method, which relied on the transfer of electrons from phenolic compounds to the FCR in alkaline medium. The content of total phenolics was calculated on the basis of a graph (r^2: 0.9706) that was prepared using gallic acid and expressed as micrograms of GAE. Total flavonoid contents of samples were estimated using a colorimetric assay as described in previous studies [23].

Sensory Analysis

The consumer acceptability properties were evaluated using a sensory evaluation of the yogurt by a jury of fifty panelists (age 20-50-years old) who were experienced and familiar with yogurt and green tea taste. Coded yogurt samples were stored at 4±1°C and were scored on the 1st, 7th, 14th and 21st days of storage. Five parameters, colour-appearance, odour, flavour, sourness and general acceptability were evaluated using a sensory rating scale of 1-9 (1 for extreme dislike, to 9 for extreme like). The tests were conducted at city center (Erzurum, Turkey), in their houses by consumers or by students and teaching staff of the Atatürk University Food Engineering Department. Sensory analysis was made at room temperature and under fluorescent lamps. Sensory analysis forms were given. After that, the yogurt samples taken from the refrigerator were given to the panelists one by one. All yogurt samples were presented to the panelists in glass jars (100 mL). The samples were presented in random order. Water was provided to the panelists for palate cleansing between the samples [26].

Statistical Analysis

The experiments were conducted in a completely randomized design in a factorial arrangement: four treatments of yogurt (C, PC, P+1% GTP and P+2% GTP), four storage periods (1st, 7th, 14th and 21st days) and two replicates. All data were analyzed statistically using SPSS 17.0 program (SPSS Inc., Chicago, IL, USA). ANOVA and Duncan's Multiple Range Test were used to determine significant differences among the results.

RESULTS

The gross chemical and physical properties of the raw milk and GTP are shown in *Table 1*. The yogurt samples were

analyzed for total solids, protein and fat at the beginning of the storage period. The addition of GTP significantly affected the gross chemical composition of the yogurt samples *(Table 2)*.

The results of microbiological analysis of the yogurt samples were shown in *Table 3*. The count of *L. acidophilus* showed significant (P<0.05) differences among the probiotic yogurt groups. In the case of 1% GTP addition to the 21st day, up to 28 days with the addition of 2% GTP, the probiotic property was maintained *(Table 2)*. On the other hand, the storage period had also a significant effect on the count of *L. acidophilus* (P<0.05) *(Table 3)*. The results showed a significant decrease in *L. acidoph*ilus in probiotic treatment during storage time. The addition of GTP to the milk before the yogurt fermentation did not have a negative effect on yogurt formation. But, the results showed that both the treatment and storage period had significant effects (P<0.05) on the counts of *L. bulgaricus* and *S. thermophilus (Table 3)*. In the present study, it was found that the treatment and storage period did not have a significant effect (P>0.05) on the counts of yeast and mold *(Table 3)*.

The yogurt samples showed potent Fe^{3+} reducing capability, and these differences were statistically important (P<0.01). The cupric ion (Cu^{2+}) reducing power of same concentration (30 µg/mL) of the yogurt samples and the standard reducing compounds is shown in *Table 4*. It was determined that Cu^{2+} reducing capacity of the yogurt samples was increased dependent on the concentration (10-30 µg/mL). In this study, the final method used to assess the reducing ability was the Fe^{3+}-TPTZ reducing power, which offers a well-known index of the antioxidant, or reducing, potential of plant samples or pure compounds. The DPPH radical scavenging of the yogurt samples and GTP are

Table 1. *The gross chemical properties of raw milk, GTP and fresh yogurt samples*

Materials and Yogurt Samples		Total Solids (%)	Fat (%)	Protein (%)	Ash (%)	Acidity (%)	pH
Material	Milk	12.37	3.45	3.40	0.64	0.17	6.65
	GTP	92.70	-	19.44	4.13	-	-
Yogurt samples	C	12.61±0.17^{ab}	3.90±0.14^{c}	3.36±0.03^{a}	-	-	-
	PC	12.37±0.04^{a}	3.70±0.14^{bc}	3.56±0.03^{a}	-	-	-
	P +1% GTP	13.13±0.23^{b}	3.50±0.14^{ab}	3.61±0.01^{a}	-	-	-
	P + 2% GTP	14.11±0.31^{c}	3.20±0.00^{a}	4.03±0.29^{b}	-	-	-

C: *Control (probiotic free and GTP free);* ***PC:*** *with probiotic and GTP free;* ***P+1% GTP:*** *probiotic + 1% GTP);* ***P+2% GTP:*** *probiotic + 2% GTP*
Mean values followed by different letters in the same column are significantly different (P<0.05)

Table 2. *The syneresis, acidity and pH of the yogurt samples during the storage*

Yogurt Samples	Storage Time (days)	Syneresis (mL/25g)	Titratable Acidity (lactic acid, %)	pH
C	1	10.95±0.7^{b,B}	0.71±0.01^{a,A}	4.71±0.01^{c,AB}
	7	11.30±0.1^{b,B}	0.74±0.01^{a,B}	4.50±0.00^{b,B}
	14	9.9±0.1^{c,A}	0.79±0.01^{a,C}	4.28±0.04^{a,A}
	21	11.3±0.4^{d,B}	0.82±0.01^{b,C}	4.31±0.01^{a,B}
PC	1	10.75±0.1^{b,B}	0.74±0.01^{a,A}	4.69±0.09^{c,A}
	7	11.45±0.2^{b,C}	0.78±0.01^{b,B}	4.42±0.01^{b,A}
	14	10.2±0.0^{d,A}	0.85±0.01^{b,C}	4.23±0.04^{a,A}
	21	10.7±0.1^{cd,B}	0.83±0.01^{c,C}	4.20±0.00^{a,A}
P+1% GTP	1	10.75±0.4^{b,BC}	0.81±0.0^{b,B}	4.80±0.01^{c,B}
	7	11.15±0.1^{b,C}	0.80±0.01^{b,B}	4.60±0.03^{b,C}
	14	9.65±0.1^{b,AB}	0.87±0.0^{c,C}	4.45±0.03^{a,AB}
	21	9.2±0.8^{a,A}	0.73±0.0^{a,A}	4.45±0.00^{a,C}
P+2% GTP	1	8.8±0.0^{a,A}	0.71±0.01^{a,A}	4.96±0.00^{c,C}
	7	9.65±0.2^{a,B}	0.80±0.01^{b,B}	4.88±0.02^{bc,D}
	14	8.8±0.0^{a,A}	0.84±0.01^{b,C}	4.58±0.17^{a,B}
	21	9.4±0.3^{bc,B}	0.83±0.01^{bc,BC}	4.72±0.00^{ab,D}

C: *Control (probiotic free and GTP free);* ***PC:*** *with probiotic and GTP free;* ***P+1% GTP:*** *probiotic + 1% GTP);* ***P+2% GTP:*** *probiotic + 2% GTP*
Horizontal column, lower cases (a-d) express differences between yogurt samples (P<0.05); Vertical column, capital letter (A-C) express differences between storage periods (P<0.05)

Table 3. *The changes in microbiological characteristics of yogurt samples during the storage period (log cfu/g)*

Yogurt Samples	Storage Time (days)	*L. bulgaricus*	*S. thermophilus*	Yeast and Molds	*L. acidophilus*	
C	1	7.55±0.10	8.96±0.01	<2	<2	
	7	7.37±010	8.91±0.01	<2	<2	
	14	8.29±0.12	9.04±0.03	<2	<2	
	21	7.69±0.10	8.66±0.05	<2	<2	
	28	7.78±0.03	8.81±0.04	<2	<2	
PC	1	6.65±0.10	8.69±0.06	<2	7.55±0.07	
	7	7.45±0.01	8.77±0.02	<2	7.89±0.06	
	14	8.04±0.01	8.78±0.08	<2	6.52±0.04	
	21	7.41±0.02	8.40±0.02	<2	5.08±0.05	
	28	6.91±0.02	8.68±0.04	<2	<4	
P+1% GTP	1	7.32±0.05	8.28±0.04	<2	6.63±0.06	
	7	8.18±0.02	8.86±0.04	<2	7.00±0.02	
	14	7.59±0.10	8.72±0.08	<2	6.95±0.02	
	21	7.75±0.04	8.72±0.02	<2	6.92±0.05	
	28	6.97±0.05	8.18±0.02	<2	5.71±0.06	
P+2% GTP	1	6.22±0.06	8.21±0.39	<2	6.59±0.10	
	7	7.95±0.05	8.25±0.44	<2	7.72±0.02	
	14	7.84±0.05	8.72±0.05	<2	6.38±0.05	
	21	7.30±0.03	8.18±0.03	<2	7.11±0.03	
	28	7.90±0.01	8.40±0.03	<2	6.95±0.06	
Yogurt samples	**n**					
C	10	7.74±0.33a	8.88±0.14a	<2±0.0a	-	
PC	10	7.29±0.51d	8.66±0.15b	<2±0.0a	6.21±1.56c	
P+1% GTP	10	7.56±0.43b	8.55±0.29b	<2±0.0a	6.64±0.51b	
P+2% GTP	10	7.44±0.69c	8.35±0.29c	<2±0.0a	6.95±0.49a	
Storage period						
1	8	6.93±0.57e	8.53±0.35b	<2±0.0a	6.92±0.49b	
7	8	7.74±0.36b	8.70±0.33a	<2±0.0a	7.54±0.43a	
14	8	7.94±0.28a	8.81±0.15a	<2±0.0a	6.62±0.27c	
21	8	7.54±0.20c	8.49±0.24b	<2±0.0a	6.37±1.01d	
28	8	7.39±0.48d	8.52±0.27b	<2±0.0a	5.56±1.33e	
Source	**D.F.**	**ANOVA**			**D.F.**	**ANOVA**
Sample (S)	3	**	**	**	2	**
Storage Period (SP)	4	**	**	**	4	**
S × SP	12	**	**	**	8	**
Error	20				15	

C: *Control (probiotic free and GTP free);* ***PC:*** *with probiotic and GTP free;* ***P+1% GTP:*** *probiotic + 1% GTP);* ***P+2% GTP:*** *probiotic + 2% GTP*
Averages of the same column values (each section separately) by the same letter did not differ significantly from Duncan's multiple range tests at 5% significance; ***(a-e)***
Mean ± SD, values followed by the same letters within a column are significantly different at P<0.05

summarized in *Table 4*, which shows the half-maximal radical scavenging concentrations (IC_{50}) of the samples and GTP and the standards, trolox and α-tocopherol. A lower IC_{50} value demonstrates a higher DPPH· scavenging activity. As shown in *Table 4*, the yogurt samples and GTP were efficient $ABTS^{\cdot+}$ scavengers in a concentration-dependent manner (10-30 µg/mL, r^2: 0.957). The content of phenolic compounds in the yogurt samples was expressed as milligrams of GAE *(Table 5)*.

The scores for the sensory properties of the yogurts are presented in *Table 6*. The supplementation of the yogurts with the *L. acidophilus* and GTP significantly affected (P<0.05) the sensory scores. Generally, the GTP yogurts received lower scores. In terms of sourness, no statistically significant differences (P>0.05) were detected among the samples. Even at the end of the 21st day, all samples were evaluated as favorable, and statistically there were no differences (P>0.05) among the yogurt samples.

Table 4. *Determination of absorbance values of reducing ability of 30 µg/mL concentration of yogurt samples and GTP by ferric ions (Fe^{3+})reducing, FRAP methods, and cupric ions (Cu^{2+}) reducing capacity, half maximal concentrations (IC_{50}, µg/mL) of yogurt samples and GTP and standards for radical scavenging abilities including $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$, $O_2^{\bullet-}$ and metal (Fe^{2+}) chelating effects*

Antioxidants, Yogurt Samples and GTP	Fe^{3+}-Fe^{2+} Reducing		Cu^{2+}-Cu^{+} Reducing		Fe^{3+}-TPTZ Reducing		DPPH• Scavenging		$ABTS^{\bullet+}$ Scavenging		$DMPD^{\bullet+}$ Scavenging		O_2 Scavenging		Fe^{2+} Chelating	
	λ_{700}	r^2	λ_{450}	r^2	λ_{593}	r^2	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2
α-tocopherol	1.078±0.004	0.9424	1.375±0.003	0.9898	1.769±0.008	0.9383	13.58	0.9901	6.18	0.9688	57.75	0.9648	31.50	0.9748	19.80	0.9373
Trolox	1.088±0.007	0.9844	1.982±0.006	0.9711	2.505±0.008	0.9441	11.01	0.9811	7.14	0.9788	22.35	0.9830	18.73	0.9277	7.96	0.9011
EDTA							-	-	-		-		-		1.86	0.9972
C	0.167±0.003	0.9388	0.303±0.004	0.9915	0.615±0.007	0.9717	99.01	0.9560	53.30	0.9923	28.07	0.9438	33.01	0.9205	53.44	0.9462
PC	0.186±0.005	0.9109	0.248±0.007	0.9689	0.697±0.006	0.9889	77.11	0.9919	49.50	0.9511	36.47	0.9385	63.01	0.9366	20.38	0.9628
P+1% GTP	0.510±0.007	0.9399	0.293±0.007	0.9616	0.528±0.006	0.9636	98.85	0.9872	43.31	0.9318	25.66	0.9952	36.47	0.9942	77.01	0.9878
P+2% GTP	0.564±0.006	0.9755	0.470±0.008	0.9528	0.469±0.010	0.9480	63.02	0.9660	31.50	0.9006	27.72	0.9910	23.10	0.9848	53.37	0.9511
GTP	1.426±0.006	0.9292	0.994±0.006	0.9770	1.601±0.011	0.9101	43.31	0.9922	23.10	0.9889	11.55	0.9458	19.25	0.9782	23.14	0.9318

Table 5. *Total phenolics and flavonoids contents of yogurt samples and GTP as gallic acid equivalent (GAE) and quercetin equivalent (QE)*

Antioxidants	Total Phenolics (µg GAE)	Total Flavonoids (µg QE)
C	2.13	2.23
PC	7.86	3.30
P+1% GTP	26.68	6.01
P+2% GTP	40.18	7.76
GTP	1275	9.30

C: *Control (probiotic free and GTP free);* ***PC:*** *with probiotic and GTP free;* ***P+1% GTP:*** *probiotic + 1% GTP);* ***P+2% GTP:*** *probiotic + 2% GTP;* ***GTP:*** *Green tea powder*

DISCUSSION

To obtain GTP, harvested green tea leaves were dried, ground and sifted. For this reason, the dryness and protein ratio of GTP is very high. Increasing the GTP level increased total solids (between 12.37 and 14.11%) and protein (between 3.36 and 4.03%) contents of all samples. The initial and final pH values showed that the pH values of the yogurt samples were higher in the samples that had been supplemented with higher levels of GTP *(Table 2)*. The lactic acid produced by Lactobacilli inhibits the growth of other organisms and lowers the pH of the product in these products. *L. acidophilus* grows in low pH (<3.5), anaerobic conditions and undergoes fermentation only [27]. Acidity was found to be higher in yogurt containing *L. acidophius* than in control. The increase in pH may have resulted from the presence of various basic compounds in the GTP. A similar result was found by Najgebauer-Lejko [2]. Conversely, lower pH values were found for tea infusion-supplemented yogurts, but the level of fortification had little effect on that parameter Najgebauer-Lejko [7]. The pH values of the yogurt samples supplemented with GTP were higher than the average value determined for the other yogurts. The result obtained here differed from the study of green tea infusions by Najgebauer-Lejko et al.[7]. The differences between the initial and final pH values decreased with increasing concentrations of GTP *(Table 2)*. During the storage period, the mean acidity values were 0.74%, 0.78%, 0.84%, and 0.80% lactic acid on the 1st, 7th, 14th and 21st days, respectively *(Table 2)*. The mean syneresis values were 10.8, 10.7, 10.2, and 9.2 mL/25 g for the C, PC, P+1% GTP, and P+2% GTP samples, respectively. Syneresis decreased with the increase of dry matter in the yogurt samples. A similar result was also reported by Cakmakci et al.[28]. *L. acidophilus* had no significant effect on the composition of yogurt samples *(Table 1, Table 2)*. The lowest syneresis value was observed on the 14th of storage *(Table 2)*.

The increasing level of GTP used in the production of probiotic samples increased the number of *L. acidophilus*. As it can be understood from these results, the increase of GTP supplementation has further stimulated the development of *L. acidophilus*. This could be due to the fact that the GTP promoted the growth of *L. acidophilus*. Indeed, Lee et al.[29] reported that tea phenolics had significant effects on the intestinal environment probably by acting as metabolic prebiotics. Similarly, Ankolekar et al.[10] and Zhao and Shah [9] reported that green tea extracts containing *L. acidophilus* did not inhibit the growth of beneficial LAB. In another study, green tea extracts were found to permitted the survival of the selected probiotic strains better than the salt solution López de Lacey [30]. It can make an important contribution to know the effect of green tea on *L. acidophilus*, in terms of its survival to gastric and intestinal conditions, its adhesion properties, and antagonisms with pathogens. Also, as the GTP rate added in yogurt production increases, these effects may have increased.

Use of *L. acidophilus* alone in yogurt production (no GTP) strain lost viability and decay to <4 log cfu/g in the 28th day. The *L. acidophilus* count was high up to the 7th day, as previously also found by Turgut and Cakmakci [31]. However, the *L. acidophilus* count tended to decrease after the 7th day of storage. Nevertheless, it maintained its probiotic properties (>10^6 cfu/g) [4,5] until the 14th day of storage. It is thought that the initial inoculum level can be increased

Table 6. *Sensory characteristics of yogurt samples during storage (score 1: poor; 9: excellent)*

Sensory Properties	Storage Period (days)	Types of Yogurt			
		C	PC	P+1% GTP	P+2% GTP
Colour & appearance	1	8.43 ± 0.53^{b}	8.71 ± 0.49^{b}	6.33 ± 1.63^{a}	6.83 ± 1.60^{a}
	7	8.56 ± 0.53^{b}	8.78 ± 0.44^{b}	7.50 ± 1.06^{a}	8.00 ± 1.00^{ab}
	14	8.50 ± 0.58^{b}	8.75 ± 0.50^{b}	7.50 ± 0.58^{ab}	6.75 ± 1.50^{a}
	21	8.33 ± 0.58^{bc}	9.00 ± 0.00^{c}	7.50 ± 0.50^{ab}	6.50 ± 1.32^{a}
Odour	1	$8.42\pm0.79^{b,A}$	$8.57\pm0.79^{b,A}$	$5.50\pm2.07^{a,A}$	$5.83\pm2.14^{a,A}$
	7	$8.22\pm0.67^{a,A}$	$8.22\pm0.67^{a,A}$	$7.89\pm0.78^{a,B}$	$8.00\pm0.87^{a,B}$
	14	$8.00\pm0.00^{a,A}$	$8.25\pm0.50^{a,A}$	$7.75\pm1.41^{a,B}$	$7.00\pm1.41^{a,AB}$
	21	$7.67\pm0.58^{a,A}$	$8.00\pm0.00^{a,A}$	$8.5\pm0.5^{a,B}$	$7.27\pm1.1^{a,AB}$
Flavour	1	$8.57\pm0.53^{b,B}$	$9.00\pm0.00^{b,C}$	$4.33\pm2.07^{a,A}$	$4.33\pm2.07^{a,A}$
	7	$7.78\pm0.67^{a,AB}$	$8.06\pm0.88^{a,AB}$	$7.63\pm0.48^{a,B}$	$8.30\pm0.80^{a,B}$
	14	$8.25\pm0.50^{ab,B}$	$8.62\pm0.48^{b,BC}$	$7.50\pm0.58^{a,B}$	$7.75\pm0.87^{ab,B}$
	21	$7.33\pm0.29^{a,A}$	$7.57\pm0.51^{a,A}$	$8.23\pm0.68^{a,B}$	$7.00\pm1.00^{a,B}$
Sourness	1	8.43 ± 0.53^{B}	8.57 ± 0.53^{A}	7.33 ± 1.97^{A}	6.50 ± 2.59^{A}
	7	7.28 ± 0.44^{A}	7.44 ± 0.68^{A}	7.44 ± 1.72^{A}	6.92 ± 2.28^{A}
	14	7.25 ± 0.96^{A}	7.50 ± 1.73^{A}	6.50 ± 1.73^{A}	6.42 ± 1.65^{A}
	21	7.33 ± 0.58^{A}	8.17 ± 0.76^{A}	8.17 ± 0.76^{A}	7.00 ± 0.00^{A}
General acceptability	1	$8.00\pm0.82^{b,A}$	$8.14\pm0.90^{b,A}$	$5.83\pm1.94^{a,A}$	$4.83\pm2.14^{a,A}$
	7	$7.67\pm0.50^{a,A}$	$8.06\pm0.88^{a,A}$	$7.67\pm0.75^{a,B}$	$8.31\pm0.58^{a,B}$
	14	$8.00\pm0.00^{ab,A}$	$8.55\pm0.53^{b,A}$	$7.75\pm0.50^{ab,B}$	$7.30\pm0.89^{a,B}$
	21	$7.50\pm0.50^{ab,A}$	$8.00\pm0.00^{b,A}$	$8.40\pm0.66^{b,B}$	$6.80\pm0.72^{a,B}$

C: *Control (probiotic free and GTP free);* ***PC:*** *with probiotic and GTP free;* ***P+1% GTP:*** *probiotic + 1% GTP);* ***P+2% GTP:*** *probiotic + 2% GTP*
Horizontal column (a-c): *differences between yogurt types, P<0.05;* ***Vertical column (A-C):*** *differences between storage period, P<0.05*

to maintain the number of *L. acidophilus* required for probiotic properties for a longer time.

Najgebauer-Lejko et al.[7] and Ankolekar et al.[10] also stated that tea polypenols have antimicrobial effects against many microorganisms including pathogens, but these compounds do not affect the development of lactic acid bacteria. The highest average *L. bulgaricus* and *S. thermophilus* counts were determined in C samples. However, the average counts of *L. bulgaricus* and *S. thermophilus* were higher than 7.0 and 8.0 log cfu/g in other yogurt groups, respectively. On the other hand, all yogurt samples maintained high levels of the starter bacteria during the 4-week cold storage. However, there was a slight decrease in the count of starter bacteria during last 2 week of storage. This decline could be due to the increase in organic acid production. Similarly, it was reported in several studies that the accumulation of organic acids as a result of growth and fermentation is an important factor in the loss of cell viability [4,32]. Yeast and mold were not counted (<2 log cfu/g) in any yogurt sample during storage periods. This result is effective in extending the shelf life of yogurt. Because the shelf life of yogurt depends on the hygienic conditions during processing and packaging [33].

Various plants are sources of functional food components. One of the most important functions of these compounds is their antioxidant effect, which increases the importance of plants. Our study supplies valuable results on the antioxidant capacity of C, PC, P+1% GTP, P+2% GTP and GTP as indicated by several bioanalytical methods including measurements including ferric ions (Fe^{3+}), Cupric ions (Cu^{2+}) and Fe^{3+}-TPTZ reducing abilities, the DPPH, ABTS, DMPD and $O_2^{\bullet-}$ radical scavenging activities, the ferrous ions (Fe^{2+})-chelating activity, and the total phenolic and flavonoid contents.

Reducing power of bioactive compounds or food components reflects the electron-donating capacity and is associated with antioxidant activity [34]. The reducing ability of food or plant materials can be determined by means of the direct reduction of Fe^{3+} to Fe^{2+}. The Fe^{3+} reducing capacity of the yogurt samples, and standard antioxidants increased consistently with increasing concentrations of the samples. The Fe^{3+}-reducing capacity of the yogurt samples and both standards (as absorbance values) demonstrated the following order: GTP > α-tocopherol ≈ trolox > P+2% GTP > P+1% GTP > PC > C) at the same concentration (30 µg/mL). The results proved that yogurt samples had marked Fe^{3+}-reducing ability. In this method, the reducing capacity of food constituents measure direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. Addition of free Fe^{3+} to the reduced product leads to the formation of the intense Perl's Prussian blue complex, $Fe_4[Fe(CN\text{-})_6]_3$, which has a strong absorbance at 700 nm [34].

The Cu^{2+} reducing capacity of the yogurt samples and standard reducing agents at the same concentration (30 µg/mL) demonstrated the following order: trolox > α-tocopherol > GTP > P+2% GTP > C ≈ P+1% GTP > PC. The results clearly showed that cupric ion (Cu^{2+})-reducing ability was similar to the ferric ion (Fe^{3+})-reducing ability. Cu^{2+} reducing assays are based on the reduction of Cu^{2+} to Cu^{+} by the combined action of all antioxidants or reducing in aqueous-ethanolic medium (pH 7.0) in the presence of neocuproine food constituents yielding a Cu^{+}-complexes with maximum absorption peak at 450 nm.

According to the results obtained from FRAP (Fe^{3+}-TPTZ) assay *(Table 4)*, the reducing power of yogurt samples, and standard antioxidants decreased in the following order: trolox > α-tocopherol > GTP > PC ≈ C > P+1% GTP) ≈ P+2% GTP. In this method, higher absorbance values indicate a greater capacity to reduce the Fe^{3+}-TPTZ complex. FRAP values are calculated by measuring the absorbance increase at 593 nm and relating it to a ferrous ions standard solution or to an antioxidant standard solution. The change in absorbance is proportional to the combined FRAP value of the antioxidants in the food constitutes [35].

Free radical chain reactions are widely accepted as a common mechanism of lipid peroxidation. Radical-scavenging compounds may directly react with and quench peroxide radicals to terminate the peroxidation chain reactions and protect the quality and stability of foods [36]. The total radical-scavenging capacities of the yogurt samples and GTP were analyzed and compared to those of α-tocopherol and trolox using the DPPH·, $ABTS^{\cdot+}$, $DMPD^{\cdot+}$ and $O_2^{\cdot-}$ radical scavenging methods. The IC_{50} values for samples in this analysis were α-tocopherol < trolox < GTP < P+2% GTP < P+1% GTP < PC < C. The results show that the concentration of $ABTS^{\cdot+}$ (P>0.01) decreases substantially due to the scavenging capacity of all samples.

Another assay that is very similar to the use of the $ABTS^{\cdot+}$ is the $DMPD^{\cdot+}$ assay. The UV-visible spectrum of $DMPD^{\cdot+}$ had a maximum absorbance at 505 nm. This assay is particularly suitable for hydrophilic antioxidants, but is less sensitive to hydrophobic bioactive compounds, while the opposite case applies for the other two tests. As in the previous both DPPH· and $ABTS^{\cdot+}$ radical scavenging methods, the samples efficiently scavenged the $DMPD^{\cdot+}$ radical in concentration-dependent manners (10-30 µg/mL). The IC_{50} values for the yogurt samples, GTP, and standards were calculated as GTP < trolox < P+1% GTP < P+2% GTP < C < PC < α-tocopherol *(Table 4)*.

Superoxide anion radicals ($O_2^{\cdot-}$) are biologically toxic and are deployed by the immune system to kill invading micro-organisms. Also, this radical species exhibits limited chemical reactivity, but can generate more dangerous species, including singled oxygen and hydroxyl radicals, which cause the peroxidation of lipids [27]. As shown in *Table 4*, the IC_{50} value for the $O_2^{\cdot-}$ radical-scavenging of the yogurt samples and GTP were found to be 33.01 µg/mL (r^2: 0.9205), 63.01 µg/mL (r^2: 0.9366), 36.47 µg/mL (r^2: 0.9385), 23.10 µg/mL (r^2: 0.9848), 19.25 µg/mL (r^2: 0.9782), respectively. On the other hand, the IC_{50} values for trolox and α-tocopherol were found to be 31.50 µg/mL (r^2: 0.9748) and 18.73 µg/mL (r^2: 0.9277), respectively. The $O_2^{\cdot-}$ scavenging effects of the samples and standards on the $O_2^{\cdot-}$ increased in the following order: Trolox ≈ GTP > P+2% GTP > α-tocopherol > C > P+1% GTP > PC. A lower value of the IC_{50} indicates a greater $O_2^{\cdot-}$ scavenging activity.

The yogurt samples and GTP were also effective in chelating metal ions. The IC_{50} values for the samples and standard metal chelator compounds including EDTA demonstrated the following order: EDTA > trolox > α-tocopherol > PC > GTP > P+2% GTP ≈ C > P+1% GTP. These results clearly show that the Fe^{2+} ion-chelating effect of the yogurt samples was similar to that of α-tocopherol but lower than that of trolox. Lower IC_{50} values indicate higher metal chelation capacity.

Polyphenols are a broad group of phytochemicals that have antioxidant properties. The total phenolic compounds in the yogurt samples demonstrated the following order: C < PC < P+1% GTP < P+2% GTP < GTP *(Table 5)*. Phenolic compounds are likely to contribute to the radical-scavenging activity of the GTP extracts [37]. Furthermore, the total flavonoid compounds in yogurt samples were determined as milligrams of quercetin equivalents (QE). As shown in *Table 5*, the total flavonoids in the samples demonstrated the following order: C < PC < P+1% GTP < P+2% GTP < GTP.

Sensory evaluation is the most important criterion for acceptance or rejection of a food [38-40]. The incorporation of GTP into the yogurt with *L. acidophilus* had a significant influence on the notes received in the sensory evaluation. Statistical analysis showed that significant differences were found between the C and PC samples and the GTP yogurts. According to the C and PC yogurt samples, the color scores of the yogurt samples with GTP added were found to be lower. Similar results were found in yogurt with kiwi marmalade, which has green color similar to GTP [41]. The GTP addition caused a significant change from the usual color of plain yogurt. In 21 days of storage, probiotic yogurt samples with 1% GTP and 2% GTP received similar scores. However, all yogurt samples received well-acceptable color and appearance scores *(Table 6)*. The panelists noted that the yogurt with *L. acidophilus* and 2% GTP had a markedly bitter and astringent taste during the storage. As a reason, it can be said that the bitter and astringent taste of GTP penetrates into the yogurt. Catechins are water-soluble, thus giving bitterness and astringency to green tea infusion. Modification of catechins is effective on the color, taste and aroma of teas [42]. For example, it has been reported that the conversion from ester catechins to non-ester catechins can reduce the bitterness and

firmness of green tea [42,43]. In particular, flavour scores in fresh yogurts with GTP were found to be very low *(Table 6)*. Thus, the increase in flavour scores in other periods can be attributed to some biochemical transformations of catechins and other phenolic compounds. In this research, compared to C, generally PC and P+1% GTP samples were more preferred by the sensory evaluation panelists during the storage period.

We concluded that GTP has antioxidant properties that could be attributed to the presence of phenolic and flavonoid compounds. A positive effect of GTP on *L. acidophilus* was observed that depended of the amount of GTP. GTP stimulated the probiotic activity. In conclusion, GTP could be successfully used as a functional additive for selected probiotic yogurts to enhance the health benefits of the yogurt. Addition of GTP in the manufacture of yogurt is recommended because GTP is a natural herbal product with a wide range of beneficial health and nutritional properties; this makes this yogurt a new functional food. The addition of GTP produced a new kind of probiotic yogurt that retained an acceptable quality during storage for 3 weeks. To our knowledge, this is the first study of the use of GTP for the supplementation of yogurt with *L. acidophilus*. Sensory properties are one of the most important factors in the acceptance of a new food product. When all the research results are taken into consideration, it can be said that probiotic yogurt with 1% GTP supplement can be consumed for 21 days.

Acknowledgements

The authors would like to thank Atatürk University Food Engineering and Biochemistry Departments for laboratory facilities (Erzurum, Turkey) and Atatürk Tea and Horticultural Research Institute (Rize, Turkey) for green tea powder (GTP).

Conflict of Interest Statement

There are no conflicts of interest to declare.

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Kafkas Univ Vet Fak Derg
25 (5): 683-688, 2019
DOI: 10.9775/kvfd.2018.21613

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

Research Article

Effects of Grit Supplementation to Diets Containing Maize and Barley as Cereal Grains on Performance and Slaughter Characteristics in Broilers

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Article ID: KVFD-2018-21613 Received: 19.12.2018 Accepted: 21.04.2019 Published Online: 21.04.2019

How to Cite This Article

Eser H, Yalçın S, Onbaşılar İ, Burçak E, Yalçın S: Effects of grit supplementation to diets containing maize and barley as cereal grains on performance and slaughter characteristics in broilers. *Kafkas Univ Vet Fak Derg,* 25 (5): 683-688, 2019. DOI: 10.9775/kvfd.2018.21613

Abstract

The aim of this study was to determine the effects of grit supplementation to the diets containing maize and barley as cereal grains on performance and slaughter characteristics in broilers. In the experiment, a total of 160 Ross 308 male broiler chicks were allocated to 2 experimental groups with 4 replicate pens containing 20 birds per each for 5 weeks of experimental period. Granite grit was added at the level of 0 and 0.8% to the basal diets containing barley and maize as cereal grains for control and treatment groups, respectively. Granite grit supplementation had no significant effect on final body weight and body weight gain. Feed intake during the experiment was significantly reduced by grit supplementation ($P<0.05$). However, no significant differences were observed with the feed conversion ratio. Granite grit supplementation to the diets of broilers increased the relative weight of gizzard ($P=0.001$) and decreased the relative weight of abdominal fat ($P<0.05$). Dietary treatments did not affect blood serum total cholesterol and triglyceride. In conclusion, granite grit supplementation might be used in broiler nutrition due to having increment in the relative weight of gizzard and reduction in relative weight of abdominal fat.

Keywords: *Broiler, Slaughter characteristics, Gizzard, Grit, Performance*

Tahıl Taneleri Olarak Mısır ve Arpa Kapsayan Karma Yemlere Grit İlavesinin Broylerlerde Performans ve Kesim Özelliklerine Etkileri

Öz

Bu çalışmada amaç, tahıl tanesi olarak mısır ve arpa kapsayan karma yemlere grit ilavesinin broylerlerde performans ve kesim özelliklerine olan etkilerinin belirlenmesini oluşturmuştur. Denemede, toplam 160 adet Ross 308 erkek broyler 5 haftalık deneme süresince her birinde 20 civciv bulunan 4 tekrarlı 2 deneme grubuna ayrılmıştır. Granit grit tahıl tanesi olarak mısır ve arpa kapsayan kontrol ve deneme grupları temel rasyonlarına sırasıyla %0 ve %0.8 düzeylerinde eklenmiştir. Granit grit ilavesinin, deneme sonu canlı ağırlık ve canlı ağırlık kazancı üzerine önemli bir etkisinin olmadığı görülmüştür. Deneme süresince yem tüketimi grit ilavesiyle önemli miktarda azalmıştır ($P<0.05$). Gruplar arasında yem dönüşüm oranı bakımından istatistik açıdan önemli bir farklılık gözlenmemiştir. Mısır ve arpa kapsayan rasyonlara granit grit katkısı relatif taşlık ağırlığını artırmış ($P=0.001$) ve relatif abdominal yağ ağırlığını azaltmıştır ($P<0.05$). Grit ilavesi kan serumu toplam kolesterol ve trigliserit düzeylerini etkilememiştir. Sonuç olarak, tahıl tanesi olarak mısır ve arpa kapsayan karma yemlere granit grit ilavesinin relatif taşlık ağırlığını artırması ve relatif abdominal yağ ağırlığını azaltması nedeniyle broyler beslenmesinde kullanılabileceği saptanmıştır.

Anahtar sözcükler: *Broyler, Grit, Kesim özellikleri, Performans, Taşlık*

INTRODUCTION

Grit, as a stone and a rock fragment derived from granite, is used by the birds to enhance the mechanical digestion in gizzard [1]. Especially, one of the main advantages of dietary grit inclusion is its positive effect on gizzard

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development and functionality. The gizzard, serves as teeth, is one of the organs of the digestive system of birds. This specialized stomach, constructed of thick muscular walls, is used for grinding up feed, often aided by particles of stone or grit [2]. However, broilers are usually fed with an easily digestible mash or pelleted diet. Pellet feeds used in poultry provide high feed consumption, but adversely affect gizzard development [3]. Insufficient stimulation of gizzard development reduces nutrient absorption and digestibility due to an increase in the rate of passage of feed from gizzard to intestines [4]. It has been reported that gizzard development is substantially stimulated if diets are consisted of whole cereals or insoluble fibre [5]. Especially, the use of grit is gaining importance in nutrition with grain or roughly milled grain [6]. Grit stones in the gizzard of the birds lead to better grinding which allows longer retention of the digesta and a better feed flow [5,7-9]. It has been reported that half of the feed had passed the gizzard within 2 h [10]. The granite grit increases the size of the gizzard and its muscular power [2,5]. Grit also provides supplementary calcium and other minerals that are important for seed-consuming animals [7].

Numerous studies have been conducted to evaluate the efficacy of granite grit in broiler diets. Yıldız et al.[6] reported that inclusion of insoluble grit to the broilers diets increased the weight and the volume of the gizzard of the broilers. Moghaddam et al.[11] showed that use of grit supplementation to diets significantly improved the growth performance in broilers fed with grit 2 mm than that of those were not given. However, Bennett and Classen [12] stated that supplying insoluble grit had no beneficial effect on production parameters to laying hens fed whole barley or mash diets. Fuerjiafu [13] also reported that feeding grit to broiler chickens did not improve the gizzard weight and the performance and did not regulate the feed flow. Garipoğlu et al.[1] showed that although optional insoluble granite-grit consumption by broilers increased the weight of gizzard and length of gut, it did not improve growth performance. On the other hand, the amounts of grit in bird gizzards depend not only on the behaviour or need of birds, but also on retention of these particles in the gizzard. For instance, hard diets may reduce grit retention in the gizzard [7]. Due to these reasons, there are controversial results about grit usage. Therefore, the purpose of this experiment was to determine the effects of granite grit supplementation to broiler diets containing maize and barley as cereal grains on performance and slaughter characteristics.

MATERIAL and METHODS

Animal Care and Use

All experimental procedures were approved by The Animal Ethics Committee of the Ankara University (2018-19-124).

Birds, Housing and Feeding

A total of 160 Ross 308 seven-day-old male broiler chicks were divided into one control and one treatment group with 4 replicates per group, each of which consisted of 20 chickens. The chickens were housed in environmentally controlled pens and provided with continuous light during the 5 week of experimental period. Temperature was adjusted to the according to the recommended conditions for Ross 308 broiler during the study [14]. Average room temperature was 30±2°C on the first week and then gradually lowered to average 22±2°C, and this temperature was maintained up to slaughter age. Each pen had wood shavings litter, feed (in mash form) and water were provided *ad libitum* throughout the experiment.

The starter diets and grower diets were mainly consisted of maize, barley and soybean, and were offered to birds from 7-21, and 21-42 days of age, respectively. The ingredients and chemical composition of the basal diets for starter and grower periods are shown in *Table 1*. Granite grit having a particle size of 1-2 mm was obtained from a commercial company in Ankara-Turkey and it was used at the level of 0 and 0.8% for the diets of control group and treatment group, respectively. Granite grit used in this experiment contained 68.22% SiO_2, 16.75% Al_2O_3, 4% K_2O, 3.7%Na_2O,

Table 1. *The ingredients and chemical composition of the basal diets*

Ingredients, g/kg	Starter Diet (7-21 d)	Grower Diet (21-42 d)
Maize	320.0	360.0
Barley	200.0	200.0
Soybean meal	300.0	263.0
Full-fat soya	70.0	75.0
Fish meal	40.0	20.0
Sunflower seed oil	41.0	53.0
Limestone	10.0	10.0
Dicalcium phosphate	10.0	10.0
Salt	2.5	2.5
Vitamin premix[a]	2.0	2.0
Mineral premix[b]	1.0	1.0
DL-methionine	1.5	1.5
Lysine	1.0	1.0
Choline chloride	1.0	1.0
Analyzed nutrient values		
ME, kcal/kg[c]	2998	3105
Crude protein, %	23.52	21.07
Calcium, %	0.99	0.91
Total phosphorus, %	0.72	0.69

[a] Contained per 2 kg: 11.000.000 IU vitamin A, 3.500.000 vitamin D_3, 100 g vitamin E, 3 g vitamin K_3, 3 g vitamin B_1, 6 g vitamin B_2, 15 g calcium D-pantothenate, 1 g vitamin B_6, 20 mg vitamin B_{12}, 35 g niacin, 1.5 g folic acid and 200 mg biotin; [b] Contained per 1 kg: 30 g Cu, 120 g Mn, 110 g Zn, 2 g I, 300 mg Se and 50 g Fe; [c] Calculated [18]

1.68% Fe_2O_3, 0.48% MgO, 0.38% TiO_2, 1.40% P_2O_5, 0.72% MnO and 1.80% CaO.

Traits Measured

Nutrient compositions of the diets were determined according to the AOAC [15]. The samples were ashed in a muffle furnace prior to the analysis of calcium and total phosphorus [16,17]. Metabolizable energy levels of diets were estimated using the Carpenter and Clegg's equation [18]. Mineralogical composition of granite grit was determined by D8 Advance Diffractometer AXS (Bruker, Germany).

Chicks were weighed individually at the beginning of the experimental period and weekly to determine the body weight and body weight gain. Feed consumption was determined weekly and the feed conversion ratio was calculated as kg feed per kg body weight gain. The birds were observed for evaluating mortality.

At day 41, 8 broilers from each subgroup were randomly selected and bled from the vena brachialis under the wing. Blood samples were taken in the tubes having no anticoagulant for estimating cholesterol and triglyceride levels. Blood samples were centrifuged at 3220 x g for 8 min. Serum was collected and stored at -20°C. Serum cholesterol and triglyceride levels were determined using a Hitachi auto-analyzer (Hitachi, Tokyo) and its accompanying commercial kits.

At the end of the study (day 42), 8 broilers from each subgroup were randomly selected for processing. Feed was removed 5 h prior to slaughtering. Broilers were weighed and slaughtered in a commercial processing plant. Hot carcass, abdominal fat, liver, heart, spleen, gizzard and bursa Fabricius were weighed and expressed as percentage of slaughter weight.

Statistical Analyses

Data were analysed using SPSS 23.0 (SPSS Inc., Chicago, IL, USA). The normality of data distribution was checked using the Kolmogorov-Smirnov test. Comparison between groups was examined with independent samples t test. Level of significance was taken as $P<0.05$. Data were given as mean±standard error of mean [19].

RESULTS

The effect of dietary grit supplementation on body weight and body weight gains of broilers is shown in *Table 2*. Granite grit supplementation to the diets containing maize and barley as cereal grains did not significantly affect the final body weight and body weight gain.

The effect of dietary grit supplementation on feed intake of broilers is shown in *Table 3*. Feed intake during the experiment was significantly reduced ($P<0.05$) by grit supplementation. Feed conversion ratio was not

Table 2. *Effects of dietary grit supplementation on body weight and body weight gain of broilers*

Period, days	Grit, %		Significance
	0	0.8	
Body weight, g			
7	148.66	148.90	0.955
14	368.88	364.34	0.435
21	713.68	718.67	0.770
28	1158.93	1158.88	0.999
35	1654.68	1662.30	0.942
42	2135.83	2157.24	0.869
Body weight gain, g			
7-14	220.21	215.44	0.353
14-21	344.80	354.33	0.649
21-28	445.26	440.21	0.918
28-35	495.75	503.43	0.857
35-42	481.15	494.94	0.698
7-21	565.01	569.77	0.812
21-42	1422.15	1438.58	0.887
7-42	1987.17	2008.34	0.874

n=4; No statistical significant differences between groups

Table 3. *Effects of dietary grit supplementation on feed intake and feed conversion ratio of broilers*

Period, days	Grit, %		Significance
	0	0.8	
Feed intake, g			
7-14	329.98	324.23	0.240
14-21	579.17^{a}	543.33^{b}	<0.001
21-28	819.59	811.27	0.232
28-35	1011.05a	957.85b	0.004
35-42	1020.33	1016.99	0.428
7-21	909.15^{a}	867.56^{b}	<0.001
21-42	2850.96^{a}	2786.10^{b}	0.004
7-42	3760.11^{a}	3653.67^{b}	<0.001
Feed conversion ratio, g feed/ g weight gain			
7-14	1.50	1.51	0.827
14-21	1.69	1.54	0.182
21-28	1.86	1.89	0.918
28-35	2.05	1.93	0.525
35-42	2.14	2.07	0.627
7-21	1.61	1.53	0.190
21-42	2.02	1.96	0.715
7-42	1.90	1.83	0.591

n=4; ^{a,b} means a row followed by different letters differ significantly ($P<0.05$)

Table 4. *Effects of dietary grit supplementation on slaughter characteristics of broilers*

Characteristics, %	Grit, %		Significance
	0	0.8	
Carcass yield	75.18	74.65	0.161
Abdominal fat	1.97[a]	1.67[b]	0.008
Heart	0.60	0.58	0.236
Liver	2.68	2.70	0.759
Gizzard	1.51[b]	1.65[a]	0.001
Spleen	0.15	0.16	0.684
Bursa Fabricius	0.20	0.21	0.728

n=32; [a,b] means a row followed by different letters differ significantly (P<0.05)

Table 5. *Effects of dietary grit supplementation on serum total cholesterol and triglyceride in broilers*

Blood Parameters, mg/dL	Grit, %		Significance
	0	0.8	
Total cholesterol	93.25	94.88	0.736
Triglyceride	82.97	82.38	0.774

n=32; No statistical significant differences between groups

affected significantly by grit supplementation during the experimental period *(Table 3)*. However, 0.8% granite grit supplementation improved total feed conversion ratio numerically by 3.68% (P>0.05).

The effects of dietary grit supplementation on carcass yield and weight percentages of internal organs in broilers are shown in *Table 4*. In this study, granite grit supplementation did not significantly affect carcass yield. In contrary, the relative weight percentage of gizzard was increased (P=0.001) and the relative weight percentage of abdominal fat was decreased (P<0.05) by grit supplementation.

No significant differences were observed among the treatment groups in terms of blood serum total cholesterol and triglyceride levels during the experiment as shown in *Table 5*.

DISCUSSION

In the present study, inclusion of granite grit to the diets containing maize and barley as cereal grains did not significantly affect the final body weight and body weight gain. Similarly, some researchers stated that inclusion of insoluble grit had no effect on body weight and body weight gain of laying hens [12] and broilers [1,20,21]. However, Moghaddam et al.[11] reported that body weight gains were significantly improved in broilers added with grit having 2 mm size compared to chickens treated by grit having 3 and 4 mm in size (P<0.05) in direct proportion to the numerical increase in gizzard volume. Erener et al.[2] reported that grit supplementation to high energy and low fiber diets increased final body weight and total body weight gain.

Bale-Therik and Sabuna [22] showed that the diets contained grit had a significant effect on the body weight gain of local chicken as a result of improved digestibility of ingesta by increasing the grinding capability in the presence of grit. The differences in literatures may be due to the differences in source and particle size of the grit, diet ingredients and the diet composition.

In our study, during the experiment, feed intake was significantly reduced (P<0.05) by grit supplementation. Although feed conversion ratio was not statistically affected, 0.8% granite grit supplementation improved total feed conversion ratio numerically by 3.68% (P>0.05) *(Table 3)*. This numerical increment for feed conversion ratio can be explained by reduced feed intake. Similarly, Yalçın et al.[21] reported that feed intake during the experiment was significantly reduced and feed conversion ratio was not affected by grit addition to the diets based on maize and soybean. Erener et al.[2] also stated that although inclusion of grit to broiler diets decreased feed intake by approximately 10 g (P<0.01), grit consumed by chicks provided better performance in terms of feed intake and feed conversion ratio. Adeniji [23] reported that inclusion of 5% grit decreased feed intake and improved feed conversion ratio of one-day old pullet chicks fed rice husk. Garipoğlu et al.[1] stated that grit supplementation to diets of broiler decreased the feed intake. In contrary, feed intake and feed conversion ratio of broilers [13] and turkey toms fed to diets containing whole barley (up to 20%) [24] were not affected by grit addition. Contrary to all findings, Jin et al.[25] reported that the addition of dietary crude fiber and grit supplementation up to 4% to gosling diets improved average daily feed intake (P<0.001) and also stated that feeding grit may probably release more nutrients such as starch to supply more energy.

In the present experiment granite grit supplementation did not significantly affect carcass yield, weight percentages of heart, liver, spleen and bursa Fabricius. However, the most important findings in our study are the results of the increment in the relative weight percentage of gizzard (P=0.001) and the reduction in abdominal fat (P<0.05) with grit supplementation. Similar results were obtained in the study with grit supplementation to the diets based on maize and soybean [21]. Decrease in abdominal fat as the main source of poultry waste is very important point in broiler production. Some researchers [1,2,11] stated that there were no differences with using grit among the groups in terms of carcass yield and relative percentages of organ weight. Some early studies [1,26-28] showed that granite grit usage increased the weight of gizzard but had no effect on performance of broilers, which is also consistent with our results. This result can be explained by a large proportion and different size of the grit stones consumed by chicks

retained in the gizzard [2,29]. Besides, it has been reported by Makivic et al.[30] that insoluble fiber supplementation stimulates gizzard function by prolonging the transit time of ingested feed from the proventriculus to the gizzard, in despite of being not any increase of gizzard relative weight. Insoluble grit such as granite grit is more resistant of the dissolving pH secreted in the proventriculus, therefore having a longer retention time in the gizzard [29]. Some researches revealed the beneficial effects of grit stones on development of gizzard and thus had better performance of broiler [2,6,25,31,32]. Liu [20] stated that broilers fed granite grit had significantly larger values of gizzard content weight, relative gizzard content and empty gizzard weight. However, Fuerjiafu [13] reported that there was no significant difference in gizzard weight between grit-fed birds and non-grit-fed birds. Larsson [29] concluded that granite grit did not have any significant effects on performance and gizzard development in broilers.

However, the granite grit supplementation had been reported to have no significant effects on the relative weight percentage of abdominal fat in broiler [2] but was increased in goslings [25]. In contrary, the chicks fed 5% grit had a better (P>0.05) fat retention than the chicks fed 0% grit [23] Similar to the present study, some researchers showed that grit had no effect on serum total cholesterol and triglyceride level in broilers [21] and goslings [25].

As a conclusion, addition of 0.8% granite grit having a particle size of 1-2 mm to the diets containing maize and barley as cereal grains may be useful supplement in broilers in the field due to the improvements in relative weight of gizzard and reduction in relative weight of abdominal fat. However, further studies are needed to test the efficiency of granite grit supplementation in broilers fed diets having different fiber composition.

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Kafkas Univ Vet Fak Derg
25 (5): 689-696, 2019
DOI: 10.9775/kvfd.2018.21628

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

Research Article

Effect of β-Casomorphin-7 on Intestinal Mucosal Immunity in Aged Mice

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Article Code: KVFD-2018-21628 Received: 24.12.2018 Accepted: 09.04.2019 Published Online: 10.04.2019

How to Cite This Article

Yin H, Liu JJ, Yang D, Xu HQ: Effect of β-casomorphin-7 on intestinal mucosal immunity in aged mice. *Kafkas Univ Vet Fak Derg,* 25 (5): 689-696, 2019. DOI: 10.9775/kvfd.2018.21628

Abstract

The immune deficiency caused by aging deserves attention, especially the weakening of intestinal mucosal immunity. The effect of β-casomorphin-7 on intestinal mucosal immunity was investigated in aged mice. Mice were treated without or with different doses of β-casomorphin-7 for 30 days. Histopathological studies showed the tissue protective role of β-casomorphin-7 in aged mice. Low-dose group could significantly increase the level of IL-2 and TNF-α in intestinal mucosa. A significant increase in the level of SIgA was observed in medium- and high-dose groups. The low and medium dose groups could significantly increase the activity of SOD in small intestine mucosa. All dose groups significantly reduced the levels of MDA. The results suggest that β-casomorphin-7 could improve intestinal mucosal immune decline which is induced by aging The mechanisms for the regulating effects were likely through balancing the cytokine level and controlling the oxidative stress.

Keywords: *β-casomorphin-7, Aging intestinal mucosal immune cytokine, Oxidative stress*

Yaşlı Farelerde Bağırsak Mukozası Bağışıklığına β-Kazomorfin-7'nin Etkisi

Öz

Yaşlanmaya bağlı bağışıklık yetersizliği özellikle bağırsak mukozası bağışıklığı olmak üzere dikkat edilmesi gereken bir husustur. Bu çalışmada, yaşlı farelerin bağırsak mukozası bağışıklığına β-kazomorfin-7'nin etkisi araştırılmıştır. Farelere 30 gün süresince farklı dozlarda β-kazomorfin-7 içeren veya içermeyen uygulamalar yapıldı. Histopatolojik incelemelerde β-kazomorfin-7'nin yaşlı farelerde doku koruyucu etkisinin olduğu gözlemlendi. Düşük doz grubunda bağırsak mukozasında IL-2 ve TNF-α seviyeleri anlamlı derecede arttı. Orta ve yüksek doz gruplarında SIgA seviyesinde anlamlı bir artma gözlemlendi. Düşük ve orta doz gruplarında ince bağırsak mukozasında SOD aktivitesi anlamlı derecede arttı. Tüm doz gruplarında, MDA aktivitesi anlamlı derecede azaldı. Elde edilen sonuçlar, β-kazomorfin-7'nin yaşlılığa bağlı olarak gelişen bağırsak mukozası bağışıklığında meydana gelen düşüşü iyileştirebileceğini gösterdi. Bu düzenleyici etkiyi muhtemelen sitokin seviyesi ve oksidatif stresi kontrol altında tutmak suretiyle oluşturmaktadır.

Anahtar sözcükler: *β-Kazomorfin-7, Yaşlanan bağırsakta mukozal bağışıklık sitokin, Oksidatif stres*

INTRODUCTION

Our population is aging, but longevity is not always associated with good health. One of the most important effects of the aging process is the significant decline in the efficacy of the adaptive and congenital immune system, especially in the intestinal immune system. Compared with the systemic immune system, the age-related changes in the intestinal mucosal immune system are earlier than those of the systemic immune system [1]. The gut plays a vital role in absorbing nutrients and drugs, preventing pathogen invasion and maintaining the health of the body. In many elderly people, gastrointestinal dysfunction, mucosal defense deficiency, and oxidative stress increase, influence the ability to absorb nutrients and maintain the balance of normal microbial flora, which leads to lower immunity and an increase in the incidence of inflammation and autoimmunity in the elderly. Experiments show that the gut may become an important target for promoting longevity intervention [2].

The multifunctional properties of biologically active

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peptides from milk are increasingly acknowledged [3]. β-casomorphins belong to a family of opioid peptides derived from milk protein. β-Casomorphin-7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile, β-CM-7) was first isolated from an enzymatic digest of bovine β-casein [4]. Current researches show that β-CM-7 can regulate glucose [5], antioxidative [6,7], immunological [8], hormonal and neurological responses.

In addition, β-CM-7 is capable of modulating gene expression of the regulatory peptides from G and D cells. Data from in situ hybridization studies indicate that β-CM-7 affects gastrin gene expression indirectly by means of the paracrine action of somatostatin, and depends on its intrinsic molecular function [9]. Thus β-CM-7 is most likely to be a signal molecule in gastrointestinal tract. However, there is no report about the effect of β-CM-7 on intestinal mucosal immunity in aged mice.

Presented here, the aims of this study were to investigate whether management with β-CM-7 has any effects of regulating intestinal mucosal immunity in aged mice and its possible mechanism.

MATERIAL and METHODS

Chemicals and Reagents

β-CM-7 was purchased from Nanjing Peptide Biotech Co., Ltd. (Nanjing, China). The assay kits for analyses of superoxide dismutase (SOD), Catalase (CAT), malondialdehyde (MDA), Secretory immunoglobulin A (SIgA), Tumor Necrosis Factor-α (TNF-α) and Interleukin-2 (IL-2) were purchased from Jiancheng Biologic Project Company (Nanjing, China). All the other chemicals and reagents were of analytical grade.

Animals

Forty elderly male KM mice (11 months old) and ten young KM mice (2 months old) were purchased from Nanjing Qinglongshan Animal Center (Nanjing, China). They were housed under controlled environmental conditions of temperature (22±2°C) with a 12 h light/12 h dark cycle, and maintained on (unless otherwise stated) standard food pellets and tap water. All animal care and procedures were performed in accordance with Jiangsu Province and institutional policies for animal health and well-being. All mice samples collection and field study were approved by guide for care and use of laboratory animals of the protocol for animal study of Animal Management Committee of Jiangsu Province and Yangzhou University. The animals were acclimatized for 1 week before the study.

Experimental Design

The young mice (n=10) were as the control group (group I) while the aged mice (n=40) were randomly divided into four groups (group II to V):

Group I (Con, n=10): Young control mice with free access to normal diet and intragastric administration of Stroke-physiological saline solution for 30 days.

Group II (A con, n=10): Aged control mice with free access to a normal diet and intragastric administration of stroke-physiological saline solution for 30 days.

Group III (L, n=10): β-CM-7 treated mice; each animal was put on a normal diet and treated with the low dose of β-CM-7 (2×10^{-7} mol·d^{-1}, intragastric administration) for 30 days.

Group IV (M, n=10): β-CM-7 treated mice, each animal was put on a normal diet and treated with the medium dose of β-CM-7 (1×10^{-6}mol·d^{-1}, intragastric administration) for 30 days.

Group V (H, n=10): β-CM-7 treated mice, each animal was put on a normal diet and treated with the high dose of β-CM-7 (5×10^{-6}mol·d^{-1}, intragastric administration) for 30 days.

Collection of Organ Tissues

All mice were sacrificed and necropsy examination was carried out immediately. Intestinal mucosa was exposed and washed with phosphate buffer saline. Small intestine samples were taken and fixed in 4% neutral-buffered Polyoxymethylene and other intestinal mucosa samples were taken instantly into liquid Nitrogen and stored at −70°C.

Analytical Methods

Histopathological Observation: Small intestine fixed in 4% neutral-buffered Polyoxymethylene were embedded in paraffin, sliced at a thickness of 5 μm and stained with hematoxylin and eosin (H&E). The histological changes were observed by light microscopic examination at a magnification of 20 ×.

Assays of SOD, CAT, MDA, SIgA, TNF-α and IL-2 in Intestinal Mucosa Samples: The 10% homogenates of mucous membrane of small intestine were prepared in the phosphate buffer (0.1 M, pH 7.4) containing 1 mmol ethylenediaminetetra acetic acid (EDTA), 0.25 mM sucrose, 10 mM potassium chloride (KCl) and 1 mM phenylmethyl sulfonyl fluoride (PMSF).

Lipid peroxidation was determined by quantifying MDA concentrations, which was spectrophotometrically measured by the absorbance of a red-colored product with thiobarbituric acid.

Briefly, the determination of SOD activity was based on the production of O_2·− anions by the xanthine/xanthine oxidase system. The amount of SOD that inhibits 50% the rate of reduction under the specified conditions was regarded as one enzyme unit.

Catalase activities were determined as described by Sozmen et al.[10] in which the degradation of H_2O_2 is recorded spectrophotometrically at 240 nm. One unit of catalase was defined as the amount of enzyme which

decomposes 1 mol H_2O_2/min under specific conditions.

SIgA, TNF-α and IL-2 were determined using a commercial ELISA kit, according to the instructions.

Statistical Analysis

Data were analyzed statistically using SPSS 16.0 for Windows and expressed as the mean ± SD of 10 mice per group. Experimental results were compared by one-way ANOVA with least significant difference (LSD) post-hoc tests used to compare individual means as appropriate. $P<0.05$ or $P<0.01$ were considered statistically significant.

RESULTS

Effect of β-CM-7 on Histological Section in Intestinal Mucosa of Aged Mice

Fig. 1 was the result of HE staining in the mouse small intestine tissue section. As shown in *Fig. 1a*, the small intestinal villi in the young control group were even slender, elongated and tidy, and the tissue structure of the small intestine in the aged control group had different degrees of damage, showing intestinal villus loss and defect, increased width, short length, sparse arrangement, reduced height and density of mucous membrane, unarranged epithelial cells and interstitial atrophy.

In the β-CM-7 intervention group, the gap of the small intestinal villi was significantly reduced, the swelling was reduced and the arrangement was neatly restored. In the middle dose group, the villi of the small intestine were fine and neat, and the integrity was the best.

According to *Table 1*, the length of intestinal mucous villus in the aged control group was significantly lower than that in the control group ($P<0.05$), and the V/C value was significantly lower than that in the control group ($P<0.01$).

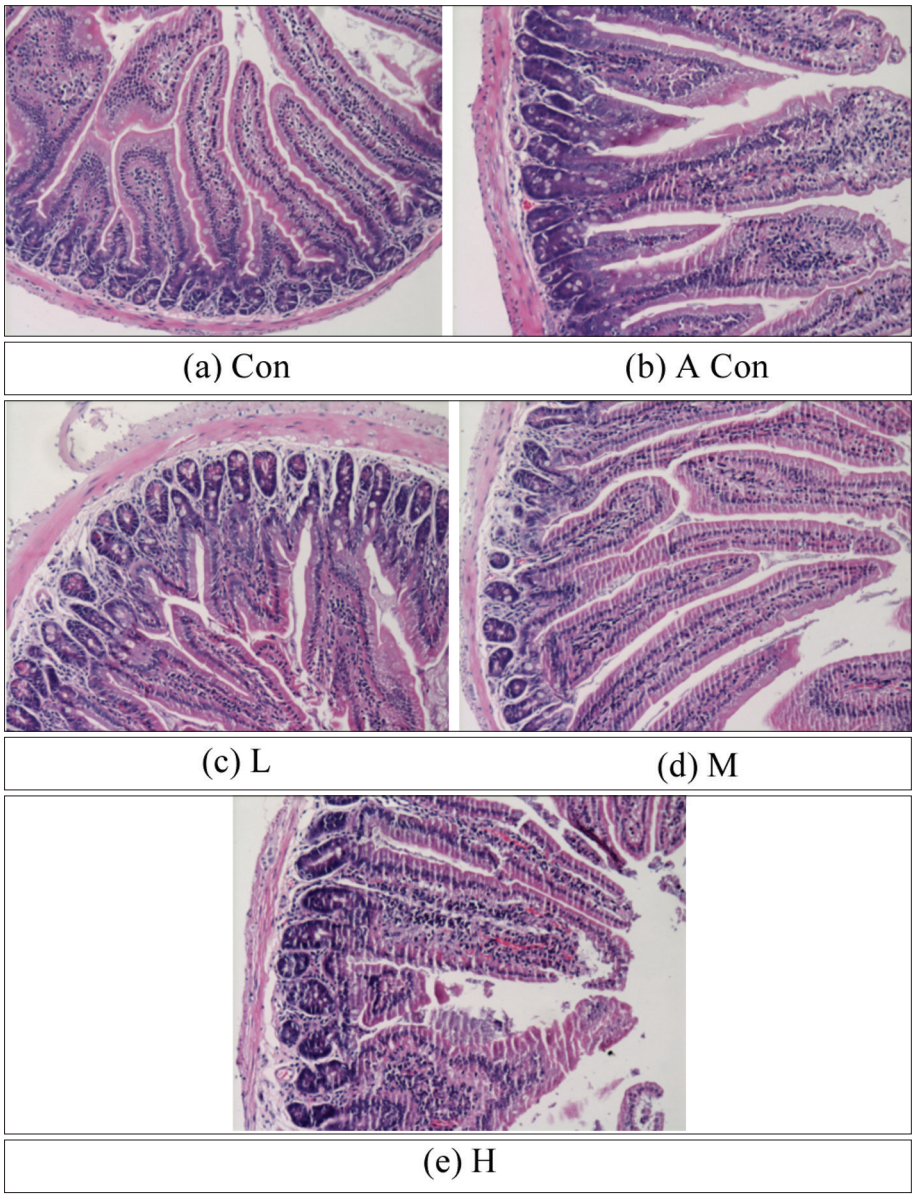


Fig 1. Histological section of small intestinal villi in mice (20×)

Table 1. *Effect of β-CM-7 on intestinal villus length, crypt depth and V/C value in mice (n=10)*

Group	Villus Length/μm	Crypt Depth/μm	Villus Length/Crypt Depth (V/C)
Con	280.49±40.34	47.30±10.80	6.25±1.78
A Con	208.92±31.83*	55.40±16.80	3.96±0.94**
L	289.50±40.12#	54.85±9.94	5.43±1.26#
M	275.00±75.11#	53.03±16.44	5.41±1.34#
H	264.62±98.68	55.40±11.81	4.88±1.81

*Compared with the Con group, P<0.05 was expressed by *, compared with the aged control group, P<0.05 was expressed with "#"*

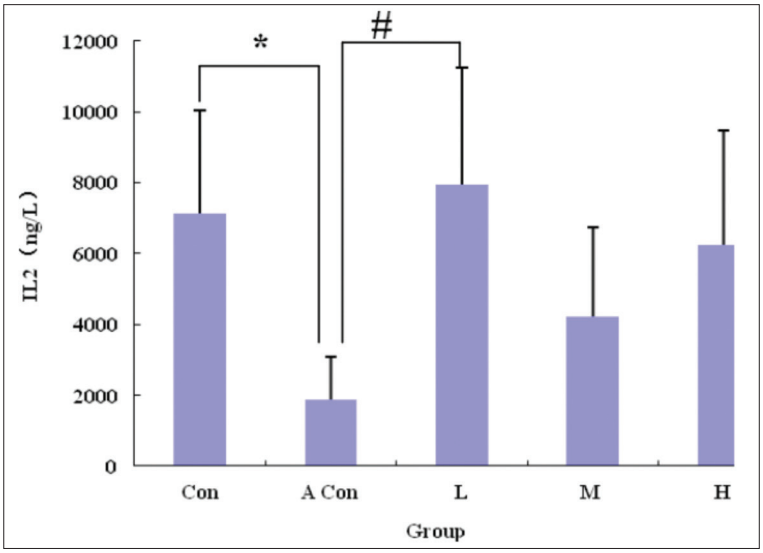
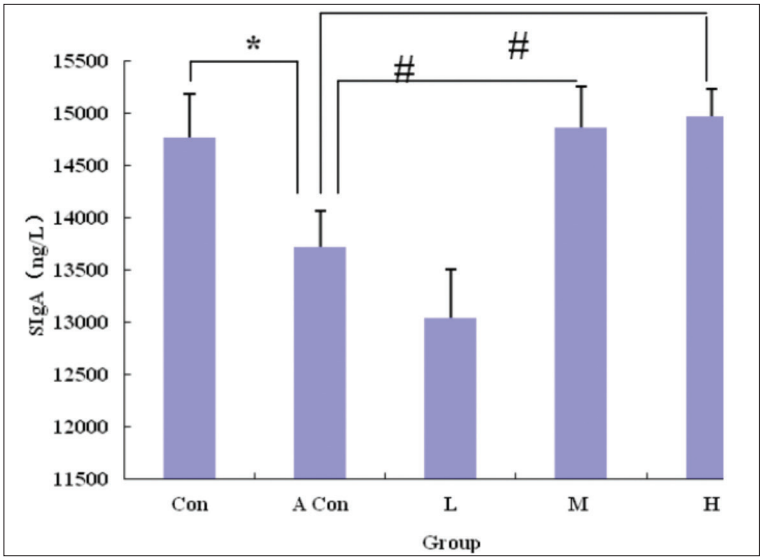


Fig 2. Effect of β-Casomorphin -7 on The level of IL-2 in intestinal mucosa of mice (n=10). Compared with the Con group, P<0.05 was expressed by *, compared with the aged control group, P<0.05 was expressed with "#"

Fig 3. Effect of β-casomorphin -7 on the level of SIgA in intestinal mucosa of mice (n=10).Compared with the Con group, P<0.05 was expressed by *, compared with the aged control group, P<0.05 was expressed with "#"



Compared with the aged control group, the dose group could increase the length of intestinal mucous villus and increase the value of V/C, and had a negative correlation with the dose. The low and middle dose groups could significantly increase the length and V/C value of intestinal mucous villus in mice, and there were statistical differences (P<0.05). However, there was no significant difference in the depth of crypt between each group, and there was no statistical difference (P>0.05).

Effect of β-CM-7 on IL-2, SIgA and TNF-α in Intestinal Mucosa of Aged Mice

According to *Fig. 2*, the level of IL-2 in intestinal mucosa of aged control group was significantly lower than that of young control group (P<0.05). Compared with the aged control group, the dose group could increase the level of IL-2 in the intestinal mucosa of mice, only the low dose group could significantly increase the level of IL-2

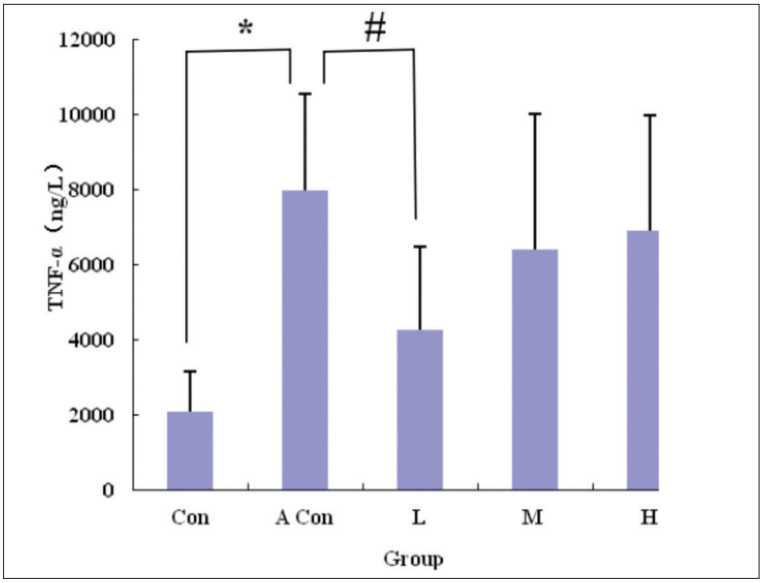


Fig 4. Effect of β-casomorphin -7 on the level of TNF-α in intestinal mucosa of mice (n=10). Compared with the Con group, P<0.05 was expressed by *, compared with the aged control group, P<0.05 was expressed with "#"

Table 2. *Effect ofβ-CM-7 on the activity of antioxidant enzymes in intestinal mucosa of mice (n=10)*

Group	SOD (U/mgprot)	MDA (nmol/mgprot)	CAT (U/mgprot)
Con	295.84±74.44	0.19±0.03	77.72±8.6
A Con	186.26±15.81**	0.40±0.15**	45.61±7.78**
L	322.97±49.93##	0.22±0.14#	60.35±17.71##
M	267.28±27.64##	0.24±0.22#	53.47±7.2
H	185.71±32.84	0.19±0.18##	47.68±14.18

*Compared with the Con group, P<0.05 was expressed by *, compared with the aged control group, P<0.05 was expressed with "#"*

in the small intestinal mucosa, and there was a statistical difference (P<0.05). In addition, the concentration of IL-2 in the intestinal mucosa of the high dose group was higher than that in the middle dose group, but there was no significant difference between the middle and high dose groups (P>0.05) compared with the aged control group. It is suggested that β-CM-7 can enhance the intestinal mucosal immunity of aged mice by increasing the IL-2 content of intestinal mucosa in mice.

The SIgA content of intestinal mucosa in aged control group was significantly lower than that in young control group (P<0.05). Compared with the aged control group, the middle dose and high dose group could significantly increase the SIgA content (P<0.01). The SIgA content in the low dose group decreased slightly, but the difference was not statistically significant (P>0.05) *(Fig. 3)*. It is suggested that β-CM-7 can improve the secretion of SIgA in the intestinal mucosa of aged mice and protect the intestinal immune barrier.

The level of TNF-α in intestinal mucosa of aged control group was significantly higher than that of young control group (P<0.01) *(Fig. 4)*. Compared with the aged control group, the dose group could reduce the content of TNF-α in the intestinal mucosa of mice in different degrees, and the low dose group could significantly reduce the content of TNF-α in the intestinal mucosa of mice, and there was a statistical difference (P<0.01). The middle dose group was lower than the high dose group, but compared with the aged control group, the difference between the middle and high dose groups was different. It was not significant (P>0.05). It is suggested that β-CM-7 can play a role in intestinal immune barrier by lowering the level of proinflammatory cytokine TNF-α.

Effect of β-CM-7 on SOD, MDA and CAT in Intestinal Mucosa of Aged Mice

According to *Table 2*, the activity of SOD in the intestinal mucosa of the aged control group was significantly lower than that in the young control group (P<0.01). Compared with the aged control group, the activity of SOD in the low dose group and the middle dose group increased significantly (P<0.01), and the low dose group increased more than the middle dose group, and there was a significant difference between the two groups (P<0.01) and the high dose group. The activity of SOD in the group was slightly lower than that in the model control group.

The content of MDA in the intestinal mucosa of the aged control group was significantly higher than that in the young control group (P<0.01). Compared with the aged control group, the content of MDA in the low dose group

and the middle dose group had a decreasing trend, and there was a difference (P<0.05), and the content of MDA in the high dose group was significantly lower than that of the aged control group (P<0.01).

The activity of CAT in the intestinal mucosa of the aged control group was significantly lower than that in the young control group (P<0.01). Compared with the model group, the activity of CAT in the low dose group increased significantly (P<0.01), and the activity of CAT in the middle and high dose groups was increased, but the difference was not significant (P>0.05).

DISCUSSION

The intestinal tract not only has the function of digestion and absorption, but also provides a natural defense barrier for the body. Adjacent intestinal epithelial cells are closely linked through intercellular junctional complexes, isolating the milieu interne of the tunicae propria and the environment outside the intestine. The intestinal barrier is divided into four parts: epithelial barrier, immune barrier, biological barrier and chemical barrier. The epithelial barrier and immune barrier are especially important. Intestinal epithelial barrier is the first line of defense against pathogens [11]. The normal structure and function of the small intestine are the basic guarantee of the nutrients being fully digested and absorbed and immune function, especially the length of intestinal villi, the depth of the recess and the thickness of the mucous membrane, which are the important indexes to measure the digestive and absorption function of the small intestine [12].

The results of this study showed that age growth could cause damage to the tissue structure of the small intestine, shorten the length of intestinal villi and decrease the ratio of the length of the villi/recess depth, which is in accordance with the results of Liang et al.[13] study. β-CM-7 can significantly increase the length of intestinal villi, increase the ratio of villi length/recess depth, alleviate the injury of intestinal mucosal tissue and protect the immune function of small intestinal mucosa.

Several studies have shown that aging can be accompanied by an increase in pro-inflammatory cytokines such as IL-6, IL-1 and TNF-α, which increase the chronic inflammation of the elderly [14,15]. It is believed that the increase of pro-inflammatory factor TNF-α causes the remodeling of actin, causing intestinal epithelial cells and close connections to be damaged, thereby damaging the intestinal mucosal barrier function and increasing the permeability of intestinal mucosa [16]. We observed a significant increase in TNF-α in the intestinal mucosa of aged mice, which is consistent with the results of the Miró et al.[17] study. After feeding different doses of β-CM-7, the content of TNF-α in intestinal mucosa of mice decreased, and the low dose had the most significant effect on TNF-α content in intestinal mucosa of mice. It indicated that β-CM-7 could reduce the damage of proinflammatory factors on intestinal mucosa and alleviate the effect of aging on intestinal mucosal immunity.

SIgA is the most immunologically active antibody on the surface of the intestinal mucosa. It is one of the main factors of intestinal mucosal immunity and an important indicator of the immune function of intestinal mucosa. As the first line of defense against potentially invasive pathogenic microbes and participating in host microbial interactions, SIgA can prevent bacteria from adhesion and proliferation on the surface of epithelial cells, prevent the uptake of bacterial toxins and other harmful substances from the mucous membrane, have synergistic bactericidal action with complement and lysozyme, and extensive immune protection [18]. This study showed that the expression of SIgA in the intestinal mucosa of the aged model mice was significantly lower than that in the young group, but after feeding β-CM-7, the content of SIgA in the middle and high dose groups could be greatly increased, and the SIgA content in the low dose group decreased slightly, but the difference was not statistically significant (P>0.05). The results showed that β-CM-7 could improve the secretion of SIgA in intestinal mucosa of aged mice and protect the intestinal immune barrier.

In 1980s, Watabe et al.[19] studies confirmed that IgA mediated response depends on the help of T lymphocytes, and the participation of cytokines is also required. IL-2 is one of the T lymphocyte growth factors. It is mainly produced by activated CD4+T and CD8+T cells. It can activate immune effect cells and produce synergistic effect factors, which can effectively remove tumor cells and virus bacteria infected cells. The results of this experiment showed that the expression of IL-2 in the intestinal mucosa of the aged model mice was significantly lower than that of the young group, but the dose group was significantly higher than the old control group. This was in accordance with the results of Zhao et al.[20] study. The study of Zhao et al.[20] showed that casein peptide could increase the serum IL-2 content in the aging model mice and have anti aging effect. Zhao et al.[20] study showed that the effect was positively correlated with the dose. However, the results of this experiment showed that the increase in the low dose group was the most obvious and did not have a positive correlation with the dose. The reasons for the inconsistency need to be further studied and analyzed.

Malondialdehyde is an oxygen free radical attacking the unsaturated fatty acid in cell membrane and triggering lipid peroxidation, which is a degrading substance [21,22]. The determination of MDA content can reflect the degree of oxidative damage in the body tissue. The increase of free radical content and the increase of MDA content indicate that the degree of tissue injury is aggravated, which is an important index of aging. According to *Table 2*, the content of MDA in the intestinal mucosa of aged mice was significantly higher than that of young mice

(P<0.01), indicating that the intestinal mucosa of old mice was in the state of oxidative stress. The content of MDA in low dose group and middle dose group had a decreasing trend (P<0.05), and the content of MDA in high dose group was significantly lower than that of model control group (P<0.01), which indicated that β-CM-7 could inhibit the damage of MDA on body tissue, and could reduce the level of oxidative stress in the elderly.

When the body constantly syntheses free radicals, the body produces some enzymes that scavenge free radicals. SOD is a specific scavenger of superoxide anion and the first line of defense against oxidation. The detection of the content of SOD can indirectly reflect the ability of scavenging oxygen free radicals [23]. As presented in *Table 2*, the SOD activity in the small intestinal mucosa of the aged mice was significantly lower than the SOD activity in the small intestinal mucosa of the young mice (P<0.01), which showed that the antioxidative ability of the aged mice was weakened. The low dose group and middle dose group could significantly increase the activity of SOD (P<0.01), indicating that β-CM-7 can up regulate the activity of SOD and enhance the antioxidant stress of small intestinal mucosa, which is consistent with the results of Yin et al.[6].

Catalase can promote the reaction of hydrogen peroxide in vivo, alleviate the damage of hydrogen peroxide on the body, and can represent the antioxidant capacity of the body [24]. According to *Table 2*, the activity of CAT in the intestinal mucosa of the aged mice was significantly lower than that of the young mice (P<0.01), and the low dose group could significantly increase the CAT activity of the small intestinal mucosa (P<0.01). The medium and high dose groups could improve the CAT activity of the small intestinal mucosa of the aged mice, but the difference was not significant (P>0.05). It indicated that β-CM-7 could improve the activity of CAT and enhances the ability of antioxidative stress in intestinal mucosa, which is consistent with the result of Yin et al.[6].

To sum up, aging can cause the increase of the concentration of inflammatory cytokines in intestinal mucosa, the damage of intestinal tissue structure and the decrease of immune function of small intestinal mucosa. β-CM-7 can significantly increase the content of IL-2 and SIgA, reduce the content of pro-inflammatory factor TNF-α and significantly improve the activity of antioxidant kinase, such as SOD and CAT in small intestinal mucosa, and then reduce the intestinal tissue damage caused by aging, maintain the normal form of intestinal tract and enhance the immunity of small intestinal mucosa. These results suggest that β-CM-7 has a certain protective effect on the intestinal mucosa of old animals, but the mechanism of β-CM-7 reconstruction of intestinal mucosal immune homeostasis and postponing inflammatory aging need further study.

In conclusion, β-CM-7 can significantly increase the content of IL-2 and SIgA, reduce the content of pro-inflammatory factor TNF-α and significantly improve the activity of antioxidant kinase in small intestinal mucosa. These results suggest that β-CM-7 has a certain protective effect on the intestinal mucosa of old animals, but the mechanism of β-CM-7 reconstruction of intestinal mucosal immune homeostasis and postponing inflammatory aging need further study.

Funding

This project was sponsored by grants from The Natural Science Foundation of the Jiangsu Higher Education Institutions of China (No: 16KJB330011 and 17KJB190001), Science and technology innovation fostering fund of Yangzhou University (No: 2016CXJ107), and Post-graduates scientific research and innovation projects (No:XKYCX18-133).

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Kafkas Univ Vet Fak Derg
25 (5): 697-701, 2019
DOI: 10.9775/kvfd.2018.21654

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

Research Article

Isolation and Molecular Identification of *Campylobacter* spp. from Vaginal Swab Sample Obtained from Sheep Herds with Abort History

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Article Code: KVFD-2018-21654 Received: 27.12.2018 Accepted: 10.04.2019 Published Online: 17.04.2019

How to Cite This Article

Gülmez Sağlam A, Akça D, Çelebi Ö, Büyük F, Çelik E, Coşkun MR, Şahin M, Otlu S: Isolation and molecular identification of *Campylobacter* spp. from vaginal swab sample obtained from sheep herds with abort history. *Kafkas Univ Vet Fak Derg,* 25 (5): 697-701, 2019. DOI: 10.9775/kvfd.2018.21654

Abstract

Campylobacteriosis is a contagious and zoonotic infection characterized by abortion and infertility in cattle and sheep. The objective of this study was to investigate *Campylobacter* spp. cause to abortion in sheep herds in Kars province. For this purpose, a total of 350 vaginal swab samples obtained from sheep with abortion were examined by cultural and molecular methods. Swab samples were inoculated on Preston Campylobacter Selective Agar for isolation of *Campylobacter* species. Following the incubation, suspected colonies were subjected to Gram staining, mobility, oxidase and catalase tests for identification. Multiplex PCR (m-PCR) was used for the identification of *Campylobacter* isolates at species level. *Campylobacter* spp. was isolated in 8 (2.28%) of the 350 vaginal swab samples examined. Of 3 isolates were identified as *Campylobacter jejuni* and 5 were *C. coli* by m-PCR. According to the data obtained from this study, it was revealed that *Campylobacter* species should be taken into consideration in the abortion cases of sheep in this region. Considering the risk of this infection both in terms of animal health and human health, it is thought that more attention should be given to protection and control measures.

Keywords: *Thermophilic Campylobacter spp., Sheep, Vaginal swab, m-PCR*

Abort Öykülü Koyun Sürülerinden Alınan Vaginal Sıvap Örneklerinden *Campylobacter* spp. İzolasyonu ve Moleküler İdentifikasyonu

Öz

Campylobacteriosis, sığır ve koyunlarda yavru atımı ve infertilite ile karakterize, bulaşıcı ve zoonotik bir infeksiyondur. Bu çalışmada, Kars ilindeki koyun sürülerinde gözlenen abort olaylarının *Campylobacter* spp. yönünden araştırılması amaçlandı. Bu amaçla, abort olgularının görüldüğü koyun sürülerinden alınan toplam 350 adet vaginal sıvap örneği kültürel ve moleküler yöntemlerle incelendi. *Campylobacter* türlerinin izolasyonu amacıyla alınan örneklerin Preston Campylobacter Selektif Agara ekimleri yapıldı. Üreme sonucu şüpheli kolonilere identifikasyon amacıyla, Gram boyama ve hareketlilik muayeneleri ile oksidaz ve katalaz testleri uygulandı. *Campylobacter* spp. olarak belirlenen şüpheli kolonilerin tür düzeyinde identifikasyonu için Multiplex PCR (m-PCR) kullanıldı. Toplamda incelenen 350 vaginal sıvap örneğinin 8 (%2.28)'inde *Campylobacter* spp. izolasyonu gerçekleştirildi. Multiplex PCR sonucunda 3'ü *Campylobacter jejuni* ve 5'i *C. coli* olarak tespit edildi. Bu çalışma sonucunda elde edilen verilere bakılarak yöremizdeki koyunlarda meydana gelen atık olgularında *Campylobacter* türlerinin de göz önüne alınması gerektiği ortaya konulmuştur. Bu infeksiyonun hem hayvan sağlığı hem de insan sağlığı açısından oluşturduğu risk göz önüne alınırsa koruma ve kontrol tedbirleri açısından daha fazla önem verilmesi gerektiği düşünülmektedir.

Anahtar sözcükler: *Termofilik Campylobacter spp., Koyun, Vaginal sıvap, m-PCR*

INTRODUCTION

Sheep breeding constitutes a significant part of the animal husbandry of Turkey. According to the data of 2017, the sheep population of Turkey is about 33 million and 450 thousand of which are farming in the Kars region [1]. One of the most important problems encountered in sheep breeding and economically damaging to the breeder is the abortion case. Bacterial, viral and protozoal infections are among the causes of abortion in animals. These

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infections are important in terms of public health as well as economically. Among the bacterial infections, brucellosis, campylobacteriosis, chlamydiosis and salmonellosis are responsible for most cases of abortion [2,3].

Campylobacteria which are pathogenic microorganisms for other animals and humans, can be found commensively in the intestinal flora of various domestic and wild animals and can cause gastrointestinal and genital infections in some cases. *Campylobacter* species cause epidemics in sheep and sporadic infections in other animals. Although healthy sheep can carry the bacteria in the intestine and gallbladder without clinical infection, some *Campylobacter* species can cause systemic infections. *Campylobacter* was first isolated from the aborted sheep fetus. Agent spreads to the environment through feces and genital secretions of infected animal and aborted fetus. When the disease first comes out, abort cases in the herd is seen 60-70%. *Campylobacter* infections are characterized by abortion, stillbirth, birth of premature and poor lambs in the 4-5th month of pregnancy and death of sheep due to metritis [4-8].

Campylobacter jejuni, *C. coli* and *C. fetus* subsp. *fetus* are species that are common in the world and cause reproductive diseases in sheep. The agent is Gram negative, motile and microaerophilic. Environmental samples such as soil and water and food can be contaminated with *Campylobacter* spp. as the result of contact with contaminants such as feces and aborted fetus. It is known that *Campylobacter* species cause cross-infection among some animal species [9]. Roug et al.[10], isolated *C. jejuni* and *C. coli* from sheep, goat, cattle and pigs in agricultural fairs in California. Results of this study are thought to show transmission *Campylobacter* species among animal species. Pao et al.[11], showed that sheep in small ruminant farms were exposed to *C. jejuni* infections at a greater risk than goats. Healthy sheep serve as reservoirs for *Campylobacter* species, especially in stressful conditions such as birth, weaning, and nutritional changes [12,13].

In this study, it was aimed to investigate the *Campylobacter* spp., which is one of the important abortion agents, from vaginal swab samples collected from sheep herds in the Kars region.

MATERIAL and METHODS

Ethical Approval

The experiment was carried out with the approval of Kafkas University Local Ethical Committee for Animal Experiments (KAÜ-HADYEK/2018-114).

Samples

Totally 350 vaginal swab samples obtained from 7 sheep herds in the Kars region were investigated for *Campylobacter* species.

Bacterial Isolation and Identification

In this study, vaginal swab samples were examined by the culture method. For pre-enrichment step, samples were inoculated in Preston Campylobacter Enrichment Broth containing 7% defibrinated horse blood and Preston Campylobacter selective supplement (SR117, OXOID) and were incubated in microaerobic conditions at 37°C and 42°C for 48 h. After incubation, 100 µL of the pre-enriched culture was plated on Preston Campylobacter Selective Agar plates and the plates were incubated at 37°C and 42°C for 48-72 h. The growth cultures were evaluated for the colony morphology, microscopic appearance, catalase and oxidase properties [14,15].

DNA Extraction and Multiplex PCR

The classical phenol-chloroform extraction method [16] was used for DNA extraction from the Campylobacter isolates. Then, the multiplex PCR (m-PCR) technique was carried out for thermophilic *Campylobacter* and the m-PCR was for *C. fetus* and *C. venerealis* [17,18]. The primer sets targeting the 23S rRNA gene of *Campylobacter* spp., the *hipO* gene of *C. jejuni*, the *glyA* gene of *C. coli*, *C. lari*, the *cstA* gene of *C. fetus*, the *virB11* gene of *C. venerealis* were used with the exception of the specific amplified products as 650, 323, 126, 251, 764 and 233 bp respectively *(Table 1)* [17,18]. Both genus and species-specific PCR was conducted in a single reaction.

Each m-PCR tube for thermophilic *Campylobacter* spp. contained 200 µM dNTP (Thermo Scientific, Lithuania); 2.5 µL of 10x reaction buffer (Thermo Scientific, Lithuania), 20 mM $MgCl_2$ (Thermo Scientific, Lithuania); 0.5 µM *C. jejuni* and *C. lari* primers; 1 µM *C. coli* and *C. fetus* primers, 2 µM *C. upsaliensis* primers; 0.2 µM 23S rRNA primer *(Table 1)*; 1.25 U of *Taq* DNA polymerase (Thermo Scientific, Lithuania), and 2.5 µL of whole-cell template DNA. The volume was adjusted with sterile distilled water to give 25 µL. DNA amplification was carried out in a thermocycler (Bio-rad, U.S.A) using an initial denaturation step at 95°C for 6 min followed by 30 cycles of amplification (denaturation at 95°C for 0.5 min, annealing at 59°C for 0.5 min, and extension at 72°C for 0.5 min), and was finalized with an extension at 72°C for 7 min.

Each m-PCR tube for *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* contained 0.5 mM of each dNTP (Thermo Scientific, Lithuania), 2 µL of 1x reaction buffer (Thermo Scientific, Lithuania), 2.5 mM $MgCl_2$ (Thermo Scientific, Lithuania), 0.625 µM MG3F/MG4R primer set, 0.375 µM nC1165g4F/nC1165g4R primer set, and 1.5 U Taq DNA polymerase (Thermo Scientific, Lithuania), 1 µL of whole-cell template DNA. The volume was adjusted with sterile distilled water to give 20 µL. For amplification, the following cycling conditions were used: initial denaturation for 3 min at 95°C followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 53°C, and extension for 1 min at 72°C.

Table 1. *Primer sequences used in the multiplex PCR assay and the expected sizes of the amplified products*

Primer	Sequence (5'–3')	Size	Target Gene
23SF 23SR	TATACCGGTAAGGAGTGCTGGAG ATCAATTAACCTTCGAGCACCG	650	23S rRNA
CJF CJR	ACTTCTTTATTGCTTGCTGC GCCACAACAAGTAAAGAAGC	323	*C. jejuni hipO*
CCF CCR	GTAAAACCAAAGCTTATCGTG TCCAGCAATGTGTGCAATG	126	C. coli glyA
CLF CLR	TAGAGAGATAGCAAAAGAGA TACACATAATAATCCCACCC	251	*C. lari glyA*
MG3F MG4R	GGTAGCCGCAGCTGCTAAGAT TAGCTACAATAACGACAACT	764	*C. fetus cstA*
nC1165g4F nC1165g4R	AGGACACAAATGGTAACTGG GATTGTATAGCGGACTTTGC	233	*C. fetus* subsp. *venerealis* virB11

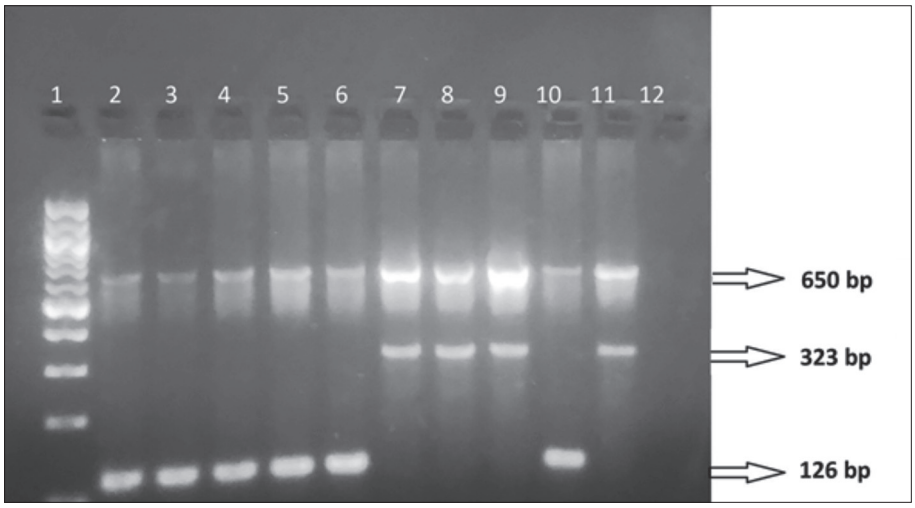

Fig 1. Gel electrophoresis image of m-PCR for thermophilic *Campylobacter* species. 1: DNA marker (Gene ruler 100 bp DNA Ladder, Fermentas); 2-9: Positive samples; 10: Positive control for *C. coli;* 11: Positive control for *C. jejuni;* 12: Negative control

The PCR reaction is accompanied by the *Campylobacter* reference strains and the amplified products were visualized by 1.5% agarose gel electrophoresis and the images were photographed under UV transilluminator (UVP, CA 91786, U.S.A.).

RESULTS

As the result of cultural examination colonies of the *Campylobacter* spp. were isolated showing microscopic characteristics such as small size, pinpoint morphology, non-hemolytic, and Gram-negative "gull-wing" shaped bacilli. Suspected isolates were subjected to biochemical tests. Thus, *Campylobacter* spp. was isolated in 8 (2.28%) of the 350 vaginal swab samples. Eight isolates, which were characterized as *Campylobacter* spp., were identified as *C. coli* (in 5 isolates) and *C. jejuni* (in 3 isolates) by using species-specific m-PCR *(Fig. 1)*.

DISCUSSION

Sheep farming has great importance for husbandry in Turkey. Abortions caused by infectious agents in sheep breeding are an important problem. These agents lead to significant economic losses, not only to a loss of an offspring but also to a decrease in milk yield, a decrease in the breeding value and in some cases infertility. Brucellosis (20-33.7%) was the first agent to be seen in the investigations of the infections causing abortion in sheep and this was followed by campylobacteriosis, chlamydiosis, listeriosis, and salmonellosis [3,19,20]. Campylobacteriosis is widely occurred all over the world and can be transmitted to people in contact with food, water, livestock and domestic animals, especially poultry [21,22]. Campylobacteriosis is the important cause of abortion in the sheep in many countries including Turkey [23-27]. Yardımcı et al.[28], reported that blood sera samples taken from sheep in Van region were analyzed by ELISA and detected *Campylobacter* antibody positivity in 39% of samples.

Many studies have been conducted to show *Campylobacter* spp. existence in sheep in many parts of the world. In the USA, Hansen et al.[29], reported as 5-17% risk of abortion due to *Campylobacter* species. Fallah et al.[25], have investigated 132 aborted sheep fetuses by PCR and showed 12 (9.09%) *C. fetus* subsp. *fetus* and 2 (1.51%) *C. jejuni* in Iran. Allsup [30], reported that Campylobacteriosis was the third responsible agent in sheep abortion and

increased from 6.8% in 1982 to 13.1% in 1984 in England. Species were determined according to the order of prevalence as *C. jejuni*, *C. fetus* subsp. *fetus* and *C. coli*.

Campylobacter species cause serious problems for animal and human health in our country as well as in the world and cause labor and economic losses. Karaman and Küçükkayan [31], have reported that *Campylobacter* spp. were isolated in 4 (1.3%) out of 297 aborted lambs obtained from different provinces between 1993-1997. In a similar study conducted by Küçükayan et al.[6], *Campylobacter* spp. were isolated in 6 (1.29%) out of 463 fetuses in 2003-2007 and all of them were identified as *C. fetus* subsp. *fetus*. Diker [32], had isolated *C. fetus* subsp. *fetus* from 15 (12,09%) out of 124 aborted sheep fetuses. Kenar et al.[33], reported that they isolated *Campylobacter* spp. in 20 (6,6%) of 303 aborted sheep fetuses. Kenar and Erganis [34], investigated 35 aborted sheep fetuses in Samsun and neighboring provinces during lambing season in 1991-1992 and *Campylobacter* spp. were isolated in 8 (22.9%) samples of which 5 (62.5%) were *C. fetus* subsp. *fetus*, 2 (25%) were *C. jejuni* and 1 (12.5%) was aerotolerant *Campylobacter*. Ekin et al.[24], investigated the presence of *Campylobacter* spp. in the gallbladder of healthy sheep in 2000 and 2002 years in Van region and found the *Campylobacter* spp. year-based prevalence as 27 (24.6%) and 24 (21.8%), respectively. Of the 27 *Campylobacter* strains isolated in 2000, 14 were identified as *C. jejuni*, 7 as *C. fetus*, 3 as *C. coli* and 3 as *C. lari*. Yeşilmen [12], have isolated the *Campylobacter* spp. in 10 (10%) out of 100 aborted sheep fetus in Diyarbakır province. Seven (70%) of the isolates were identified as *C. fetus* subsp. *fetus* and 3 (30%) were determined as *C. jejuni*. Büyük et al.[2], isolated *Campylobacter* spp. from 4 (10.25%) of 39 vaginal swab samples taken from sheep in Kars region. In a study conducted by Karakus [35], in Kars region, while both cattle and sheep have an important role as a source of *C. jejuni*, it was found that sheep played a more important role especially in the spread of *C. coli* to the environment.

In the present study, vaginal swab samples collected from sheep herds with abortion were examined in terms of *Campylobacter* species. *Campylobacter* spp. isolation was achieved in 8 (2.28%) vaginal swab samples. As the result of species-specific PCR analysis of isolates, 5 (62.5%) were identified as *C. coli* and 3 (37.5%) were *C. jejuni*. *Campylobacter* spp. isolation rate has varied between 1.2% and 92% in sheep in the world and in Turkey [2,26,36,37]. The results of this study were consistent with lots of researches. It is suggested that the factors cause the differences among the studies are the transport conditions of samples to laboratory, age and number of sampled animals, sampling season, isolation method and selective media used, hygiene and geographic structure [8,38].

In this study, it was revealed that *Campylobacter* infections should be taken into consideration in abortion cases occurring in sheep. It is also important since sheep can contaminate the environment and food with secreting the *C. jejuni* and *C. coli* and may play important role in human beings. Increased rate of isolation of *C. coli* from sheep will need more epidemiological investigations on this species as the *C. jejuni* is the primarily thermophilic agent in abortion cases.

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Kafkas Univ Vet Fak Derg
25 (5): 703-708, 2019
DOI: 10.9775/kvfd.2019.21702

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

Research Article

The Effect of Green Tea Extract Supplementation in Bull Semen Cryopreservation

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Article ID: KVFD-2019-21702 Received: 08.01.2019 Accepted: 07.05.2019 Published Online: 07.05.2019

How to Cite This Article

İnanç ME, Çil B, Yeni D, Avdatek F, Orakçı D, Tuncer PB, Türkmen R, Taşdemir U: The effect of green tea extract supplementation in bull bemen cryopreservation. *Kafkas Univ Vet Fak Derg,* 25 (5): 703-708, 2019. DOI: 10.9775/kvfd.2018.21702

Abstract

This study aims to investigate the effect of catechin (CT), green tea extract, as a supplement to Tris extender on semen quality parameters in frozen-thawed of bull sperm. Ejaculates were taken with artificial vagina from Holstein bulls and divided equal five aliquots, diluted to containing different amounts of CT (5, 10, 25 and 50 µg/mL) and no-additive (control). All samples were equilibrated at 4°C for 4 h and were frozen using a digital freezing machine. Post-thawed sperm motility and kinetic parameters were determined using the sperm analyser system. Spermatozoa DNA integrity was evaluated with the single cell gel electrophoresis, abnormal spermatozoa rate was evaluated by fluid fixation test and lipid peroxidation status was evaluated colorimetrically. CT supplementation did not improve motility and kinetic parameters. However, the higher morphological integrity was detected in CT10, 25 and 50 groups compared to control (P<0.05). Regarding chromatin integrity, positive effects of catechin were observed in the treatment groups while in CT 50 group adverse effects were found (P<0.05). Although there was no improvement in malondialdehyde levels, the highest total antioxidant activity was seen in the CT50 group (P<0.05). In conclusion, CT supplementation could be used the protection of morphological and DNA integrity from cryodamage and it has increased the total antioxidant activity depending of the dose in bull semen.

Keywords: *Bull semen freezing, Catechin, DNA Integrity, Lipid peroxidation*

Yeşil Çay Ekstraktı İlavesinin Boğa Sperması Dondurulmasına Etkisi

Öz

Bu çalışmanın amacı yeşil çay ekstraktı olan kateşinin (CT) tris sulandırıcısına eklenerek boğa sperma dondurulmasında dondurma çözdürme sonucu sperma kalitesinin incelenmesidir. Ejakülatlar Holstein boğalardan alınarak eşit beş kısma ayrıldı ve farklı oranlarda (5, 10, 25 and 50 µg/mL) CT içeren ve içermeyen (kontrol) sulandırıcı ile sulandırıldı. Bütün örnekler 4°C'de 4 saat ekilibrasyona bırakıldı ve daha sonra otomatik dondurma makinası ile dondurulduu. Çözüm sonu spermatozoa motilitesi ve kinetik parametreler sperma analiz sistemi ile değerlendirildi. DNA bütünlüğü tek hücre jel elektroforezi, anormal spermatozoa oranı sıvı fiksasyon testi ile, lipit peroksidasyon seviyesi ise kolorimetrik olarak ölçüldü. CT ilavesinin motilite ve kinetik parametreleri olumlu olarak etkilemediği görüldü. Fakat CT10 ve 25 ve 50 gruplarında kontrol grubuna göre daha yüksek morfolojik bütünlük tespit edildi (P<0.05). DNA bütünlüğü açısından CT'nin olumlu etkisi görülürken; CT 50 grubunda olumsuz etki tespit edildi (P<0.05). Malondialdehid seviyesi açısından herhangi bir iyileşme tespit edilememesine rağmen en yüksek total antioksidan kapasite CT50 grubunda görüldü. CT ilavesinin morfolojik ve DNA bütünlüğünü total antioksidan kapasitesini yükselterek soğuktan koruduğu tespit edildiği için boğa spermasında doza bağlı olarak kullanılabileceği sonucuna varıldı.

Anahtar sözcükler: *Boğa sperma dondurulması, Kateşin, DNA bütünlüğü, Lipid peroksidasyon*

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INTRODUCTION

Sperm cryopreservation is essential for preserving the genetic diversity, conservation of wild and domestic species, world-wide dissemination of genetic progress and livestock management [1]. However, it causes detrimental effects on sperm quality parameters, such as motility, morphology, viability and DNA integrity through the cryoinjury and it may also lead to the production of reactive oxygen species (ROS) [2]. Notably, the cold shock and atmospheric oxygen exposition during the semen collection and freezing/thawing procedures render the semen vulnerable for lipid peroxidation and lead to further damage to spermatozoa [3]. The primary sources of ROS in semen are the immature spermatozoa and leucocytes. Although adequate levels of ROS are required for some cellular processes as the capacitation, hyperactivation and binding of spermatozoa to the zona pellucida, excess amounts of ROS can adversely affect the motility, morphology and concentration of sperm as well as it can cause DNA damage and lipid peroxidation in the sperm [4]. Thus, to prevent the damage caused by ROS on spermatozoa, there has been a growing interest in the use of plant-based substances as antioxidants in assisted reproductive technologies [5]. Up to now, many plant-derived compounds, notably including carotenoids and flavonoids, have been studied for their antioxidant capacity to improve the fertility as components of *in vitro* culture media and through their intake as dietary supplements [6]. Some polyphenols have been found to exhibit higher antioxidant activity and lower toxicity than synthetic antioxidants [7]. As one of the natural antioxidants, green tea polyphenols are water-soluble, phytochemical flavonoids and include epigallocatechin gallate, epicatechin gallate, epicatechin and epigallocatechin [8]. They are found in high density in a variety of plant-based beverages and foods such as apricots, strawberries, black grapes and broad beans [3,9]. Consumption of catechin (CT) has been associated with the increased plasma antioxidant activity (the ability of plasma to scavenge free radicals), the resistance of LDL to oxidation and fat oxidation, while decreasing the plasma lipid peroxide and malondialdehyde (MDA) concentrations [10]. Recently, various effects of CT buck, ram and boar semen [11,12] have been presented by several research groups however, there were very few studies regarding its effect on the cryopreservation process in bulls [13]. Thus, the current study aims to investigate the effect of CT addition into Tris extender on sperm quality parameters following the cryopreservation of bull sperm.

MATERIAL and METHODS

Animal Experiments and Semen Collection

Semen from five bulls (Holstein breed) with proven fertility, aged 3-5 years, from Sultansuyu Agribusiness (Sultansuyu, Malatya, Turkey) was used for this study. Ejaculates taken by an artificial vagina once a week, the ejaculates were pooled to eliminate variability among the evaluated samples. This trial was replicated ten times for each group. All samples were kept in 37°C water bath for further evaluation of motility, concentration and progressive motility. All experiments were carried out in accordance with the approval of the Animal Care Committee of Afyon Kocatepe University Veterinary Medicine Faculty regarding ethics, with the authorisation number 49533702/29.

Semen Processing and Freezing

Semen volume was determined via a graded collection tube, and concentration was calculated with a photometer (Minitube GmbH, Tiefenbach, Germany). Samples were showing a minimum of 80% progressive motile and of 80% morphologically normal spermatozoa were used. A Tris-based extender was used to the primary medium in this study [5]. Extracted CT (10 mg) was diluted with 1 mL ethanol (Merck, 99%) to create the CT stock solution. Ejaculates were divided into five aliquots and extended $15x10^6$ spermatozoa/straw with the Tris extender containing no-additive (control) and CT (5, 10, 25 and 50 µg/mL), and subsequently, sperm was loaded into mini straws. The experiment semen samples were cooled (4°C) and equilibrated for 4 h. After, every group was frozen with controlled semen freezing machine (SY LAB Gerate GmbH, Neupurkersdorf, Austria) with Avdatek et al.[5] protocol. Finally, the straws were immersed in liquid nitrogen at -196°C. Frozen straws were thawed individually at 37°C for 30 s in a water bath for post-thawed spermological evaluations.

Assessment of Sperm Motility

Spermatozoa motilities were assessed using Computer-Assisted Sperm Analysis (CASA) system (Microptic S.L., Barcelona, Spain). A 5 µL diluted semen sample was put onto a slide (pre-warmed) put on to cover slide and percentages of progressive and non-progressive motility, as well as total motility, were recorded. Besides, motility kinetic parameters (curvilinear velocity µm/s (VCL), Straight linear velocity µm/s (VSL), average path velocity µm/s (VAP), amplitude of lateral head displacement, µm (ALH), Wobble (WOB, [VAP/VCL] × 100), beat cross frequency (BCF), Linearity (LIN, [VSL/VCL] ×100) and Strainess (STR, [VSL/VAP] ×100) and were determined. The spermatozoa motilities were calculated set as static, slow >20 µm/s, medium >60 µm/s and fast >80 µm/s protocols. For each assessment, between 220 and 370 spermatozoa were analysed in six different fields in microscope [5].

Evaluation of Sperm Morphology

Spermatozoa morphologies were evaluated Schafer and Holzmann [14] protocols. Hancock solution (500 mL double-distilled water with 150 mL buffer solution, 150 mL saline solution and 62.5 mL formalin 37%) was used. 10 µL sample was added to 1000 µL Hancock solution to examine spermatozoa morphological integrity. 5 µL mix was put on a slide and mounted with a cover slide. The morphological

integrity (tail, acrosome, head and total abnormality) were evaluated under phase-contrast microscopy (1.000×) by evaluated minimum 200 spermatozoa.

Evaluation of DNA Integrity

Spermatozoa DNA integrity was evaluated by the comet (single cell gel electrophoresis) assay kit using a (Trevigen, Gaithersburg, MD, USA). Slides were examined under fluorescent microscopy (Olympus CX31, Tokyo, Japan), and images were reflected for following scoring analysis with TriTek Comet Score software (V. 1.5). On each sample, a total of 100 spermatozoa cells from five different fields were evaluated for analysis [15].

Assessment of Oxidative Stress

Total antioxidant (TA) status was evaluated by using a colourimetrically commercial kit (RelAssay®, Gaziantep, Turkey). Glutathione peroxidase (GPx) activity was identified using a GPx assay (OxisResearch™, Bioxytech® GPx-340™, Portland, USA). Levels of lipid peroxidation, which depends on MDA, were measured using a commercial kit (MDA-586; OxisResearch, Portland, USA). The results were indicated in µmol/mL [16].

Statistical Analysis

One-way analysis of variance (ANOVA) and Duncan's post hoc test were used to state the differences among the treatment groups in terms all spermatological and biochemical parameters. Data are presented as a mean ± standard error of means (SEM). The degree of significance was set at P<0.05. SPSS/PC (Version 10.0; SPSS, Chicago, IL) software package program was used for all analysis.

RESULTS

As presented in *Table 1*, CT supplementation did not enhance the motility or the kinetic parameters of sperm. In other respects, CT50 had led to a significant decrease in motility and sperm motion characteristics (P<0.05). CT10, 25 and 50 concentrations have shown lower total abnormalities compared the control *(Table 2;* P<0.05) however, CT50 has produced unfavorable results regarding the chromatin integrity *(Table 3;* P<0.05). The other treatment groups had shown the lowest tail moment values, indicating the minimal DNA damage *(Table 3*; P<0.05). As shown in *Table 4*, the increased antioxidant activity of the CT was determined to start from the CT10 concentration (P<0.05). A dose-depending positive effect was observed regarding the GPx and total antioxidant capacity values however, MDA values were increased as well within the higher dose groups.

DISCUSSION

As various authors well documented, freezing and thawing steps of cryopreservation reduce the motility

Table 1. *Mean (±SEM) sperm motility values in frozen thawed bull semen*

Analysis	Control	CT5	CT10	CT25	CT50	P
Non progressive motility (%)	26.14±3.23^{a}	37.00±3.71^{a}	32.31±3.26^{a}	36.88±3.83^{a}	14.10±3.51^{b}	*
Progressive motility (%)	21.71±2.80^{a}	23.03±2.44^{a}	19.17±2.21^{ab}	14.88±1.90^{b}	3.47±1.26^{c}	*
Total motility (%)	47.88±5.73^{a}	60.04±5.91^{a}	51.49±5.37^{a}	51.76±5.34^{a}	17.55±4.72^{b}	*
VAP (µm/s)	78.98±2.65^{a}	73.55±2.27^{ab}	76.04±2.42^{ab}	67.11±3.76^{b}	49.14±3.58^{c}	*
VSL (µm/s)	55.84±2.10^{a}	48.50±1.47^{b}	47.60±1.20^{b}	39.20±2.42^{c}	28.16±2.36^{d}	*
VCL (µm/s)	111.34±2.78^{a}	108.91±2.45^{a}	112.86±2.98^{a}	106.99±4.50^{a}	83.77±5.95^{b}	*
ALH (µm/s)	4.10±0.08	4.12±0.06	4.04± 0.07	4.14±0.05	3.52±0.58	-
BCF (Hz)	11.86±0.45^{a}	11.03±0.23^{a}	11.56±0.17^{a}	10.98±0.34^{a}	8.21±1.28^{b}	*
LIN (%)	50.16±1.37^{a}	44.53±0.86^{b}	42.31±1.17^{b}	36.70±1.72^{c}	33.82±1.92^{c}	*
STR (%)	70.68±1.16^{a}	66.01±1.01^{b}	62.87±1.71^{bc}	58.62±1.97^{cd}	57.01±1.60^{d}	*
WOB µm s^{-1}	70.85±1.01^{a}	67.47±0.89^{a}	67.32±0.68^{a}	62.51±1.35^{b}	58.97±1.88^{b}	*

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

Table 2. *Mean (±SEM) sperm abnormality values in frozen thawed bull semen*

Analysis	Control	CT5	CT10	CT25	CT50	P
Head abnormalities (%)	2.95±2.83	3.86±2.41	1.75±1.77	2.59±2.08	2.44±1.73	-
Mid-piece abnormalities (%)	7.34±4.86^{a}	5.63±1.47^{ab}	3.89±3.51^{b}	4.34±3.39^{ab}	2.65±1.83^{b}	*
Tail abnormalities (%)	6.21±4.35^{a}	4.03±4.16^{ab}	6.01±4.95^{a}	3.62±2.61^{ab}	1.97±0.78^{b}	*
Total abnormalities (%)	16.50±5.85^{a}	13.53±6.18^{ab}	11.65±5.96^{abc}	10.56±4.65^{bc}	7.07±3.96^{c}	*

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

Table 3. *Mean (±SEM) chromatin integrity values in frozen thawed bull semen*

Analysis	Control	CT5	CT10	CT25	CT50	P
Tail length (µm/s)	19.27±3.18^{b}	14.26±3.12^{bc}	11.71±3.97^{bc}	8.75±2.61^{c}	37.60±4.07^{a}	*
Tail DNA (%)	19.11±3.06^{b}	12.36±1.59^{b}	23.04±5.58^{b}	17.85±1.77^{b}	45.10±4.90^{a}	*
Tail moment (µm/s)	29.01±4.06^{a}	12.59±2.06^{b}	11.16±2.04^{b}	7.43±2.77^{b}	22.30±3.17^{a}	*

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

Table 4. *Mean (±SEM) glutathione peroxidase (GPx), malondialdehyde (MDA) and total antioxidant (TA) activities in frozen thawed bull semen*

Analysis	Control	CT5	CT10	CT25	CT50	P
GPx (mU/mL)	12.53±0.12^{d}	12.91±0.15^{cd}	13.22±0.20^{bc}	13.69±0.29^{b}	15.83±0.14^{a}	*
MDA (µmol/mL)	2.51±0.02^{d}	2.57±0.02^{d}	2.67±0.03^{c}	2.82±0.02^{b}	3.12±0.02^{a}	*
Total antioxidant activities (mmol/trolox/mL-10^{9} cell/mL)	0.14±0.01^{d}	0.15±0.02^{d}	0.16±0.02^{c}	0.24±0.01^{b}	0.27±0.01^{a}	*

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

and fertilizing ability of spermatozoa leads to damage of plasma membrane [17], induce premature capacitation and nuclear decondensation [18]. ROS production during these cycles is considered to be one of the major cause that is accountable for the emerging impairment in the functionality of the sperm cell [19]. Whereas the problem is well-being pointed, the solution to prevent these damage is still being researched by many scientific groups. In recent years, numerous antioxidants have been introduced to the cryopreservation process on account of enhancing the post-thawed quality of sperm. Due to lack of carbonyl group in the epicatechin molecules, it is considered as a less potent antioxidant than other flavonoids such as quercetin or naringenin [12,20]. In the meantime, the emerging use of plant-based scavengers has resulted in controversial effects [7,21,22]. In the present study, CT supplementation did not enhance the post-thawed motility or kinetic parameters, but rather, adverse effects were observed in the highest dose group (P<0.05). In accordance with the delivered study, Gale et al.[23], the addition of green tea extract to the cryo-medium of boar semen extender did not produce any beneficial effects on motility, viability, acrosome integrity or membrane integrity. Another boar sperm study, in which, the toxicity of green tea extract on chilled spermatozoa was evaluated. Although no toxic effect was observed, sperm quality parameters did not differ between the control and different concentrations of green tea extract supplementation [11]. Additionally, in several studies on different species, the inclusion of natural antioxidants did not produce any positive effects [24-26]. On the other hand, several previous studies in different species generated results inconsistent to those found in current study, namely in chilled dog semen, the addition of green tea polyphenols into the extender has shown a significant protective effect on the motility and viability parameters up to four weeks of semen [8]. Also on boar semen [11] and at low concentration on human sperm [27] motility can be improved by green tea extract. Khan et al.[28] have cryopreserved the bull semen with different rates of (0.25; 0.5; 0.75; 1.0%) green tea extract and evaluated *in vitro* spermological parameters (motility, viability and membrane integrity). They found the highest progress in 0.75% green tea groups. Besides, in bull semen cryopreservation, in which motility and membrane integrity were improved with the supplementation of green tea extract [13]. Considering our results, we can hypothesize that the discrepancy in the results may be due to the density of the other substances (Tris, egg yolk) used in the extender.

Since catechins are unstable molecules, ROS formation can occur due to auto-oxidation. The stability of these molecules can be altered depending on the environmental temperature, pH or oxygen level [29]. When the antioxidant capacity of the samples was evaluated, decreased lipid peroxidation level was observed in CT concentrations starting from the 10 µg/mL (P<0.05). A dose-dependent positive effect was detected regarding the GPx and total antioxidant capacity; however, MDA values were increased as well within the higher dose groups. El- Seadawy et al. [30] have found that addition of pomegranate peel methanolic extract enriched with CT into chilled rabbit semen has decreased the lipid peroxidation level and increased the antioxidant activity. Similar results were observed in a rat [31], ram [12] and stallion [32] semen in which, were extended by CT supplementations. Also, Sugiyama et al.[33] reported that epigallocatechin gallate promotes protection against testicular ischemia-reperfusion injury due to its antioxidant activity since epigallocatechin gallate is the major CT compound of green tea extract with a 52% ratio of the total CT content [34]. The results demonstrated that polyphenols might interact with components of the spermatozoa and would have decreased the lipid peroxidation induced by free radicals [35]. On the contrary with the present study, Moretti et al.[20] supplemented swim-up selected human semen with 200 µM epicatechin and they could not find any improvement regarding the antioxidant capacity. This

result might have seen due to the chemical structure of epicatechin which does not contain the carbonyl group.

The COMET assay is widely used for analysing DNA damages in multiple cell [36]. It is a suitable cell evaluation method while maintaining the integrity of genetic material in biological evaluations [37]. Zini et al.[38] reported that DNA damage of spermatozoa has a high impact on the fertilization rate, embryo quality, and the rate of miscarriages [39]. Green tea extract might also have a promoting effect on *in vitro* maturation and embryo development [40-42]. In the current study highest dose of CT had adversely affected the DNA integrity; however, the lower doses were able to protect the DNA integrity compared to control ($P<0.05$). Besides CT10, 25 and 50 concentrations have shown lower total abnormalities. This results might be due to phenolic compounds of CT that improve morphological and DNA integrity if an appropriate amount is used. Various research supports the present study with obtained improvement on morphological integrity in different animal models [11,32] in which, the effects were observed with the supplementation of different rates green tea extracts. On the contrary of our results, Bucci et al.[42] did not find any improvement in supplementing the thawing medium of boar semen with epigallocatechin gallate (50 µM). On the basis of our results, we might hypothesize that the diferences among our results may be associated with the amounts and types of antioxidants that were used.

In conclusion, CT supplementation has provided the protection of morphological and DNA integrity from cryo-damage and it has increased the total antioxidant activity depending of the dose in bull semen. Addition of 25 µg/mL CT concentration in Tris extender can be beneficial when the overall parameters are considered. Further research is required to understand the cellular mechanisms involved in antioxidant activity.

Acknowledgement

This study was supported by General Directorate of Agribusiness, Ankara, Turkey.

Conflict of Interest

The authors confirm that they have no conflict of interest to declare

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Kafkas Univ Vet Fak Derg
25 (5): 709-716, 2019
DOI: 10.9775/kvfd.2019.21726

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

Research Article

Amelioration Effects of Vitamin E, Melatonin, L-carnitine, and Atorvastatin, on Destructive Effects of Busulfan in the Testes of Male Rats: A Gene Expression Evaluation

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Article ID: KVFD-2019-21726 Received: 11.01.2019 Accepted: 06.05.2019 Published Online: 06.05.2019

How to Cite This Article

Salehinezhad F, Eshraghi H, Kadivar A, Shirian S, Asghari A, Aali E, Davoodian N: Amelioration effects of vitamin E, melatonin, L-carnitine, and atorvastatin, on destructive effects of busulfan in the testes of male rats: A gene expression evaluation. *Kafkas Univ Vet Fak Derg,* 25 (5): 709-716, 2019. DOI: 10.9775/kvfd.2019.21726

Abstract

According to toxicity of various types of cancer treatments on different kind of cells with high division activities such as germ cells, antioxidants may protect these cells in testes against the toxic effects of the chemotherapeutic drugs. For this purpose, 24 h after busulfan treatment, 30 adult male wistar-rats were divided to six groups. Intra-peritoneally administrations of normal saline in control group and DMSO (as a busulfan solvent) in DMSO group were performed daily for 6 weeks beside the treatment contain vitamin E (Vit-E group), L-carnitine and melatonin (LM group), atorvastatin and melatonin (AM group), atorvastatin, L-carnitine, and melatonin (ALM group). After decapitation and removal of the testes, molecular evaluations were performed by the relative abundance measurement of *DAZL*, *Bcl2*, and *Casp3* transcripts. The results of this study exhibited high level of expression of DAZL in Vit-E treated rats compared to control counterparts (P<0.01). The expression level of *Bcl2* is significantly down-regulated in LM (P<0.008), and ALM groups (P<0.001), and the relative abundance of *Casp3* transcripts was significantly lower in AM (P<0.001) and ALM (P<0.007) than that of control group. As well as, there was significant high expression of this gene in Vit E-treated rats compared to the rats of control group. In conclusion, busulfan destructive effects were moderated with Vit-E administration through regulation of the expression of *DAZL*. The other antioxidants used in different combinations had not amelioration effects on spermatogenesis in busulfan-induced male rats, though the positive effects of some of these antioxidants on apoptosis reduction.

Keywords: *Rat, Busulfan, Vitamin E, Melatonin, L-carnitine, Atorvastatin, DAZL, Bcl2, Casp3*

Erkek Rat Testislerinde Busulfan Kaynaklı Hasara Karşı Vitamin E, Melatonin, L-karnitin ve Atorvastatin'in Koruyucu Etkisi: Gen Ekspresyonunun Değerlendirilmesi

Öz

Çeşitli kanser ilaçlarının toksisitelerine bağlı olarak eşey hücreleri gibi yüksek bölünme kapasitesine sahip çeşitli hücrelerde meydana gelen hasara karşı antioksidanlar koruyucu olabilir ve bu durum testis dokusunda kemoterapötik ilaçlarla oluşan hasarda etkili olabilir. Bu amaçla, 24 saat busulfan uygulaması sonucunda 30 ergin erkek Wistar rat altı gruba ayrıldı. İntraperitoneal olarak 6 hafta süresince DMSO grubuna fizyolojik tuzlu su ve DMSO (busulfan çözücüsü olarak) uygulandı. Diğer gruplarda busulfan uygulamasına ilave olarak E (Vit-E grubu), L-karnitin ve melatonin (LM grubu), atorvastatin ve melatonin (AM grubu), atorvastatin, L-karnitin ve melatonin (ALM grubu) uygulamaları gerçekleştirildi. Hayvanlarda dekapitasyonu takiben testis dokuları alındı ve *DAZL, Bcl2, ve Casp3* miktarları karşılaştırmalı olarak moleküler açıdan değerlendirildi. Çalışma sonucu, Vit-E uygulanan ratlarda DAZL ekspresyonunun kontrol grubuna oranla daha fazla olduğu gözlemlendi (P<0.01). LM (P<0.008) ve ALM (P<0.001) gruplarında *Bcl2* ekspresyonu aşağı yönde regule edilirken, AM (P<0.001) ve ALM (P<0.007) gruplarında *Casp3* ekspresyonu anlamlı derecede control grubuna oranla daha azdı. Kontrol ile karşılaştırıldığında Vit-E uygulanan grupta *Casp3* ekspresyonunun anlamlı derecede yüksek olduğu belirlendi. Sonuç olarak, busulfan kaynaklı hasarın Vit-E uygulaması sonucu *DAZL* ekspresyonu yolu ile normalize edildiği tespit edildi. Busulfan ile erkek ratlarda spermatogenez üzerine oluşturulan hasara karşı çalışmada uygulanan diğer antioksidanların hasarı azaltmada etkili olmadığı ancak bazılarının apoptozis üzerine etkisinin olduğu belirlendi.

Anahtar sözcükler: *Rat, Busulfan, Vitamin E, Melatonin, L-karnitin, Atorvastatin, DAZL, Bcl2, Casp3*

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INTRODUCTION

Alkylating agents as chemotherapeutic drugs are often used to treat cancer and increase the survival rates of patients. Alkylating agents, affect cell division by adhesion to one strands of the DNA [1]. Therefore, cells or tissues with high division activities such as germ cells and testes are more susceptible to these agents' side effects. Spermato-genesis makes round haploid spermatids through the reductive divisions of meiosis [2]. It is clear that certain chemo-therapeutic drugs specially alkylating agents influence spermatogenesis at least temporarily and in some cases permanently. Single doses of busulfan as an alkylating agent can permanently sterilize rats at non-lethal doses and cause long-term morphological damage to sperm produced by surviving spermatogonia [3]. Busulfan toxicity occurs by several different mechanisms, including reactive oxygen species (ROS) formation and protein damage (oxidation) [4]. ROS are directly involved in precarious oxidative damage of cellular macromolecules such as proteins and nucleic acids in germ cells, which can lead to cell death [5]. Indeed, the overproduction of ROS and the consequent oxidative stress has a critical role in inhibition of development of germ cells. Forasmuch as, the rats treated with Busulfan exhibited a defined increase in apoptosis, using cytoprotective and anti-apoptotic agents such as antioxidants can neutralize both ROS and apoptosis procedure caused by them [6]. ROS are scavenged by some antioxidants such as L-carnitine, Vitamin E (α-tocopherol), and Atorvastatin via their interfering with the lipid peroxidation chain reaction. Another small molecule antioxidant, Melatonin, which can detoxify highly reactive hydroxyl free radicals (OH) is involved in the detoxification of free radicals [7]. Co-administration of these components also change produced ROS profile, prevented lipid peroxidation and improved antioxidant status, synergistically [8]. Therefore, strategies that effectively preserve fertility during the course of cancer treatment especially through the decrease in apoptosis should be developed.

In this study all mentioned antioxidants, singly or in different combinations, were used to amelioration busulfan side effects in treated male rats and then the expression amount of deleted in azoospermia-like *(DAZL)* as a gene related to spermatogenesis resumption and B-cell lymphoma 2 *(Bcl2)* and Caspase 3 *(Casp3)* as the genes related to apoptosis was measured.

DAZL is a gene cluster which gets deletions in at least 10% of males with oligozoospermia or azoospermia [9].This gene is a DAZ autosomal homologue. As a result, *DAZL* has always been seen as a promising candidate for male infertility. *DAZL* plays an important role in the spermatogenic processes.

Bcl2 gene is an anti-apoptotic member of *Bcl2* Family, regulators of the cellular life-or-death switch, that prevents cytochrome C release, and hence caspase-9 activation and subsequently several other caspases, independently of mitochondrial damage [10]. Besides, caspase-3 is a cysteine protease that is activated early in a sequence of events associated with apoptosis [11].

The aim of the present study was reduction the amount of apoptosis and subsequently improves the spermato-genesis and preserves fertility of azoospermic animal model using busulfan in rat, with a focus on related molecular pathways.

MATERIAL and METHODS

Animals

Thirty adult male wistar-Rats (2-3 months old and 200-250 g) were purchased from Faculty of Veterinary Medicine of Shahrekord University and were housed for 2 weeks at the animal lab of Veterinary Clinic under the Standard laboratory conditions (12 h dark and 12 h light cycle, temperature of 23±3°C, and 50±5% humidity) for adaptability of rats to new living environment. Animal cages were kept clean, and commercial food (pellet) and water were provided *ad libitum*.

Experimental Design

In total, all rats were treated with two doses of 25 and 10 mg/kg busulfan with 14 days interval and after 24 h were randomly assigned to the following groups: Sham group that treated with DMSO as busulfan solvent, treated with busulfan (control group), and treatment groups including: treated with 100 mg/kg vitamin E (Vit E group), treated with 100 mg/kg L-carnitine and 1 mg/kg melatonin (LM group), treated with 20 mg/kg atorvastatin and 1 mg/kg melatonin (AM group), treated with 20 mg/kg atorvastatin, 100 mg/kg L-carnitine, and 1 mg/kg Melatonin (ALM group). Each group assigned by 5 rats. In all groups all administrations were performed intra-peritoneally daily for 6 weeks. Busulfan dose was assigned based on previous studies that established the toxic effect of busulfan on rat testes [12]. The atorvastatin [13], L-carnitine [13], melatonin [14] and Vit E [15] doses were also selected based on previous reports demonstrating their anti-oxidative effect. This study was approved by the Institutional Ethics Committee for Animal Experimentation and was conducted in accordance with the international guidelines [16].

Organ Removal and Tissue Processing

Animals were killed by decapitation under ether anesthesia, and testes of the animals were removed. One testis was used for sperm collection and the other one was preserved in -80°C freezer until molecular evaluations.

Sperm Collection and Evaluation

Sperm was collected from tail of left epididymis for all the

experimental rats. The left epididymis was immediately minced with scalpels and placed in pre-warmed microtube, containing 1 mL of Human Tubal Fluid (HTF) medium without BSA and placed in a 37°C incubator for 10 min. For sperm counting, 50 µL of the solution was diluted 10 times with 0.9% saline and a drop was transferred into chamber of Neubauer hemocytometer. Sperm counted under a standard optical microscope in order to determine the number of spermatozoa.

After 10 min of incubation, sperm motility was assessed by putting one drop of the on a warmed microscope slide. A cover slip was placed and at least 5 microscopic fields was observed at 400-fold magnifications. The percentage of nonmotile sperm was recorded for each rat.

Sperm viability was assessed with eosin–nigrosin staining. For this, 10 µL of sperm suspension were mixed with equal volume of eosin-nigrosin on a warmed microscope slide and a thin smear was prepared. Samples were observed under light microscope at a magnification of 1000×. At least 100 sperm were evaluated to discriminate death sperm (red stained) of live (not stained).

Molecular Evaluation

Total RNA Extraction, DNAase Treatment and cDNA Synthesis: To extract total RNA, tissues of testes (100 mg) were mechanical fragmented with a scalpel. Half mL RNX-PLUS solution (Sinaclon Bioscience, Karaj, Iran) containing phenol and guanidine was added and placed at room temperature for 5 min to homogenized samples. After adding 120 µL chloroform, the mixture was centrifuged at 8.000 g and 4°C for 5 min. The upper aqueous phase of supernatant was separated, and after addition of 400 µL isopropanol (100%), it was centrifuged (8000 g, 4°C, 5 min) and the RNA pellet was washed with 80% ethanol. Upon centrifugation at the same condition, the pellet was suspended in DEPC-treated water. To remove genomic DNA contamination, the extracted RNA was treated by RNase-free DNase I and its buffer (Sinaclon Bioscience, Karaj, Iran) and incubated at 37°C for 30 min. The reaction was stopped by EDTA at 65°C for 10 min, and the amount and quality of RNA were determined by spectrophotometry (Micro-volume Spectrophotometer System, Nano Mabna Iranian, Tehran, Iran). Only RNA of sufficient purity, having an absorbance ratio (A260/280) between 1.8 and 2.2 was considered for synthesis of cDNA.

Shortly after extraction, total RNA (1 µg) was reversely transcribed into cDNA (less than 2 h) in a 5 µL reaction volume using the Easy cDNA synthesis kit, offered by the manufacturer (Pars tous biotechnology, Mashhad, Iran). The thermal program for cDNA synthesis included the following 3 steps: 25°C for 10 min (activation of the reverse transcriptase), 47°C for 60 min (reverse transcription), and 85°C for 5 min (inactivation of the reverse transcriptase). The synthesized cDNA was then stored at -20°C.

Quantitative Real-Time PCR: Real-time PCR was performed in two replicates for each sample (Rotor Gene Q 6000, Qiagen, USA). Primer sequences, the GenBank accession numbers, the size of amplified products, and annealing temperature of each primer are shown in *Table 1*. Half µL DNase I treated cDNA was added to 5 µL SYBR Premix Ex Taq II Mix and 0.5 µM of each specific primer in a total volume of 10 µL. The PCR program was comprised of 45 cycles of 94°C for 5 s (denaturation), and 54-60°C for 30 s (annealing & extension; *Table 1)* and 72°C for 30 s.

Considering the selection of an appropriate housekeeping gene as a reference gene for normalization, there are several studies demonstrating that *Actb* is highly reliable reference genes among the other genes used for RT-qPCR normalization and analysis of relative gene expression in the mouse testes [17].

Melt curve analysis was conducted to confirm the specificity of each product. The no-template control and no-reverse transcriptase control were considered to check contamination of the PCR reagents. Data were analyzed using LinReg PCR software version 2012.0 (USA), to give the threshold cycle number (Ct). Mean efficiency values (E) for each gene were also determined from the amplification profiles of individual samples using the same software [7]. The following formula was applied to determine the relative gene expression in tissue testes of treated rat compared to the control group [18,19].

Statistical Analysis

The differences in relative abundance of gene expression

Table 1. *Sequence and annealing temperature of primers used for Real Time-PCR*

Gene	Gene Bank Accession No.	Primer Sequence (5′-3′)	Product Size (bp)	Annealing Temp (°C)	Reference
Casp3	NM_012922.2	F-GGACCTGTGGACCTGAAAAA R-GCATGCCATATCATCGTCAG	159	54	[20]
Bcl2	NM_016993.1	F-GGTGAACTGGGGGAGGATTG R-GCATGCTGGGGCCATATAGT	197	60	[20]
DAZL	NM_001109414.1	F-TCTTCATCAGCAACCACCAG R-GACAAATCCATAGCCCTTCG	195	60	[12]
B-Actin (Actb)	NM_031144.3	F- CACCCGCGAGTACAACCTTC R- GAAGCCGGCCTTGCACAT	127	60	Designed by writers

between groups were analyzed using one sample t-test student after ArcSin transformation with SPSS software version 20.0.0 (IBM Corp.; USA). Data were expressed as mean±SEM. Differences were considered significant at P<0.05.

RESULTS

The results of sperm evaluation showed that the mean percent of live and non-motile sperm and the sperm concentration were not different significantly between treatment groups. The difference was only significant between control and sham for all three parameters *(Table 2)*.

The results of gene expression were presented by relative comparison of all of treatments groups with control group. The relative abundance (RA) of transcripts in tissue of treated rat testes has been shown in *Fig. 1A-B* and *Fig. 2*. As shown in *Fig. 1A*, the expression level of *Bcl2* is significantly down regulated in those rats received both L-carnitine and melatonin (LM) (P<0.008), and those that received all three antioxidants, Atorvastatin, L-carnitine and melatonin (ALM) (P<0.001) in comparison with control group. No significant difference was observed between each of other treatment groups and control group in *Bcl2* expression.

According to *Fig. 1B*, the relative abundance of *Casp3* transcripts was significantly lower in AM (P<0.001) and ALM (P<0.007) than control group. As well as, there was significant high expression of this gene in Vit E-treated rats compared to the rats of control group. There was no significant difference between each of other groups (DMSO, and LM) and control group.

Table 2. *The mean±SE percent of live and non-motile sperm and the sperm concentration*

Sperm Characteristics in Treatment Groups		N	Mean	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Live sperm (%)	Sham	8	67±1.52[a]	63.40	70.60
	ALM	8	52±1.92[ab]	47.46	56.54
	vitamin E	8	50.25±2.6[ab]	44.09	56.41
	Control	8	24.75±2.23[b]	19.47	30.03
	Total	32	48.5±2.9	42.58	54.42
Sperm count	Sham	8	564.38±15.63[a]	527.40	601.35
	ALM	8	461.63±18.78[ab]	417.21	506.04
	vitamin E	8	424.38±11.7[ab]	389.93	458.82
	Control	8	225±23.28[b]	197.35	252.65
	Total	32	418.84±131.72	371.35	466.34
Non-motile sperm	Sham	8	18.88±1.31[a]	15.76	21.99
	ALM	8	28±1.45[ab]	24.57	31.43
	vitamin E	8	31.13±1.7[ab]	27.09	35.16
	Control	8	34.5±3.15[b]	27.05	41.95
	Total	32	28.13±1.42	25.22	31.03

ALM: *atorvastatin, L-carnitine, and melatonin group,* ***Sham:*** *treated with DMSO as busulfan solvent*

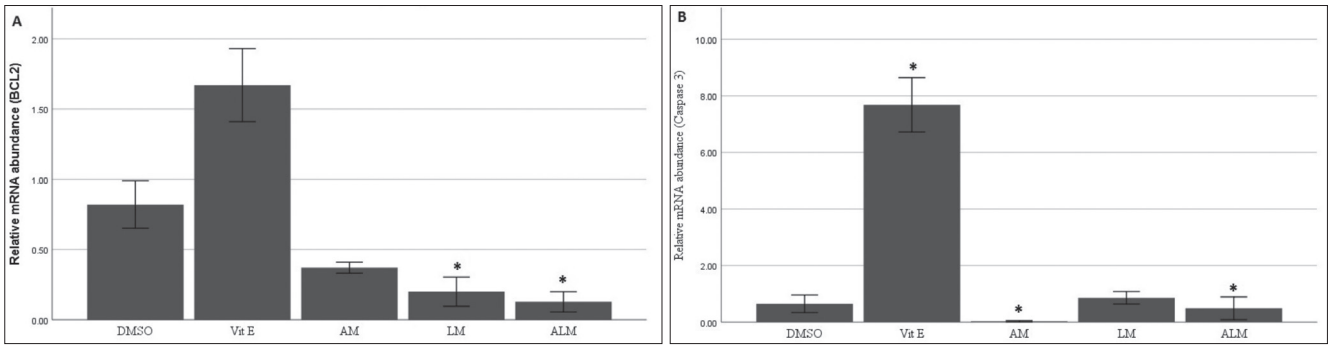

Fig 1. Relative abundance of *Bcl2* (A) and *Casp3* (B) transcripts in rat testes tissue derived from treated rats with busulfan solvent (DMSO), Vitamin E (Vit E), L-carnitine and Melatonin (LM), atorvastatin and melatonin (AM), atorvastatin, L-carnitine, and Melatonin (ALM) compared to control group (busulfun treated rats). All reactions were normalized for β-Actin mRNA expression. Values with superscripts "*" refers to significant (P<0.05) differences in relative transcript abundance of each group compared to control group

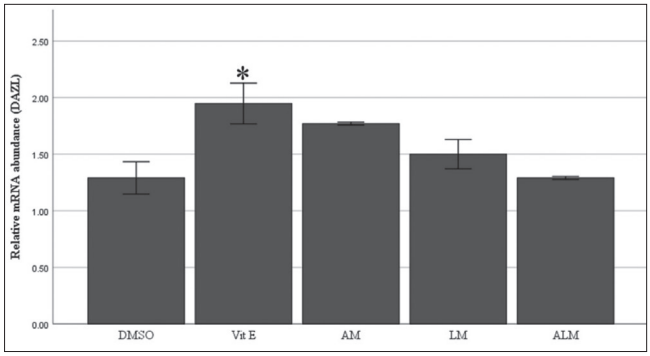

Fig 2. Relative abundance of DAZL transcripts in rat testes tissue derived from treated rats with busulfan solvent (DMSO), Vitamin E (Vit E), L-carnitine and Melatonin (LM), atorvastatin and melatonin (AM), atorvastatin, L-carnitine, and Melatonin (ALM) compared to control group (busulfun treated rats). All reactions were normalized for β-Actin mRNA expression. Values with superscripts "*" refers to significant (P<0.05) differences in relative transcript abundance of each group compared to control group

Table 3. *Mean±SEM of relative abundance of Bcl2, Casp3, DAZL mRNA in treatment groups in comparison with control group*

Groups	*Bcl2*	*Casp3*	*DAZL*
DMSO	0.82±0.13	0.65±0.24	1.29±0.11
Vit E	1.67±0.20	7.68±0.74*	1.97±0.08*
AM	0.37±0.03	0.03±0.02*	1.77±0.01
LM	0.2±0.08*	0.86±0.17	1.50±0.10
ALM	0.13±0.05*	0.49±0.31*	1.29±0.01

All reactions were normalized for β-Actin mRNA expression. "" refers to significant (P<0.05) differences in relative transcript abundance of each group compared to control group*

Deleted in azoospermia-like, exhibited high level of expression in Vit E treated rats compared to control counterparts (P<0.01) and no differences were found between each of other treatment groups (DMSO, AM, LM, and ALM) and control group *(Fig. 2)*.

The relative abundance of *Bcl2*, *Casp3*, and *DAZL* transcripts in rat testes tissue derived from treated rats in all treatment groups compared to control group (Busulfun treated rats) are briefly shown in *Table 3*.

DISCUSSION

One of the most important problems in the life of couples' is infertility and it's complications and the causes of some infertilities are related to men. The most common cause of male infertility is inability to produce sufficient numbers of active and healthy sperms [21]. Many factors can affect sperm production and infertility risks. Among these factors are using chemotherapy drugs for cancer, antibiotics, toxic substances, s, stress, pesticides, radiation, vitamin deficiency, and air pollution. These factors can reduce sperm concentration with generation of free radicals and oxidation of germ cells in the testes [21].

Busulfan, as a chemotherapy drug, is a bifunctional alkylating agent and is used for the treatment of various malignant diseases, such as polycythemia vera and chronic myelogenous leukemia [3]. Busulfan is a highly cytotoxic and genotoxic agent [3] that can induce various adverse effects, both acute and chronic, such as DNA damage and subsequently activates apoptosis or senescence in a cell type-dependent manner probably due to oxidative stress [4] in several biological organs such as hematologic [22], nervous [23] and reproductive organs [24]. It has been confirmed that chemotherapy with busulfan can induce apoptosis in sperm [6] and increase ROS generation and resulting in death of spermatozoa [25]. As well as, accurance of teratospermia, the presence of spermatozoa with abnormal morphology in semen is the other possible result of busulfan treatment in mice [3]. These studies showed that busulfan is involved in the arrest of spermatogenesis, though some of the changes are reversible and dose dependent. In this study two dose of 25 and 10 mg/kg busulfan with 14 days interval were used which can destruct the spermatogenic process in rat as was shown in other studies [3,26,27].

Antioxidants neutralize free radicals and subsequently the oxidative reactions caused by them. The dietary antioxidants may be beneficial in reducing the lipid peroxidation and DNA damage in sperm during the treatment with busulfan [28]. Different antioxidants have examined in challenging procedures to reduce the adverse effects of ROS on rat spermatozoa [16,29].

Here, we studied the protective effect of vitamin E, melatonin, L-carnitine, and atorvastatin treatment, alone and in different combinations, against busulfan-mediated sperm damage in mice. Our results demonstrated that perform all treatments 48 h after the beginning of chemotherapy with busulfan and daily administration of antioxidants for 8 weeks can slightly reduce busulfan-mediated destructions.

To confirm the role of busulfan in germ cells destruction and apoptosis, respectively, *DAZL* activation and anti-apoptotic molecule *Bcl2* as well as activation of *Casp3* were measured in testes tissue after busulfan treatment.

Consistent with busulfan-induced increased mitochondrial membrane depolarization, significantly increased expression of *Bcl2*, was observed in testes tissue treated with vitamin E compared to other antioxidants-treated rats, suggesting busulfan-induced apoptosis is moderated with Vitamin E administration through regulation of the expression of *Bcl2*, but did not lead to subsequent decrease caspase-3 activation.

Vitamin E (α-tocopherol) is an organic fat soluble compound located generally in cell membranes. This dominant antioxidant extinguishes superoxide anions and free hydroxyl radicals thereby reducing lipid peroxidation initiated by

ROS in plasma membranes [30] and thus protects the cell membrane from ROS-induced damages. This antioxidant reduces testicular tissue damages cause by cytotoxic agents through increasing the expression of antioxidant related genes. In the male reproductive tract, the antioxidant property of this vitamin in inhibition of destructive effects of free radicals in testes [31] and sperm [32] was confirmed. As well as, some studies showed vitamin E is efficient in protecting testes from induced damage by oxidative stress and mitigation this damage can be achieved by vitamin E treatment [33].

It was expected high expression of *Bcl2* reduces *Casp3* expression after administration of Vitamin E. But, it should be considered that there are at least two principal pathways for activating *Casp3*. The more ancient, which is induced by diverse intracellular stresses, including cytokine deprivation and genotoxic damage, is regulated by *Bcl2* and its relatives. Progression through the pathway usually leads to the activation of *Casp9* and subsequently *Casp3*. A more-recently evolved pathway is triggered when 'death receptors' on the plasma membrane engaged by cognate ligands of the tumour-necrosis factor (TNF) family, recruit *Casp8* through bind the adaptor protein FAS-associated death domain (FADD) (also called MORT1). This pathway eventually increase *Casp3* [10]. Therefore, high expression of *Casp3* in vitamin E-treated rats may be due to activation of second mentioned pathway.

Among the groups, Vitamin E more moderated spermatogenesis resumption as showed in increasing *DAZL* gene expression. This study was the first study in directly determination of the effect of vitamin E on *DAZL* expression as a worthy candidate for male infertility that plays an important role in the spermatogenesis.

However, a number of studies suggested that supplementation with oral antioxidants such as vitamin E and carnitine could improve the sperm quality in infertile patients which can certainly be through the stimulation of related gene expression such as *DAZL*.

However, we observed lower *Casp3* gene expression in other treatments, especially in those rats treated with both AM and ALM. When combination groups were compared to each other, it was defined that down-regulation of *Casp3* mRNA in AM and ALM groups might be more due to the presence of melatonin, and on the other hand, high expression of this gene in LM in comparison of AM group and then ALM group was created by L-carnitine. This is consistent with Fan et al.[34] study that expressed mRNA and protein levels caspase-8 was increased by L-carnitine treatment in hepa1c1c7 mouse cancer cells. As well as, it was noted that despite the inhibition effect of L-carnitine on the activity of recombinant caspases 3 in Jurkat cells (a human T lymphocyte cells line), its long-chain fatty acid derivative palmitoylcarnitine increases the activity of all the caspases. It was suggested that reversed effect of palmitoylcarnitine on the inhibition of caspase activity by carnitine, may be regulated in part by the balance of palmitoylcarnitine and carnitine under physiological conditions [35].

Many studies demonstrated that melatonin has an anti-apoptotic effect in somatic and germ cells [36-38]. Melatonin has been reported to be protective in male reproductive health, which readily crosses the blood-testes barrier and has a very low toxicity [39]. Studies have investigated the use of melatonin to relieve the side effects of environmental toxins and chemotherapy drugs during spermatogenesis [36,40]. However, few systematic studies have investigated whether melatonin employs a protective role in the psychological stress-induced impairment of spermatogenesis as well as the mechanisms by which melatonin mitigates the damage in testes.

Many studies have found that L-carnitine and its derivatives can optimize sperm motion parameters [41,42]. On the other hand, other studies failed to detect significant increases in sperm concentration following L-carnitine treatment [43-45]. The relatively small doses and short duration of treatment employed may be the main reason why no substantial increases were detected. By the large, it has be found that L-carnitine further improves sperm motility and chromatin quality via antioxidant properties, the enhanced glucose uptake by sperm [46], and long chain fatty acids transportation across the inner membrane of the mitochondria for use in metabolism [47]. On the other hand, its effects in reducing some of the side effects of busulfan on the testes is lower than other used compounds [25]. This maybe the reason of low expression of *DAZL* in the L-carnitine-contained groups in comparison the other groups.

In this study the lowest expression of *Bcl2* was seen in ALM group. The main reason of this event can be atorvastatin. Atorvastatin improves the lipid profile, lipid oxidation, and oxidative/antioxidative status. These positive effects may be attributed to the antioxidant properties of statins. It has been suggested that statins increase apoptosis and change levels of *Bcl2* family members (e.g., *Bax* increasing and *Bcl2* reduction). Several reports found that statins reduce levels of the anti-apoptotic protein *Bcl2*, and increase apoptosis and cell death [48-50]. Though, there is evidence that statins increase *Bcl2* abundance which would desirable and in some instances reduce apoptosis and cell death [51,52].

Generally at high statin concentrations apoptosis is increased, and *Bcl2* expression levels and cell viability are reduced [53]. The mechanisms for the statin-induced reduction of Bcl2 protein levels have not been forthcoming. Statins reduce cholesterol, protein prenylation, and two isoprenoids FPP (farnesyl pyrophosphate) and GGPP (geranylgeranyl pyrophosphate but how those reductions trigger a weakening of the anti-apoptotic protein Bcl2 and increase abundance of pro-apoptotic proteins such as Bax and Bim is not understood. There is evidence that statins has

function outside of the mevalonate pathway. Statins for example bind to a heterodimeric glycoprotein, lymphocyte function-associated antigen-1 (LFA-1) which is a member of the β2 integrin family [54]. Directly related to the subject of statins, these compounds increase *Bcl2* gene expression and protein levels, which do not involve the mevalonate pathway.

It can be concluded that, according to the antiapoptotic effects of these antioxidants, vitamin E retrieved spermatogenesis potential of *busulfan*-induced infertile male rats better than the other antioxidants used in different combinations. It was approved by increasing *DAZL* gene expression, a worthy candidate for male infertility evaluation.

Acknowledgment

The authors would like to thank Islamic Azad University, Science and Research Branch, Tehran, and Shahrekord University for their supports. We also thank Behnam Bakhtiarimoghadam for keeping rats and injection of drugs in treatment groups.

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Kafkas Univ Vet Fak Derg
25 (5): 717-720, 2019
DOI: 10.9775/kvfd.2018.21606

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

Case Report

Dog Massacre with Pesticide for Theft: Methomyl Poisoning [1]

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Article Code: KVFD-2018-21606 Received: 20.12.2018 Accepted: 25.03.2019 Published Online: 26.03.2019

How to Cite This Article

Özdemir Ö, Ateş MB, Ortatatli M, Terzi F, Avci T, Hatipoğlu F, Çiftçi MK: Dog massacre with pesticide for theft: methomyl poisoning. *Kafkas Univ Vet Fak Derg,* 25 (5): 717-720, 2019. DOI: 10.9775/kvfd.2018.21606

Abstract

In this case report, acute methomyl poisoning in dogs is described by pathological and toxicological findings for the first time in Turkey. Five of the thirty stray dogs that were found dead within 1-2 days after eating the foodstuffs in an industrial area in Konya were necropsied. Grossly, undigested pieces of chicken meat with a mild insecticidal smell in stomach of all dogs were found and toxicological analysis by GC-MS revealed toxic levels of methomyl in the stomach contents (15.7-17.8 ppm) and, intestines, livers and kidneys (1.2-2.9 ppm) also. In addition, histopathologically degenerative and necrotic changes were observed in liver, brain, lung, kidneys and gastrointestinal system mucosa. Postmortem and histopathological examinations and toxicological analyses revealed that deaths were related to methomyl poisoning and it has been subsequently learned from the police officers and printed media that this poison had been deliberately infected to dog food for the purpose of theft committed in the industrial area. Publishing these intentional poisoning events, we have wanted to draw attention to that more serious measures and statutory regulations should be taken by governments about animal rights.

Keywords: *Methomyl poisoning, Histopathology, Toxicology, Dog massacre, Intentional poisoning*

Hırsızlık İçin Tarım İlacıyla Köpek Katliamı: Metomil Zehirlenmesi

Öz

Bu olgu sunumunda, Türkiye'de ilk kez köpeklerde akut metomil zehirlenmesi patolojik ve toksikolojik bulgularıyla tanımlanmıştır. Konya'daki bir sanayi bölgesinde, yemlerini yedikten sonraki 1-2 gün içerisinde ölü olarak bulunan 30 adet sokak köpeğinden 5 tanesine nekropsi yapılmıştır. Nekropside köpeklerin midelerinde hafif insektisit kokusu içeren sindirilmemiş tavuk parçalarına rastlanılmış olup, GC-MS cihazıyla yapılan toksikolojik analiz sonucu mide içeriğinde (15,7-17.8 ppm) ve ayrıca bağırsaklar, karaciğer ve böbreklerde (1.2-2.9 ppm) toksik düzeyde metomil tespit edilmiştir. Ayrıca histopatolojik olarak karaciğer, beyin, akciğer, kalp, böbrek ve gastrointestinal sistem mukozalarında dejeneratif değişikler gözlenmiştir. Postmortem ve histopatolojik bulgular ile toksikolojik analizler neticesinde ölümlerin metomil zehirlenmesine bağlı olarak şekillendiği ortaya konulmuş olup, daha sonra adli makamlar ile yazılı basından alınan bilgilerden bu zehirin köpek yiyeceklerine bölgede gerçekleştirilen hırsızlık amacıyla kasten bulaştırıldığı öğrenilmiştir. Bu kasıtlı zehirlenme vakası ile, hükümetler tarafından hayvan hakları konusunda daha ciddi ve yasal önlemlerin alınması gerektiğine dikkat çekilmek istenilmiştir.

Anahtar sözcükler: *Metomil zehirlenmesi, Histopatoloji, Toksikoloji, Köpek katliamı, Kasıtlı zehirleme*

INTRODUCTION

Methomyl is a carbamate insecticide active ingredient that is widely used in agricultural applications and animal shelters against insects all over the world [1,2]. Absorption of methomyl may be from skin, respiratory tract or gastrointestinal tract. It is highly toxic to birds and mammals when taken orally and shows its effect by inhibiting the enzyme Acetylcholinesterase (AChE). Acetylcholine is one of the most important neurotransmitter substances involved in the autonomic and somatic nervous system. Acetylcholine activates muscles by acting in the chemical pathway of nerve impulses at neuromuscular junctions. This neurotransmitter substance is destroyed by the AChE enzyme after nerve stimulation. The inhibition of AChE in methomyl poisoning results in a toxicity table in which cholinergic symptoms are predominant due to the accumulation of acetylcholine at neuromuscular junctions and nerve

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synapses [2-4]. Methomyl is classified in Class Ib as a highly toxic substance by World Health Organization (WHO) [5,6]. Methomyl oral LD50 is 17-24 mg/kg in rats, 10 mg/kg in mice, and 15 mg/kg in guinea pigs. In dogs, it has been reported that 10-20 mg/kg oral dose may lead to death [7]. Methomyl was found to be less detected in organs and tissues, although it was found to be high in the blood after oral administration [8-10].

Pathological and toxicological data related to methomyl poisoning are very limited in field of veterinary medicine and there is no published data in Turkey although there may be probably similar poisoning incidents. Therefore, the aim of this study is to present the results of macroscopic and histopathological findings along with toxicological analysis on methomyl poisoning, to assist in taking rapid diagnostic and preventive measures and to promote the sensitivity to the subject such deliberate poisonings.

CASE HISTORY

In this case presented, 30 street dogs were found dead in the next 1-2 days after having eaten their food in the nutrition centers built for the feeding of stray animals in Konya Industry area in Turkey. Five of them were brought to Selcuk University, Faculty of Veterinary Medicine, Pathology Laboratory for the investigation of the cause of deaths. After taking the relevant records, systemic necropsies were performed. Organ samples and gastro-intestinal contents were taken for histopathological and toxicological examinations.

For histopathological examination, tissue samples taken from gastrointestinal tracts, liver, kidney, lung, heart, and brain were fixed in 10% formaldehyde solution for one day and then routine tissue follow-up procedures were performed. Five µm thick sections were obtained from the tissues embedded in paraffin blocks and stained with hematoxylin-eosin (HxE). Prepared slides were examined under light microscope (Olympus BX51, Tokyo, Japan). For toxicological analyses, stomach and intestinal contents, liver and kidney samples were examined by GC-MS device at Konya Veterinary Control Institute.

At necropsy, macroscopic findings were the same in all dogs. Grossly, serosanguinous fluids in the thorax and abdominal cavity, and undigested chicken meat and bone fragments *(Fig. 1A)* with a mild insecticidal smell in the stomachs were found. It was observed that the liver was pale yellow, swollen and easily fragilable. Semi-clotted blood in the left ventricle and endocardial hemorrhages were noted in the heart *(Fig.1B-C)*. There was edema in the lungs, and the kidneys were pale. In the brain meninges, mattening and hyperemia were determined. In addition, postmortal clotting deficiency of blood in the vessels were observed in two of the dogs.

In histopathological examination, severe and diffuse vacuolar degeneration of hepatocytes and congestion in the liver *(Fig. 2A-B)*, hyperemia and tubular degeneration in kidneys *(Fig. 2C)*, hyperemia, edema and perivascular hemorrhage in the brain *(Fig. 2D)*, epithelial necrosis in stomach *(Fig. 2E)* and congestion and alveolar edema in lung *(Fig. 2F)* were determined.

According to the toxicological analysis by GC-MS, methomyl was found as 15.7 to 17.8 ppm in stomach contents, 4.5 ppm in the intestinal contents, 2.25 to 2.90 ppm in livers and 1.2-1.5 ppm in kidneys of 5 dogs.

DISCUSSION

Intentional or accidental toxications are very important reasons in animal and human deaths. In a study, incidence of domestic carnivorous poisoning in Italy between 1996 and 2003 was 40% (260 cases) of all agricultural drug poisoning cases (650 cases) originating from insecticides in dogs and 32% of them were carbamate insecticides, and that the dogs were mostly affected by carbamates (39 cases), especially by methomyl [9]. In another retrospective study in Italy by Caloni et al.[11], it was reported that dogs accounted for 71.1% of all 304 cases of domestic animal poisonings between 2011 and 2013. In addition, insecticides (40.8%) are mostly important ones among

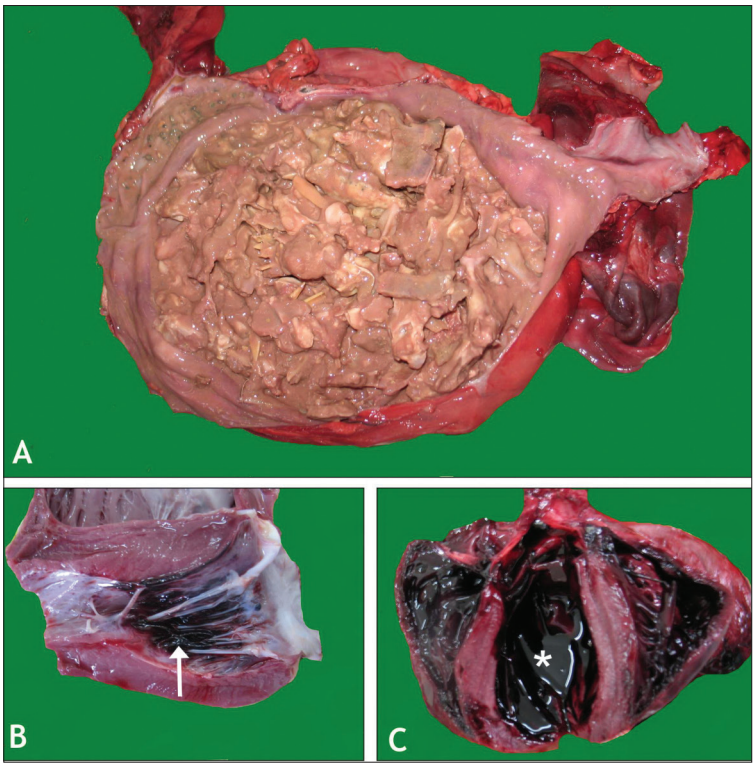

Fig 1. A) Undigested pieces of chicken in stomach, **B)** Endocardial haemorrhages *(arrow)*, **C)** Semi-clotted blood in ventricle *(asterisk)*

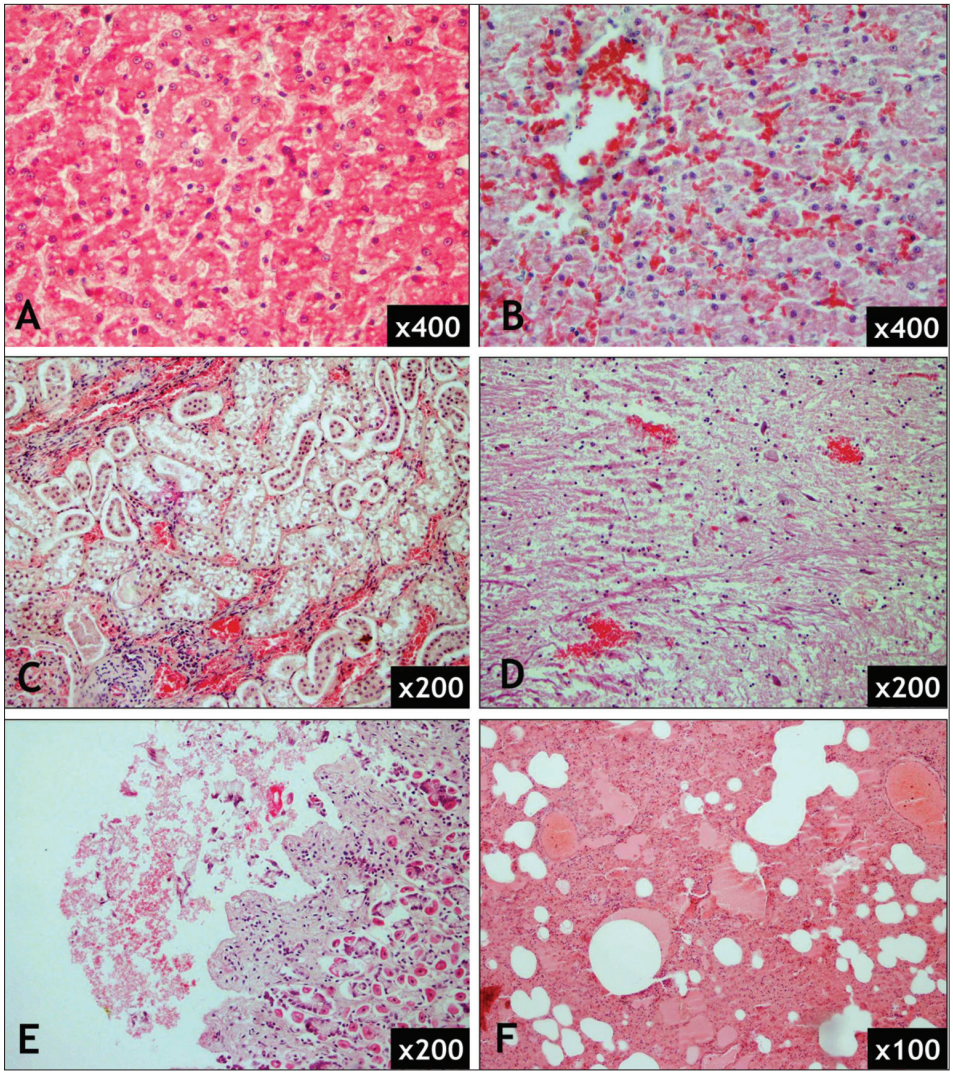

Fig 2. A-B) Vacuolar degeneration of hepatocytes and congestion in the liver, **C)** Hyperemia and tubular degeneration in kidneys, **D)** Perivascular hemorrhage in the brain, **E)** Epithelial necrosis in stomach, **F)** Hyperemia and alveolar edema in lung (H&E)

pesticide poisoning events and methomyl is the most common insecticide seen in both dog and cat poisoning cases. In another study conducted by Martinez-Haro et al.[12] in Spain, 260 cases of intentional or accidental poisoning were dealt with. According to this study, it was reported that most of the recorded animal poisonings in Spain originated from insecticides (66.1%) and that all methomyl poisoning cases were made intentionally.

In these studies where numerical data related to intoxication in domestic carnivores were given, it has been shown that dogs are more exposed to intoxication cases and methomyl has been highly effective on dogs. In addition, it was emphasized that methomyl has used intensively in intentional poisonings. In this case report by supporting data above, the presence of methomyl-induced toxicity in 30 dogs simultaneously and rapid deaths of them point out that the dogs are highly sensitive to methomyl. At the same time, deliberately administration of this poisoning by thieves, because they perceive street or guard dogs as threats, helps to explain why dogs are more likely to be exposed to such poisoning cases. In addition, it is thought that methomyl active substance preparations are used extensively in poisoning cases due to the fact that they are easily obtained without supervision in anywhere and that their effect is acute.

In an experimental study conducted by Radad et al.[13], rats were given methomyl (2 mg/kg) three times a week for three months and histopathologically liver, kidney, lung, testis, and spleen were significantly affected. In another study performed in rats, degenerative changes were observed in the brains of the methomyl-administered groups (10 mg/kg, 2 mg/kg) compared to the control groups [14]. In a study of dog poisoning cases in Macedonia in 2007-2017, it was reported that the most important necropsy findings in methomyl poisoning events were inflammatory changes in the gastrointestinal tract and histopathologically the most affected organs were the kidney, stomach, intestine, and lung [15].

There are very limited studies describing, macroscopic and microscopic findings related to the methomyl toxicity encountered in dogs. Most of the methomyl poisonings cases are to be toxicoepidemiological. Therefore, the pathological

data in our cases are important. Pulmonary edema and congestion detected macroscopically and microscopically are thought to be caused by increased permeability of the pulmonary capillary membranes with bradycardia as a result of inhibition of acetylcholinesterase enzyme by the methomyl. The presence of semi-clotted blood in the left ventricle of the heart suggests that methomyl may cause blood clotting disorder. In addition, it has been reported that hemorrhages of various tissues in carbamate insecticide poisonings may be the result of convulsions. In our cases, the occurrence of hemorrhages in the endocardium and especially in the brains may also be the result of damage to the vascular endothelium by methomyl as well as convulsions.

Vacuolar degeneration of hepatocytes in the liver and tubular degeneration in the kidney may be attributed to the toxic degenerative effect of methomyl on these organs. In addition, although excessive cholinergic stimulation in the parasympathetic nervous system causes hypermotility in the gastrointestinal tract, the presence of undigested food in the stomach may be the result of sudden death by the acute toxic effect of methomyl. When all these findings are evaluated, macroscopic and histopathological lesions for methomyl toxication are important and revealing the pathogenesis of death. In addition, generally the crime scene where intentional toxications were carried out is not extensively investigated on the spot and the chain of inquiry usually starts with the necropsy and the records taken. In the present case, because of the suspicion of poisoning thanks to the results of systemic necropsy of the dogs, toxicological samples were taken. Eventually, the damages caused by methomyl, which was also toxicologically determined, to the organs were shown histopathologically, too.

There is no the data on the amount of toxic methomyl present in the organs and gastrointestinal contents of dogs. Therefore, the amounts of methomyl detected in our case (15.7 to 17.8 ppm in stomach contents, 4.5 ppm in the intestinal contents, 2.25 to 2.90 ppm in livers and 1.2-1.5 ppm in kidneys) could not be compared with other fatal cases. However, these data can provide valuable research opportunities for the determination of methomyl fatal levels in dogs. It is also possible that the amount of methomyl given orally with food may be much higher at first.

In conclusion in this case, it was revealed that deaths were due to methomyl intoxication in the light of postmortem findings, histopathological investigations and toxicological results. In addition, according to the information received later from the police officers and the printed media, it was confirmed that these poisonings were intentionally carried out in this industrial area for theft.

In this study, the pathological and toxicological aspects of methomyl poisoning in dogs were investigated for the first time in Turkey. As with other toxicoepidemiological studies [9,11,12], it was found that especially stray dogs or security dogs are more exposed to intentional poisonings. Methomyl is preferred in intentional poisonings since it is cheap and can be easily supplied without any supervision in Turkey, and has a high and rapid fatal effect. The case findings may provide a wider knowledge of the causes and types of poisoning in animals, assist the veterinarians in making a diagnosis and help both the animal owners and the concerned persons from taking preventive measures. With this case presentation, it has been drawn attention to that more stronger legal regulations should be taken by governments in access and abuse of such toxic drugs and about animal rights.

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Kafkas Univ Vet Fak Derg
25 (5): 721-724, 2019
DOI: 10.9775/kvfd.2018.21609

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

Case Report

The First Case of Anal Myiasis Caused by *Chrysomya albiceps* (Wiedemann, 1819) in a Dog Infested with *Rhiphicephalus sanguineus* (Latreille, 1806) Ticks Suspected to Cause Paralysis in Turkey [1]

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[1] *Presented as poster in 20th National Parasitology Congress, September 23-27, 2017, Eskişehir, Turkey*

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Article ID: KVFD-2018-21609 Received: 20.12.2018 Accepted: 17.04.2019 Published Online: 17.04.2019

How to Cite This Article

Ceylan O, Dik B, İlhan C, İder M, Gülersoy E: The first case of anal myiasis caused by *Chrysomya albiceps* (Wiedemann, 1819) in a dog infested with *Rhiphicephalus sanguineus* (Latreille, 1806) ticks suspected to cause paralysis in Turkey. *Kafkas Univ Vet Fak Derg,* 25 (5): 721-724, 2019. DOI: 10.9775/kvfd.2018.21609

Abstract

A four-year-old Kangal dog with hundreds of ixodid ticks in the head and neck regions and a large number of myiasis larvae in the anal region were brought to Selcuk University, Faculty of Veterinary Medicine, Department of Internal Medicine. A paralysis covering the anterior and posterior extremites, except the head region was determined in the dog. It was suspected that the paralysis was caused by ticks on the dog after elimination of some other disease. However, no definitive diagnosis was made for paralysis. Collected ticks were identified as *Rhiphicephalus sanguineus* (Latreille, 1806). The myiasis larvae collected from the anal region were identified as second and third instars of *Chrysomya albiceps* (Wiedemann, 1819). As a result, it is suggested that *C. albiceps* and *Rh. sanguineus* should be considered in the etiology of myiasis and tick paralysis cases, respectively.

Keywords: *Rhiphicephalus sanguineus, Calliphoridae, Chrysomya, Paralysis, Myiasis*

Türkiye'de *Rhiphicephalus sanguineus* (Latreille, 1806) Kenelerinin Paralize Sebep Olduğundan Şüphe Edilen Bir Köpekte *Chrysomya albiceps* (Wiedemann, 1819)'in Neden Olduğu İlk Anal Miyaz Olgusu

Öz

Baş ve boyun bölgesinde yüzlerce kene ve anal bölgesinde çok sayıda miyaz larvası bulunan dört yaşında kangal köpek Selçuk Üniversitesi Veteriner Fakültesi, İç Hastalıkları Anabilim Dalına getirilmiştir. Köpekte baş bölgesi dışında ön ve arka ekstremiteleri kapsayan bir paraliz tespit edilmiştir. Bu paraliz durumuna diğer bazı hastalıkların eliminasyonundan sonra kenelerin sebep olduğundan şüphe edilmiş, fakat kesin bir tanı konulamamıştır. Toplanan keneler *Rhiphicephalus sanguineus* (Latreille, 1806) olarak teşhis edilmiştir. Anal bölgeden toplanan miyaz larvaları ise ikinci ve üçüncü dönem *Chrysomya albiceps* (Wiedemann, 1819) olarak teşhis edilmişlerdir. Sonuç olarak, *C. albiceps*'in miyaz vakalarının, *Rh. sanguineus*'un ise paraliz vakalarının etiyolojisinde düşünülmesi gerektiği ileri sürülmüştür.

Anahtar sözcükler: *Rhiphicephalus sanguineus, Calliphoridae, Chrysomya, Paraliz, Miyaz*

INTRODUCTION

The tick paralysis is caused by inoculation of neurotoxins found in the salivary glands of some tick species during the feeding by sucking blood. Inoculated neurotoxins act by blocking acetyl choline release at neuromuscular junctions and cause paralysis in hosts [1]. Paralysis caused by the ticks are commonly seen and sometimes cause deaths in dogs [2].

Myiasis is a larva infestation situation in which the fly larvae of the order Diptera are found on vertebrate host at least for a certain period of time and are fed with dead or live tissues, body fluids, and digested foods [3]. Although the species causing myiasis in the family of Calliphoridae are mostly found in the genera *Lucilia* and *Calliphora*, the other species in the other genera such as *Chrysomya, Cordylobia, Phormia* and *Cochliomyia (Callitroga)* can rarely cause myiasis. The greater part of the larvae in this genus-

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related species, also known as hairy maggot, are fed with rotten organic matter and cause facultative wound myiasis. However, species that cause obligator myiasis such as *C. bezziana* are in this genus [3-5].

Chrysomya albiceps has a widespread distribution throughout the world, especially in the Mediterranean region, Transcaucasus, Middle East, Mid-Asia and a large part of Africa. It is reported that the species has a wider distribution compared to the first half of the 20th century and that the distribution has become cosmopolitan day by day [3,6,7]. There are some literatures about the existence of the species in Turkey [8,9].

The female *C. albiceps* lay eggs on rotten animal carcass, the first instar larvae newly hatched from the egg are fed exudate where they are left. The second and third instars become predators for the other Dipteran larvae. For this reason, the larvae usually cause myiasis in live mammals, thanks to predator effects, following myiasis cases initiated by *Lucilia* species. This behaviour possibly lead to a decline in the population numbers of native species [3,10,11].

This case was found worthy of publication due to the first detection of the anal myiasis caused by *C. albiceps* in a dog in Turkey. It also became more important because of the presence of intense *Rh.sanguineus* ticks infestation and paralysis suspected to be caused by these ticks.

CASE HISTORY

A four-year-old male Kangal dog was brought to the Clinic of Internal Medicine, Veterinary Faculty, with complaints of weakness, gait disorder, lying in a horizontal position for 15 days *(Fig. 1)* and decubitus injuries depending on this. Lethargy, hyperpnea, regurgitation, urinary incontinence and tetraplegia were detected as clinical symptoms. Due to lying in the lateral position continuously, rhabdomyolysis occured in the dog, and an increase in aspartate aminotransferase (128 U/L) and creatine phosphokinase (2636 U/L) enzyme levels in serum biochemistry was detected. An increase in the level of pO_2 (52.1 mmHg) was observed in blood gases because of hyperpnea. No specific findings were observed in other blood gases, hemogram and serum biochemical parameters. In order to determine the ethiology of paralysis, some diseases and traumatic injuries were evaluated. Among these, snake poisoning was excluded because of the absence of snake bite marks on dog's body. Spinal traumas were excluded because of the absence of trauma in patient's history. Myasthenia gravis was dismissed because of the lack of atrophy of the muscles of mastication and constant ptyalism in physical examination. Botulism was also exluded in the diagnosis. Tick paralysis was suspected because of the presence of hundreds of ixodid ticks in the head region of the dog. Etiology of the paralysis could not exactly determined, because the owner took away the dog in the same day. In addition, the dipteran larvae were found in the anal and perianal areas of the dog *(Fig. 1)*. Collected ticks were stored in 70% ethanol, examined under a stereo zoom microscope and identified.

A few dipteran larvae washed in distilled water were transferred into 70% alcohol. Some of the remaining larvae were fed with chicken liver in a jar which it's tubulure covered with a cheesecloth to obtain pupae and adult flies. Then, some larvae were left to be transparent in 10% potassium hydroxide (KOH) for 48-72 h. They were rinsed in distilled water and taken into 70 and 99% alcohol, respectively. Later, they were examined under a stereo zoom microscope. Some of the larvae were dissected and mounted on the slides in Canada balsam. The larvae were identified as second and third instar larvae of *C. albiceps* according to their morphological characteristics such as cephalo-pharyngeal skeleton, anterior and posterior spiracles. It was determined that the second instar larvae *(Fig. 2A, B)* were white color and 5-10 mm in length *(Fig. 2A)*, and the third instar larvae of *C. albiceps* were about 15 mm in length *(Fig. 2C)*. Thorn-like fleshy projections which had setiferous apex were observed *(Fig. 3B)*. These projections were found to be relatively longer in the dorsal and lateral parts in the third instars than the second instars. It was seen that anterior stigmas have 9-10 finger-shaped projections in the second instars, and this number is up to 11-12 in the third instars *(Fig. 3A)*. No buttons on posterior stigma was observed *(Fig. 2D)*. It was observed that the pupa were dark brown and found to be easily recognizable due to their projections *(Fig. 3D)*. Adult *C. albiceps* was metallic green and 5-10 mm in length *(Fig. 3C)*.

DISCUSSION

Tick paralysis is a neural syndrome caused by neurotoxins transmitted by ticks during blood-sucking on host [1]. The

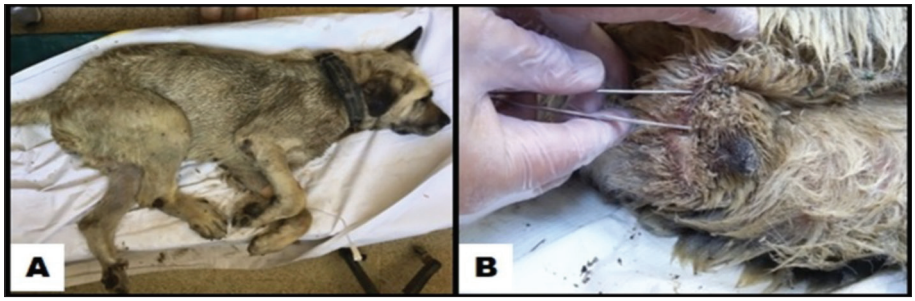

Fig 1. Clinical appearence of dog. **A-** Tick paralysis, lying on a horizontal position, **B-** Myiasis larvae in the anal and perianal regions

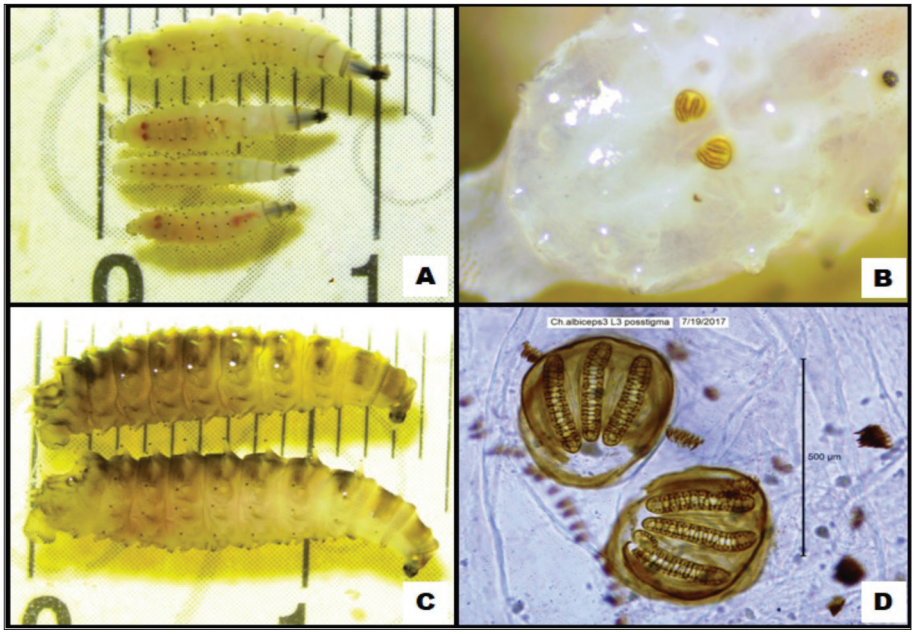

Fig 2. Second and third instars of *C. albiceps* **A-** Second instars of *C. albiceps*, **B-** Posterior stigma of second instar, **C-** Third instars of *C. albiceps*, **D-** Posterior stigma of third instar

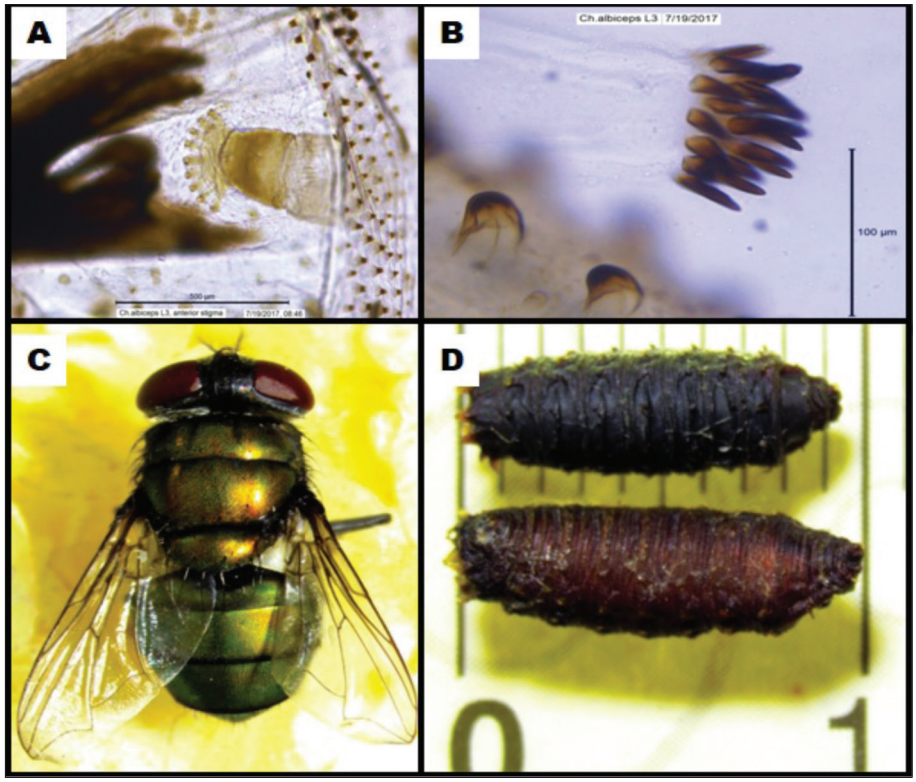

Fig 3. Morphological characteristics of third instar, adult and pupa of *C. albieps*, **A-** Anterior stigma of third instar, **B-** Setiferous fleshy overhang on third instar, **C-** *C. albiceps,* adult, **D-** *C. albiceps,* pupa

cause of neurological changes in the dog have been suspected to be intensive tick infestation and possibly neurotoxins that inoculated by tick vectors during the blood sucking. Paralysis cases caused by the ixodid ticks are common in dogs and can cause deaths [2]. In Australia, it was reported that *Ixodes cornuatus* and *I. holocyclus* are the most common etiological agents of tick paralysis in dogs [12]. However, these species are not found in Turkey as well as in Europe. In the present study, it was thought that *Rh. sanguineus* was detected as the possible causative agent of the tick paralysis in the dog. However, the ethiology of paralysis has not been clearly revealed. Although, *Rh. sanguineus* has a wide distribution throughout the world, it has been reported that there are not enough studies on whether paralysis in dogs are caused by these ticks. Only one paralysis case originating from *Rh. sanguineus* in a dog was reported by Viloria in Venezuela [13]. As a result of the literature search, tick paralysis case in dogs has not been encountered in Turkey.

Forty three tick species belonging to 10 genera, which were previously proven or suspected to cause tick paralysis, have been reported. *Rh. sanguineus* is one of them. There is little information about the toxicity of some of these species. The published studies on this issue is inadequate or some points have not been sufficiently clarified [14,15]. The number of studies related to tick paralysis have been found to be insufficient in Turkey. Eventhough no exact diagnosis was made in this study, tick paralysis was suspected due to the elimination of some of the diseases that could be confused with tick paralysis and the presence of a large number of ticks on the dog.

The anal myiasis phenomenon which we encounter in this study is thought to be the result of the lying in a horizontal position without movement for a long time due to paralysis. As a result of the literature search, many studies has been found related to several *Chrysomya* species. However, there are few studies on myiasis due to *C. albiceps* species. Forensic entomology studies related to *C. albiceps* were conducted in Turkey and various places in the world [16]. Kökdener and Polat [17] reported some Calliphorid species including *C. albiceps* in the north of the Turkey. In addition, they emphasized the importance of such regional faunistic studies in the future forensic studies. Açıkgöz et al.[16] focused on the predator effect of this species in the study conducted in Turkey. They claimed that the larvae of this species attacked to other larvae species on the wound and they have pointed out that there can be mistakes during the calculation of the time of death in the case of this larvae species involved. In a study conducted in Macedonia, *C. albiceps* was used for the calculation of the time between death and the discovery of the corpse [18]. Many cases were presented in the human and veterinary medicine fields related to *C. albiceps* in foreign countries. Sinha et al.[19] reported that *C. albiceps* and *C. megacephala* species were responsible for wound myiasis in the knee and ankle region of a patient. A case of myiasis originating from *C. albiceps* was reported in a deer in India [20]. Such a myiasis case caused by *C. albiceps* has not been encountered in the field of veterinary medicine up to date in Turkey. This is the first anal myiasis case report caused by *C. albiceps* in Turkey.

Dik et al.[4] encountered myiasis cases in the anal and perianal regions different kinds of animals and reported that *Eristalix tenax*, *Lucilia sericata* and *Wohlfartia magnifica* were responsible in these myiasis cases. Furthermore, they reported that the dominant species causing myiasis were *W. magnifica* and *L. sericata*. *C. albiceps*, a species different from the species that encountered in many cases, caused myiasis in this study. Schnur et al.[7] reported a myiasis case in which *L. sericata* and *C. albiceps* were seen together in a dog in Israel. In the present study, no other dipteran larvae except for *C. albiceps* were found.

In this case report, paralysis possibly originating from *Rh. sanguineus* ticks together with anal myiasis caused by *C. albiceps* case is not a very common occurence. Information on this topic was given and the obtained results were discussed in the light of related literatures.

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Kafkas Univ Vet Fak Derg
25 (5): 725-728, 2019
DOI: 10.9775/kvfd.2019.21766

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

Case Report

Scanning Electron Microscopy Images of *Rhipicephalus (Boophilus) kohlsi* from a Wild Goat in Northeastern Anatolia, Turkey

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Article ID: KVFD-2019-21766 Received: 16.01.2019 Accepted: 02.05.2019 Published Online: 02.05.2019

How to Cite This Article

Güven E, Kirman R, Akyüz M: Scanning electron microscopy images of *Rhipicephalus (Boophilus) kohlsi* from a wild goat in Northeastern Anatolia, Turkey. *Kafkas Univ Vet Fak Derg,* 25 (5): 725-728, 2019. DOI: 10.9775/kvfd.2019.21766

Abstract

Tick samples were collected from a wild goat *(Capra aegagrus)* found in Uzundere province of Erzurum, Turkey. Species identification performed based on morphological criteria, and 3 males and 1 female tick were identified as *Rhipicephalus (Boophilus) kohlsi.* Scanning electron microscopy (SEM) used to view the characteristic morphologic features of the ticks. Although previous studies report the presence of *R. kohlsi* in Turkey and in the world, this study represents the first SEM images of male and female *R. kohlsi.*

Keywords: *Wild goat, Rhipicephalus (Boophilus) kohlsi, Scanning electron microscopy*

Türkiye'nin Kuzeydoğusunda Bir Yaban Keçisinden Elde Edilen *Rhipicephalus (Boophilus) kohlsi*'nin Taramalı Elektron Mikroskobu Görüntüleri

Öz

Erzurum'un Uzundere ilçesinde bulunan bir yaban keçisinden *(Capra aegagrus)* kene örnekleri toplandı. Morfolojik kriterlere göre yapılan tür identifikasyonunda, toplanan 3 erkek ve 1 dişi kene *Rhipicephalus (Boophilus) kohlsi* olarak tanımlandı. Kenelerin karakteristik morfolojik özelliklerini incelemek için taramalı elektron mikroskobu (SEM) kullanıldı. Önceki çalışmalarda *R. kohlsi*'nin Türkiye ve dünyadaki varlığı bildirilmiş olmakla beraber bu çalışma erkek ve dişi *R. kohlsi*'nin ilk SEM görüntülerini sunmaktadır.

Anahtar sözcükler: *Yaban keçisi, Rhipicephalus (Boophilus) kohlsi, Taramalı elektron mikroskobu*

INTRODUCTION

Wild goat *(Capra aegagrus)* exists intermittently at south-west Turkey, southwest and central Asia and southern Russia [1-6]. In Turkey, wild goats inhabit rugged, mountainous area with altitude between 1500 and 3500 meters in southern, southeastern, eastern and northeastern Anatolia especially in arid habitats.

Rhipicephalus (Boophilus) kohlsi was first recorded and described by Hoogstraal and Kaiser [7] from sheep and goats in Jordan, in 1970. In the following years, this species reported from Israel [8,9], Western Saudi Arabia [10], Iraq [11], West Africa [12], Iran [13], Uzbekistan [14], and Turkey [15,16]. This tick is primarily defined as a parasite of goats and sheep but also reported from cattle, horse, mule, pig and camel [8,14]. The preferred body sites by the tick are especially neck and ears in goats, but it also attaches to the tail in fat-tailed sheep [8].

In this case report, we provide morphological data for the male and female *R. kohlsi* collected from a wild goat by using scanning electron microscopy (SEM).

CASE HISTORY

An injured wild goat was found in Uzundere, Erzurum (40° 32′ 11¨ N and 41° 32′ 54¨ E, 1140 m above sea level, northeastern of Turkey) in April, 2017 and taken to the Animal Hospital of the Faculty of Veterinary Medicine for treatment. The female and young (<1-year-old) wild goat was checked for ectoparasites during inspection. Four tick samples were taken from neck and ear, and kept in tubes containing 70% ethanol until identification. The ticks were

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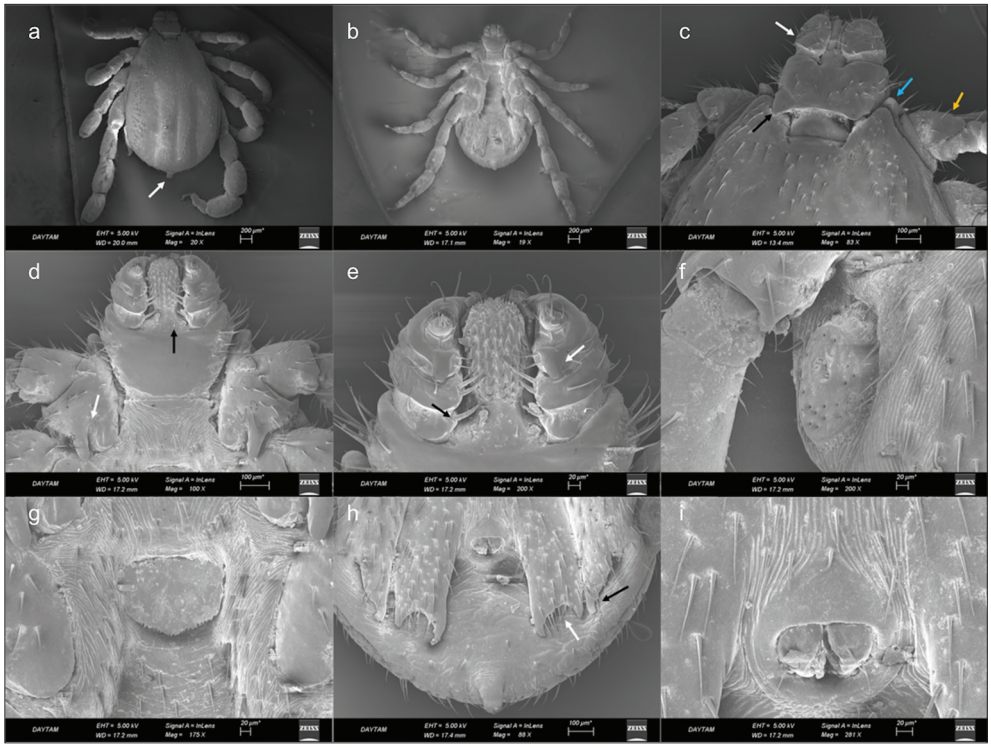

Fig 1. *Rhipicephalus kohlsi* male. (a) Dorsal view, arrow indicates caudal appendage, (b) Ventral view, (c) Dorsal view of capitulum, white arrow indicates palp article 2, blue arrow indicates coxa 1 anterior spur, orange arrow indicates trochanter 1, black arrow indicates cornua, (d) Ventral view of capitulum, white arrow indicates coxa 1 spur, black arrow indicates post hypostomal setae, (e) Ventral view of palpi and hypostome, black arrow indicates the lobe and seta on palp article 1, white arrow indicates inner spur of palp article 3, (f) Spiracular plate, (g) Genital aperture, (h) Adanal and accessory adanal plates, white arrow indicates posterior margin of adanal plate, black arrow indicates the bulge at accessory plates, (i) Anus

identified based on their morphological criteria using taxonomic keys described by Hoogstraal and Kaiser [7] under the stereomicroscope (Nikon SMZ 745T, Japan). Body dimensions of ticks are given as millimeter (mm). In the cleaning process of the ticks, an ultrasonic cleaner was used for 5 min while they were immersed in 5% potassium hydroxide solution in water. Also, three samples (one female and 2 males) were prepared for SEM imagining. Ticks were dehydrated through a graded series of ethanol (50, 70, 80 and 100%), sputtered with gold in the sputter coater (Quorum Q150R S, Germany) and examined with SEM (Zeiss Sigma 300, Germany).

Body length of male ticks vary between 3.1 and 3.3 (from apex of palpi to apex of caudal appendage), width vary between 1.7 and 1.9 *(Fig. 1a,b)*. The color is yellowish brown.

Basis capituli subrectangular, more than twice as wide as long (average width/length: 0.5/0.2). Lateral margins slightly convex, posterior margin slightly concave. Triangle-shaped cornua with rounded margin. The base of hypostome deprived of setae. Setae on lateral and anterolateral parts but probably due to breakage during process exact number uncounted *(Fig. 1c)*. At the ventral side, setae on posterior of palpi and posterolateral corners. Also, a pair of setae on the base level of palpal segment 1. Hypostome and palpi equal in length, hypostome dental formula 4/4 *(Fig. 1d)*. Palpal article 1 visible from ventral, bearing an apparent lobe with a large, thick seta at the inner margin *(Fig. 1e)*. Article 2 with narrow outer and wider inner margin visible from dorsal *(Fig. 1c)*, 2 thick setae at the inner margin visible from ventral. Article 3 bearing a conspicuous inner spur visible from ventral, with 2 narrow setae at the inner margin. Article 4 located on the apex of article 3and bearing thick setae *(Fig. 1e)*.

The length of scutum vary between 2.5 and 2.7 (from apex of scapulae to apex of caudal appendage), and the width between 1.7 and 1.9. Caudal appendage short but wide, and truncated cone like in shape *(Fig. 1a)*. Eyes inconspicuous. Cervical grooves deep, extending to level of between coxa 2 and coxa 3. Posteromedian and paramedian grooves apparent, paramedian grooves longer than posteromedian groove *(Fig. 1a)*. Spiracular plate nearly circular with very small pores *(Fig.1f)*. Genital aperture wide; located on the anterior margin of coxa 2; and small, irregular serrations on the posterior margin *(Fig. 1g)*. Adanal plates extending to the body margin but not passing over. Posterior margin of adanal plate concave thoroughly with a longer inner spur and a short outer spur. Accessory adanal plate nearly triangular, extending to level of outer spur of adanal plates. A spur like bulge at the terminal part of the accessory plates *(Fig. 1h)*. Anus nearly circular, with 2 setae on each

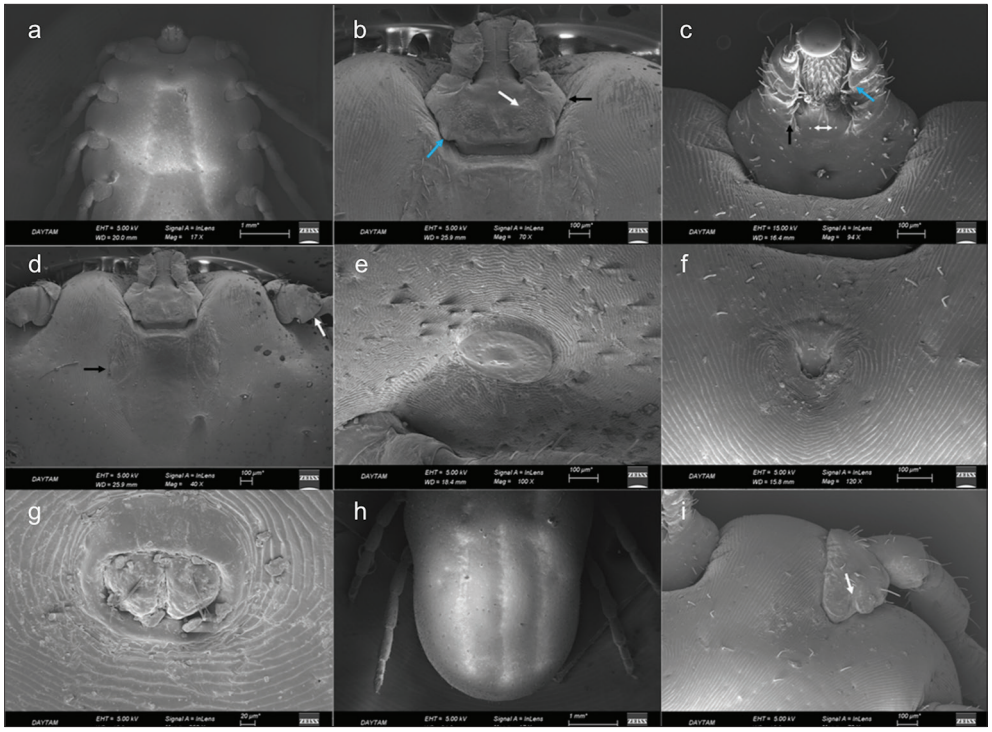

Fig 2. *Rhipicephalus kohlsi* female. (a) Ventral view, (b) Dorsal view of capitulum, white arrow indicates porose area, black arrow indicates lateral angle, blue arrow indicates cornua, (c) Ventral view of capitulum, white double headed arrow post hypostomal setae, black arrow indicates the lobe and seta on palp article 1, blue arrow indicates inner spur of palp article 3, (d) Black arrow indicates eyes; white arrow indicates trochanter 1, (e) Spiracular plate, (f) Genital aperture, (g) Anus, (h) Dorsal view of idiosoma, (i) Ventral view of coxa 1 spur

valve *(Fig. 1i)*. Idiosoma covered with large, seta bearing punctuations regularly *(Fig. 1a,b)*.

Legs strong and long. Coxa 1 elongate a large anterior spur visible from dorsal view *(Fig. 1c)*. From ventral view coxa 1 bearing a wider and round shaped inner spur and a narrow, a bit longer and sharper outer spur *(Fig. 1d)*. A large, triangular shield on trochanter 1 visible from dorsal view *(Fig. 1c)*. Coxa 2 with a bulge like outer spur, and a spur located at the junction point of inner and posterior margins. The structure of coxa 3 similar with coxa 2 but inconspicuous. Coxa 4 with a small, triangular shaped and median located spur *(Fig. 1b)*.

Body length of partially fed female tick 7.3, and width 4.1 *(Fig. 2a)*.

Basis capituli hexagonal, with sharp lateral angles; the length 0.7 and the width 0.3. Posterior margin slightly convex. Cornua mildly subtriangle in shape but small, with rounded margin. Porose areas pear shaped, medium size, located separately *(Fig. 2b)*. Setae on the ventral side could not be completely counted because of occurrence of setae breakage in these parts. A pair of setae visible at the post hypostomal region. Hypostome dental formula 4/4 *(Fig. 2c)*. Palpal article 1 visible from ventral, with a lobe with a thick seta at the inner margin. Article 2 straight, with 2 or 3 setae at inner margin; article 3 bearing a conspicuous inner spur visible from dorsal, with 2 narrow setae at the inner margin. Article 4 located on the apex of article 3 and bearing thick setae *(Fig. 2c)*.

The length of scutum 1.3 (from apex of scapulae to posterior margin), and the width 1 (at widest point). Posterior margin slightly sinuous with a convex curve towards eyes. Eyes large, flat. Cervical grooves extending to posterolateral margin. At the proximal side of scutum, 13-14 setae on lateral parts *(Fig. 2d)*. Spiracular plate nearly circular with very small pores *(Fig. 2e)*. Genital aperture located on the posterior of coxa 1, posterior lips in a broad U shape and lateral margins diverging *(Fig. 2f)*. Anus nearly circular, with 2 setae on each valve *(Fig. 2g)*. Idiosoma covered with setae *(Fig. 2h)*.

Legs strong and long *(Fig. 2a)*. Coxa 1 spurs nearly equal, coxa 2-4 spurs indistinct *(Fig. 2i)*. Trochanter 1 with a large, triangular shield visible from dorsal view *(Fig. 2d)*.

DISCUSSION

Distribution area of *R. kohlsi* is known to be restricted in Middle East, Central Asia and West Africa [9-14]. In Turkey, reports were from southeastern Anatolia region [15,16] but with our study from northeastern Anatolia, it is understood that *R. kohlsi* possibly has a wider geographical distribution in our country than considered before.

Worldwide, records of this species are rare maybe because

of having a restricted distribution. Also, it is an unregarded tick species due to not having a known vectorial capacity until now [11]. One of the outcomes of global warming is the spread of tick species and tick-borne diseases to new areas. Although there is not a known vectorial role of *R. kohlsi* yet, but we cannot be sure from forthcoming years. Nonetheless, knowledge about the presence of tick species in a region is beneficial to consider possible emerging or re-emerging tick-borne diseases.

The ticks are confirmed to be *R. kohlsi* by using taxonomic keys of Hoogstraal and Kaiser [7]. The keys for the differential diagnosis of *R. kohlsi* are as follows: setae-bearing lobe on the inner margin of palpal article 1 visible from ventral, and dental formula 4/4 for both sexes. Male tick with caudal appendage, adanal plates not passing over the body margin and with a concave posterior margin. The size of accessory plates' inner spur is intermediate, between adanal plates' size. The genital aperture is wide in males and shield-shaped in female. Coxa 1 has a deep cleft in both sexes. Porose areas are pear shaped. Although Hoogstraal and Kaiser [7] stated that porose areas fairly small, our sample has medium sized porose areas.

This case report represents the presence and morphological data of *R. kohlsi* obtained from a wild goat. Also, the first SEM records of male and female *R. kohlsi* were presented. This species may have a wider distribution than expected so it has to be taken into consideration in tick identification studies.

Acknowledgments

Scanning electron microscopy observations were carried out using facilities of the East Anatolia High Technology Application and Research Center (DAYTAM).

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Kafkas Univ Vet Fak Derg
25 (5): 729-730, 2019
DOI: 10.9775/kvfd.2019.22616

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
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Letter to the Editor

Ventricular Septal Defect and Pulmonic Stenosis in a Dog (Bir Köpekte Ventriküler Septal Defekt ve Pulmonik Stenozis)

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Article Code: KVFD-2019-22616 Published Online: 12.06.2019

How to Cite This Article

Yilmaz Z, Levent P, Saril A, Uemura A, Kocatürk M, Tanaka R: Ventricular septal defect and pulmonic stenosis in a dog. *Kafkas Univ Vet Fak Derg,* 25 (5): 729-730, 2019. DOI: 10.9775/kvfd.2019.22616

Dear Editor,

Ventricular septal defect (VSD) is characterized by an abnormal communication between left (LV) and right ventricles (RV) with left-to-right and than right-to-left cardiac shunts [1,2]. Pulmonic stenosis (PS) is a congenital heart defect of the semilunar valve between the RV and the pulmonary artery (PA). When the leaflets of PA valves are thickened and/or the annulus is narrowed, these findings give rise to PS causing very mild to severe obstruction of the blood flow from heart to the lungs [2]. PS may also be associated with other congenital defects such as VSD and aortic stenosis (AS). Dogs with small VSD and/or mild PS have a normal lifespan without clinical signs. In dog with large VSD, in the physical examination, loud cardiac murmur, exercise intolerance, and severe panting can be observed. However, dogs with severe PS may have exercise-induced syncope, arrhythmias or heart failure [1]. These observations show that timing in the diagnosis of congenital heart defects is important on whether medical therapy and/or surgical approach could be suggested. Thus, we have described here the presence of two congenital cardiac defects (VSD and PS), and their diagnostic algorithm by use of conventional diagnostic techniques with three-dimensional computed tomographic angiography (3D-CTA) in a dog.

A Doberman Pincher dog (5 years, female, and 7.1 kg) was referred for further examination of heart diseases from a small animal clinic to Animal Hospital (Faculty of Veterinary Medicine, Bursa Uludag University) with a history of exercise intolerance during strenuous exercise for 2.5 months. Before admission, the patient was treated with furosemide for 7 days due to suspicious of pulmonary oedema. Body temperature (38.5°C), capillary refilling time (<2 sec) and respiratory rates (22/min.) were within reference ranges. Colour of mucous membranes was normal, and external lymph nodes and abdominal palpation were unremarkable. Soft systolic cardiac murmur - grade 1/6 was auscultated over left 3-5 intercostal spaces. Thoracic radiography showed PA bulging with a normal lung tissue appearance suggestive for aorta-pulmonary window (APW), patent ductus arteriosus (PDA) or PS. ECG examination showed qrS complex and sinus tachycardia (150 bpm). Complete blood cell counts and serum biochemistry profile were non-specific. Serum BUN (29.4 mg/dL) was observed at maximum level of its reference (10-30 mg/dL) and serum Na level (141 mEq/L) was of its lower reference level (141-152 mEq/L) due to furosemide administration. Serum cardiac troponin I was 0.06 ng/mL (reference: <0.07 ng/mL), as well.

Standard echocardiographic images showed that LV related geometric (LV dimension and thickness of inter-ventricular septum and LV post-wall) and functional measurements (fractional shortening - FS% and ejection fraction - EF%) were within reference ranges. Two-dimensional left atrium (LA) to aortic root (Ao) ratio was 1.2, and Ao to PA ratio was 1.4, suggestive for narrowing PA. There was also post-stenotic dilation of main PA. Colour Doppler showed a severe turbulence just below the PA valve, and CW Doppler indicated the presence of PS based on the high PA velocity (4.48 m/s) and pressure gradient (PG: 80.1 mmHg) *(Fig. 1)*. Based on the PG, PS has been defined as mild (<50 mmHg), moderate (50-80 mmHg) and severe (>80 mmHg) [2]. In this dog, PS was further classified as a valvular type B due to hypoplastic pulmonic valve annulus and thickening of valvular leaflets [1,2]. PDA and APW were rule-out the differential diagnosis list because there were not continuous type machinery murmur in thoracic auscultation, typical ECG abnormalities (tall R wave and P-mitrale) and continuous systolic and diastolic pulmonary flows with CW Doppler echocardiography [1].

A small VSD was identified due to less than 1/3rd of Ao diameter (0.6 cm *vs* 1.4 cm). The severity of VSD is mainly determined by the size of VSD and PG between ventricles. A small VSD (restrictive) causes a louder murmur and higher PG (usually around maximum velocity of 5 m/s and PG of 80 mmHg)

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because LV systemic pressure is five times larger than RV pressure (around 100 mmHg to 20 mmHg, respectively) which is also the reason of left to right shunt than right to left shunt in small defects [1]. Thus, the possible reason why we observed the soft and lower intensity murmur and measured low intensity velocity (2.9 m/s) and PG (35.8 mmHg) of ductal flow *(Fig. 2)* in this dog is that shunt rate of VSD might be controlled by RV pressure overload arising from PS, as reported previously [3]. In this case, Qp:Qs ratio was 2.1, in compatible with left to right shunt. VSD is considered as small (restrictive), moderate (moderately restrictive), or large (nonrestrictive) if Qp:Qs was <1.5, between 1.5 and 2.5, and >2.5, respectively [4]. Thus, since VSD peak velocity was measured between 2.5 m/s and 4.0 m/s with lower PG of 25-60 mmHg, moderately restrictive VSD could be possible in this case. Perimembraneous VSD and PS were confirmed by use of 3D-CTA *(Fig. 3)*, as reported in a previous case [5]. Based on the diastolic (mitral and tricuspid inflows - E/A ratio and E/e'_{septal}) and systolic indices (LV - FS%, EF%, ejection time and pre-ejection period), diastolic and systolic dysfunctions were not possible yet in this dog.

For this patient, medical support did not suggested because there were not a cardiac dysfunction and chamber enlargement due to moderate PS and small VSD. The dog was re-examined two times in 3-month intervals, and did not need still any medication. The possible mechanism of the non-clinical signs in this dog is that PS could be prevent left heart failure caused by VSD [3]. In dogs with clinical symptoms and having a cardiac remodelling due to PS, medical support such as pimobendan, sildenafil or bosentan is used to reduce PA hypertension [1,5]. In severe cases of PS, balloon valvuloplasty is performed as well. Dogs with mild VSD should be monitored without medication if the patients do not show the clinical signs because it could be closed spontaneously in time. If not, VSD can cause left heart failure, and then classical drug therapy should be initiated. VSD could be improved by surgical approaches [1].

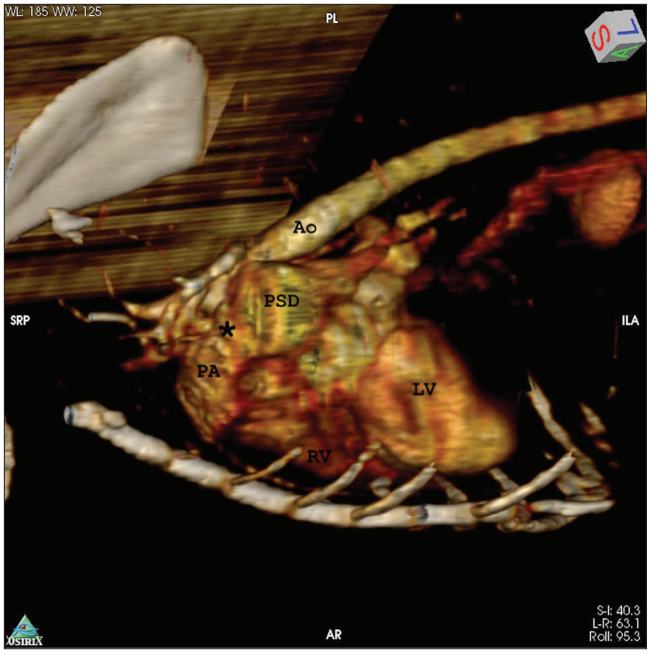

Fig 3. 3D-CTA showed the pulmonic stenosis *(asterix)* and post-stenotic dilation (PSD) of pulmonary artery (PA). Ao: Aorta, LV: Left ventricle, RV: Right ventricle

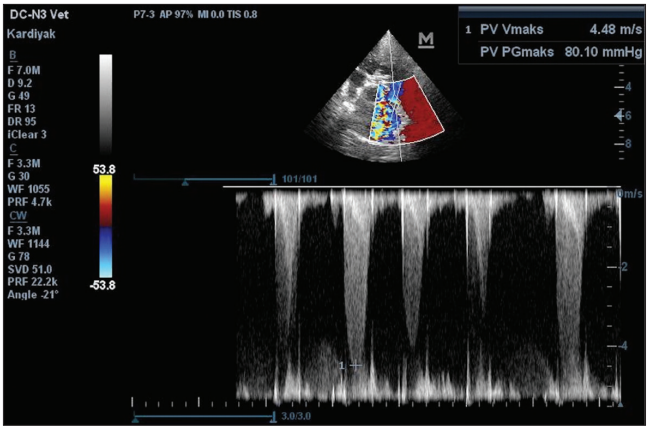

Fig 1. Right parasternal short axis view, CW Doppler indicated pulmonic stenosis (Vmax: 4.48 m/s and pressure gradient: 80.1 mmHg)

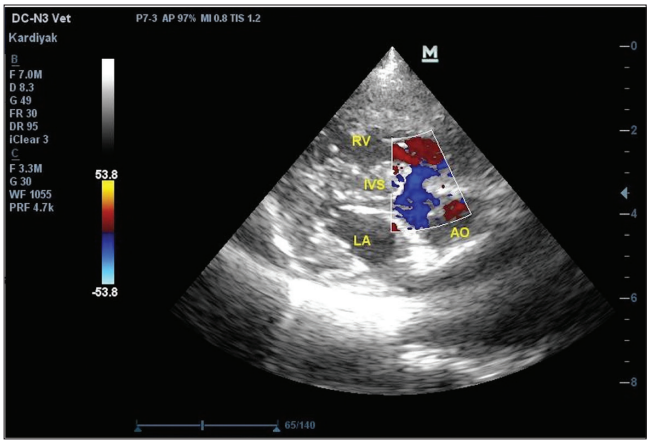

Fig 2. Right parasternal long axis 5 chamber view and color Doppler indicated a defect just below the aortic valve, in compatible with perimembraneous ventricular septal defect (VSD)

In conclusion, radiography only provides information regarding the size and shape of the silhouette of the heart but angiocardiography (3D-CTA) could be used to see vasculatures, stenosis, shunts and multiple congenital cardiac defects in dogs. Doppler echocardiography is superior to describe severity of PS, compared to 3D-CTA. Medical support should only be thought if the patients have shown clinical signs related with VSD and PS.

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Kafkas Univ Vet Fak Derg
25 (5): 731-740, 2019
DOI: 10.9775/kvfd.2018.21341

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

Review

Relationship Between Polyunsaturated Fatty Acids and Animal Production: A Review

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Article ID: KVFD-2018-21341 Received: 13.11.2018 Accepted: 06.05.2019 Published Online: 08.05.2019

How to Cite This Article

Tang S, Guo S, Wang J, Wang Y, Fu S, Shen Z: Relationship between polyunsaturated fatty acids and animal production: A review. *Kafkas Univ Vet Fak Derg,* 25 (5): 731-740, 2019. DOI: 10.9775/kvfd.2018.21341

Abstract

Polyunsaturated fatty acids (PUFAs) play important roles in maintaining normal physiological conditions and, consequently, in animal health. Two PUFAs families, n-6 and n-3 fatty acids (FAs), are physiologically and metabolically distinct. The focus on PUFAs has been gradually extended from the apparent properties of growth performance, antioxidant ability and immune function to the mechanism of molecular regulation and mechanism such as regulating the expression of related genes and the anticancer action mechanism, which strengthen the understanding of the theoretical basis and application of animal husbandry in a range of animal species. The present review focuses on current knowledge related to the origin, biological function and practical application of PUFAs in animal production.

Keywords: *Polyunsaturated fatty acids Biological function Animal production Application*

Çoklu Doymamış Yağ Asitleri ve Hayvansal Üretim Arasındaki İlişki: Derleme

Öz

Çoklu doymamış yağ asitleri normal fizyolojik süreçlerin ve bunun sonucu olarak da hayvan sağlığının sağlanmasında önemli rol oynamaktadır. İki çoklu doymamış yağ asitleri ailesi olan n-6 ve n-3 yağ asitleri fizyolojik ve metabolik olarak önemlidir. Büyüme performansı, antioksidan kabiliyeti ve immun fonksiyonlarından moleküler regülasyon ve ilgili genlerin ekspresyonunun ve anti kanser mekanizmaları gibi mekanizmalardaki belirgin özellikleri nedeniyle çoklu doymamış yağ asitlerine ilgi artmıştır. Bu durum pek çok hayvan türünde yetiştiricilik konusunda teorik temellerin anlaşılmasına katkıda bulunmuştur. Bu derleme, çoklu doymamış yağ asitlerinin hayvansal üretimdeki yeri, biyolojik fonksiyonları ve pratik uygulamaları üzerine güncel bilgiye odaklanmıştır.

Anahtar sözcükler: *Çoklu doymamış yağ asitleri, Biyolojik fonksiyon, Hayvansal üretim, Uygulama*

INTRODUCTION

Feeding fat has nutritional and economic advantages to satisfy energy requirement and replace carbohydrates with inexpensive lipid sources. Fatty acids (FAs) are important ingredients and essential substances of fats and oils. Based on their chemical structure FA can be differentiated into three groups: saturated, monounsaturated and polyunsaturated fatty acids (PUFAs). The biological reactivity of FA is defined by the length of the carbon chain and by both the number and position of any double bonds present. While saturated fatty acids (SFAs) do not contain double bonds within the acyl chain, unsaturated fatty acids (UFAs) contain at least one double bond. When two or more double bonds are present, these UFAs are referred to as polyunsaturated fatty acids (PUFAs). Dietary imbalance of the n-6/n-3 PUFAs ratio can affect human health, especially with high n-6/n-3 PUFAs ratios [1].

FAs supplies have been demonstrated to alter immune cells function because FAs alter the composition of the cells' membrane phospholipids, which relates to the membrane lipid fluidity, signal transcription factors, and bioactive synthesis of lipid mediators [2]. Several studies have documented that PUFAs not only play an important role in maintaining structure and function of cell membrane, enhancing immune function, promoting growth and development, regulating lipid metabolism and related gene

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expression, but also reducing thrombosis, reducing the incidence of cardiovascular diseases as well as resisting cancer [3]. With the reduction of production cost and continuous pursuit of high quality animal product, the application of PUFAs in animal production has been a subject of great public attention and concern. The focus on PUFAs has been gradually extended from the apparent properties of growth performance, antioxidant ability and immune function to the mechanism of molecular regulation and mechanism such as regulating the expression of related genes and the anticancer action mechanism, which strengthen the understanding of the theoretical basis and application of animal husbandry in a range of animal species. This paper briefly described the origin, biological function and practical application of PUFAs in animal production.

SOURCES OF PUFAs

Polyunsaturated fatty acids are mainly found in all natural foods in varying amounts, but fatty foods contain the most. Generally speaking, vegetable oils are the most concentrated sources of PUFAs in the Western diet and include sunflower oil, safflower oil, corn oil, flax oil, sesame seed oil, pumpkin seed oil and canola (or rapeseed) oil. The exceptions include plants that grow in tropical climates, such as the oils extracted from chocolate and coconuts. These oils are highly saturated, and so are very stable and undoubtedly safe and beneficial.

Table 1 illustrated the members and typical sources of the n-6 and n-3 PUFA families. Linoleic acid (LA) is the parent FA of the n-6 family, which occurs in almost every dietary fat and attains major proportions in some common vegetable oils. Due to its wide distribution and abundance in many common dietary fats, many populations over-consume LA, and consequently the intake of n-3 FAs fatty acids is very often lower than ideal. γ-linolenic acid (18:3n-6 or GLA) is a trace constituent in some animal PLs and ruminant fats. It is more readily available from seed oils of evening primrose (8-10%), borage (20-25%) and blackcurrant (15-18%). Arachidonic acid (AA) is present in lean meat, especially in free-living animals, liver and egg lipids. It is rare in the plant kingdom, but it has been reported in marine algae and other aquatic plants.

Alpha-linolenic acid (ALA), the parent FA of the n-3 family, is primarily present in the plant kingdom. Among the common vegetable oils, it is readily available in canola (6-10%) and soybean (5-8%) oils. Marine fish such as mackerel, salmon, sardine, herring and smelt are excellent sources of eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA). Fish oil (FO) containing 60% of EPA and DHA is usually sold as sources of these important n-3 PUFAs. Algal oils and single-cell oil sources of the long-chain PUFAs are now becoming available to provide EPA+DHA+AA. Furthermore, genetically modified oil sources are now being developed and will be widely available in the near future by the genetic manipulation of soy and other plants.

BIOLOGICAL FUNCTIONS OF PUFAs

Influence on Membrane Fluidity

Polyunsaturated fatty acids play an important role in the composition of all cell membranes where they maintain homeostasis for correct membrane protein function and influence membrane fluidity, thus regulating cell signaling processes, cellular functions and gene expression. The fluidity

Table 1. *Typical sources of n-6 and n-3 PUFAs*

Family	Common Name	Systematic Name	Abbreviation	Typical Sources
n-6 family	Linoleic acid	cis-9,cis-12-octadecadienoic	18:2n-6 (LA)	most vegetable oils
	γ-Linolenic acid	cis-6,cis-9,cis-12-octadecatrienoic acid	18:3n-6 (GLA)	evening primrose, borage and black currant seed oils
	Homo-γ-linolenic acid	cis-8,cis-11,cis-14-eicosatetrienoic acid	20:3n-6 (DGLA)	very minor component in animal tissues
	Arachidonic acid	cis-5,cis-8,cis-11,cis-14-eicosatetraenoic acid	20:4n-6 (AA)	animal fats, liver, egg lipids, fish
	Docosatetraenoic acid	cis-7,cis-10,cis-13,cis-16-docosapentaenoic acid	22:4n-6	very minor component in animal tissues
	Docosapentaenoic acid	cis-4,cis-7,cis-10,cis-13,cis-16-docosapentaenoic acid	22:5n-6	very minor component in animal tissues
n-3 family	α-Linolenic acid	cis-9,cis-12-cis-15-octadecatrienoic acid	18:3n-3 (ALA)	flaxseed oil, perilla oil, canola oil, soybean oil
	Stearidonic acid	cis-6,cis-9,cis-12,cis-15-octadecatetraenoic acid	18:4n-3 (SA)	fish oils, genetically enhanced soybean oil, blackcurrant seed oil, hemp oil
	Eicosapentaenoic acid	cis-5,cis-8,cis-11,cis-14,cis-17-eicosapentaenoic acid	20:5n-3 (EPA)	fish, especially oily fish (salmon, herring, anchovy, smelt and mackerel)
	Docosapentaenoic acid	cis-7,cis-10,cis-13,cis-16, cis-19-docosapentaenoic acid	22:5n-3 (n-3 DPA)	fish, especially oily fish (salmon, herring, anchovy, smelt and mackerel)
	Docosahexaenoic acid	cis-4,cis-7,cis-10,cis-13,cis-16,cis-19-docosapentaenoic acid	22:6n-3 (DHA)	fish, especially oily fish (salmon, herring, anchovy, smelt and mackerel)

of the membrane is dependent on the lipid composition of the membrane and among the significant components of cell membranes are the phospholipids. The types of FAs in the diet determine the types of FAs that are available to the composition of cell membranes. A phospholipid made from a saturated fat has a different structure and is less fluid than the one that incorporates an essential FA. PUFAs are important constituents of all cell membranes, which confer on membranes properties of fluidity, and thus determine and influence the behavior of membrane-bound enzymes and receptors [4]. PUFAs had an inhibitory effect on cell proliferation/viability and strongly increased tumor cell lipid peroxidation in a dose-dependent manner [5]. n-3 PUFAs supplementation can modify the membrane phospholipid fatty acid composition and modulate the second messenger signaling pathways in the adult cardiac myocytes. However, because the unsaturated double bonds of PUFAs are susceptible to the oxidation of free radicals, excessive intake of PUFAs can lead to the enhanced lipid peroxidation *in vivo* and reduce cell membrane fluidity. They are able to decrease the cholesterol level in the neuronal membrane, which would otherwise decrease membrane fluidity, which in turn would make it difficult for the cell to carry out its normal functions and increase the cell's susceptibility to injury and death. These consequences for cell function are not restricted to absolute levels of FAs alone; rather it appears that the relative amounts of n-3 PUFAs and n-6 PUFAs in the cell membranes are responsible for affecting cellular function. In addition, n-3 PUFAs had ameliorative effects on the oxidative tissue degeneration and inflammatory processes induced by CCl_4 treatment in rats [6].

Polyunsaturated fatty acids are important components of phospholipid in neuronal membranes, which play important roles in signal transduction of cellular surface. DHA accounts for 10% of the brain phospholipids, and it also has a high content in the central nervous system, mainly distributing in acetylcholine, amino phospholipids and serine glycerol [7]. DHA is instrumental in the function of brain cell membranes, which are important for the transmission of brain signals. By making cell membranes more fluid, n-3 PUFAs, especially DHA, improve communication between the brain cells and lack of n-3 PUFAs in the body can cause a communication breakdown in the brain. EPA and DHA could affect signal transduction in brain cells by activating peroxisomal proliferator-activated receptors (PPARs), inhibiting G-proteins and protein kinase C, as well as calcium, sodium, and potassium ion channels. In addition, DHA and EPA can affect the membrane structure of sperm cells, making the sperm membrane with good fluidity and participating in cell response mediated in protein, which could influence the production of lipid-mediated conductor, cell signal transduction as well as gene expression [8].

Lipid Metabolism

Polyunsaturated fatty acids, particularly those of the n-3 family, play essential roles in the maintenance of energy balance and glucose metabolism. n-3 PUFAs, in addition to affecting general properties of cells as membrane components, play a role in modulating the production of both lipid (eicosanoids) and protein (cytokines) mediators. The n-3 family of PUFAs appear to have the unique ability to enhance thermogenesis and thereby reduce the efficiency of body fat deposition. PUFAs exert their effects on lipid metabolism and thermogenesis by upregulating the transcription of the mitochondrial uncoupling protein-3, and inducing genes encoding proteins involved in fatty acid oxidation (e.g. carnitine palmitoyltransferase and acyl-CoA oxidase) while simultaneously down-regulating the transcription of genes encoding proteins involved in lipid synthesis (e.g. fatty acid synthase). n-3 PUFAs can inhibit the activity of fatty acid synthase (FAS), diacylglycerol acylase and hydroxymethyl glutarate coenzyme A reductase to promote the oxidative decomposition of fatty acids, thus depress the synthesis of triglyceride (TG) and reduce the content of low density lipoprotein (LDL) receptor in the liver to inhibit total cholesterol (TC) synthesis and absorption, finally reducing the level of TG and TC in the serum [9].

Lipid metabolism, inflammation, oxidative stress and endothelial function play important roles in the pathogenesis of cardiovascular disease (CVD), which may be affected by an imbalance in the n-6/n-3 PUFAs ratio [10]. Low n-6/n-3 PUFAs ratio (1:1 and 5:1) had a beneficial effect on cardiovascular risk factors by enhancing favorable lipid profiles, having anti-inflammatory and anti-oxidative stress effects, and improving endothelial function. In contrast, a high n-6/n-3 PUFAs ratio (20:1) had adverse effects.

Regulation of Gene Expression

Supplementation of PUFAs has become an important dietary strategy to reduce hepatic lipogenesis in rodents and humans by inducing the regulation of gene expression. PUFAs can affect gene expression through various mechanisms including, but not limited to, changes in membrane composition, intracellular calcium levels, and eicosanoid production. Furthermore, PUFAs and their various metabolites can act at the level of the nucleus, in conjunction with nuclear receptors and transcription factors, to affect the transcription of a variety of genes. Several of these transcription mediators have been identified and include the nuclear receptors peroxisome proliferator-activated receptor (PPAR), hepatocyte nuclear factor (HNF)-4α, and liver X receptor (LXR) and the transcription factors sterol-regulatory element binding protein (SREBP) and nuclear factor-κB (NFκB). Their interaction with PUFAs has been shown to be critical to the regulation of several key genes of lipid metabolism [11].

The presence of high concentrations of n-3 PUFAs, or shifts in n-6/n-3 ratios may modulate the expression of genes known to be critical to inflammatory processes [12]. The majority of studies examining PUFAs and gene expression

have been carried out in isolated cell systems and a few animal studies. Curtis et al.[13] had shown that α-linolenic acid (LNA, 18:3, n-3), EPA, or DHA reduce the expression of genes for TNFα and IL-1β in bovine chondrocytes. DHA played different regulating roles in lipid metabolism in different tissues to reduce body fat mass through regulating lipogenesis and lipolysis genes. In adipose tissue, DHA increased the expression of lipogenesis and lipolysis genes. In liver lipogenesis genes were decreased, but lipolysis genes were increased by DHA [14]. Similarly, mice fed FO had decreased mRNA levels for numerous inflammatory cytokines including IL-1β, IL-6, and TNFα in kidney, spleen, and peritoneal macrophages [15]. Feeding 6% α-LA-enriched camelina meal to lactating dairy cows reduced expression of IL-1β, IL-8, and TNF-α in peripheral blood mononuclear cells [16].

Immune Function

The beneficial effects of PUFAs are of obvious therapeutic interest, they are believed to be preventive in various chronic diseases, including rheumatoid arthritis, coronary heart disease, and stroke, and certain types of cancer, including breast, prostate, and colorectal cancers. Preclinical studies in cell culture and rodent models show that the EPA and DHA acids are immunomodulatory and can influence both innate and adaptive immunity [17]. EPA and DHA are usually used in the treatment of inflammatory diseases such as rheumatoid arthritis, psoriasis and ulcerative colitis. In the eye, deficiency of DHA is associated with abnormalities in the visual system, including retinitis pigmentosa, peroxisomal disorders and compromised growth and development in infants [18]. Dietary PUFAs can modulate the immune system to improve growth performance and health. Research showed that milk replacer containing FO weakened acute phase responses and the effect was linear in response as the FO FA replacement increased from 5 to 10% [19].

Various n-3 PUFAs have been reported to exert beneficial effects, such as protection against liver diseases, regulation of cholesterol, and reduction of blood pressure, which prevents cardiovascular diseases (CVDs) [20]. n-3 PUFAs can modify B-cell activation, antigen presentation to helper T cells, antibody production, surface expression of select molecules, development in bone marrow, and the relative percentage or frequency of B cells in specific tissues [21-23]. In addition, n-3 PUFAs can also exert immunomodulatory effects on lymphocytes by targeting plasma membrane molecular organization [24]. Several studies have indicated that n-3 PUFAs exert anti-inflammatory effects by regulating the expression of peroxisome proliferator activated receptors (PPARs) and nuclear factor kappa B (NF-κB) [25-27]. The inflammatory response is triggered by activation of NF-κB, which induces the expression of pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes, such as cyclooxygenase 2 (COX-2) and nitric oxide synthase (NOS) [28].

APPLICATION OF PUFAs IN ANIMAL PRODUCTION

Application in Poultry Diets

Previous studies in broiler chickens have shown different relationships between the fatty acid contents of diets and tissues, especially for breast and thigh meat [29,30]. Comparable levels of n-3 PUFAs in the meat can be achieved by only feeding the flaxseed oil diet in the last 3-4 weeks of the growth period [31]. The growth performance of broilers fed on n-3 PUFA-enriched diets (linseed oil) was not different from those fed on a control diet [32]. Dietary incorporation of linseed oil and pig lard during starter, grower and finisher phases can enrich broiler chickens meat with n-3 PUFAs [33].

Eggs enriched with n-3 PUFAs can be used to increase the n-3 PUFAs content of the human diet. In recent decades, the consumption of chicken meat has steadily increased and many studies have focused on the use of dietary modifications to improve the quality of the poultry egg and meat. More specifically, enhancing the functional value of poultry egg and meat through dietary intervention appears to be the most justified, safe, and efficient method [34]. The most efficient way of promoting the functional value of meat was to feed chickens a diet containing FO and rapeseed (10 g/L and 60 g/L diet, respectively) for the last three weeks before slaughter [35]. Such a dietary intervention not only increased the share of long-chain PUFAs and decreased the PUFAs n-6/n-3 ratio of meat lipids, but also increased the content of EPA and DHA in the edible parts of the carcass. López-Ferrer et al.[36] assessed the effect of a diet supplemented with FO on the FAs composition and quality of broiler meat. The results showed that high FO concentrations decreased the saturated and monoenoic FAs contents in the thigh samples. The amount of PUFAs (mainly as EPA, DPA and DHA) increased when FO added in the diets and the levels of total n-6 PUFAs resulted in slight changes, mostly in LA. The total egg yolk n-3 PUFAs was increased from 135.4 mg/egg to 344.5 mg/egg after 18-day feeding with the diet containing 15% Lin-PRO (flaxseed:pea = 1:1; wt/wt) [37]. For EPA and DHA, but not for LA, the diet effect was more distinct in the extensor carpi radialis compared to *longissimus* thoracis and *biceps femoris* [38]. The emulsified fat powder can improve serum SOD and GSH-Px the activity and decrease the content of MDA, improving the antioxidant performance of laying hens [39,40]. The effectiveness of the canola oil on the some metabolites ostriches was evaluated and indicated that glucose and total protein levels increased significantly, whereas total immunoglobulins insulin, albumin, ALT and AST did not change [41]. The main immune organs in poultry are the thymus, spleen, and bursa of *Fabricius*. During an immune response, mature lymphocytes and other immune cells interact with antigens in these tissues. Wang et al.[42] observed that feeding laying chickens diets rich in

n-3 PUFAs promoted the growth of the thymus, spleen, and bursa up to 4 weeks of age. However, from the age of 4 weeks onward, immune tissue weights began to decline, and the bursa degenerated between 4 and 8 week of age. Dietary n-3 PUFAs could decrease phagocytosis and lymphocyte proliferation in broiler chickens, highlighting the need for the poultry industry to consider the health status of poultry when poultry meat is being enriched with FO [43]. An increased ratio of membrane n-6 to n-3 is involved in the pathogenesis of depression and n-3 supplementation has shown positive effects in clinical trials [44]. Typical formulated broiler diets are deficient in n-3 PUFAs due to widening n-6/n-3 PUFAs ratio which could greatly affect performance, immune system of birds and meat quality. Ibrahim et al.[45] evaluated the effect of modifying dietary n-6 and n-3 PUFAs ratio from plant and animal oil sources on performance, behavior, cytokine mRNA expression, antioxidative status and meat FAs profile of broiler chickens. The results indicated that narrowing n-6/n-3 ratio through the addition of FO or LO improved growth performance and immune response of broilers and resulted in healthy chicken meat, enriched with long chain n-3 PUFAs.

The proportion of respective PUFAs in the chicken partly depends on diet and an increase in the proportion of n-3 PUFAs in the diet has been shown to improve fertility. Diet supplementation with linseed oil has capability to make the suitable changes in the lipid contents of sperm as well as the improvement in live percentage of sperms [46]. Blesbois et al.[47] measured the effects of dietary n-3 PUFAs supplementation on the reproductive capacity of adult male turkeys in industrial flocks. The FO diet very effectively increased the percentage of n-3 PUFAs (22:5n-3 and 22:6n-3) in spermatozoa and correspondingly decreased the percentage of n-6 PUFAs (20:4-6 and 22:4n-6). These changes did not affect the spermatozoa content of n-9 PUFAs, particularly of 22:3n-9 which is abundant in turkey spermatozoa (9-12% of the total FAs). The supplementation was effective in the middle as at the end of the reproductive period. The reproductive capacity of males was modified by the diet and the positive effect of the n-3 supplemented diet increased with age (increase in hatching rates of nearly 2 points at 48-58 weeks for males fed FO diet). These results indicated that n-3 PUFAs enrichment of the turkey diet induced changes in spermatozoa PUFAs, with an overall positive effect on the reproductive capacity of adult males, especially during the decreasing phase of the annual reproductive period.

Application in Swine Diets

Sow Nutrition: Addition of fat to the diets of sows during late gestation and (or) lactation can increase milk production and fat content of colostrum and milk. The maternal dietary fat during the perinatal period affected the type and quantity of the FAs in milk, which was one of the most important pathways to afford nutrition for neonates [48]. For sows, PUFAs can not only influence the composition of FAs in sows, but also affect the composition of FAs in the body tissues of piglets through the composition of FAs in colostrum and milk, which can provide plenty of FAs and energy supply for piglets with the aims to improve the survival rate and growth of piglets as well as the reproductive performance of sows. Supplementation of the maternal diet with FO or linseed oil increased the level of n-3 PUFAs of the piglets in a tissue-specific manner. The response of Δ6-desaturase and Δ5-desaturase protein expression in female piglets to the dietary manipulation was also tissue-specific, suggesting that the increase in n-3 PUFAs content in the progeny was related, at least partially, to the activation of Δ6-desaturase and Δ5-desaturase expression [49].

The administration of different oils to sows during lactation can alter the FAs composition of the offspring piglets at weaning, although the type of FAs was not consistent [50]. The percentage of solids in milk was greater for sows fed the tallow diet, due to an increase in the fat and ash content. Compared with percentages of FAs in milk of sows fed the control diet, the percentages of C10:0, C14:0, C16:0, C16:1, and C18:3 FAs were lower and the percentages of C18:0 and C18:1 FAs were higher in milk of sows fed tallow diets. Litter weaning weight was greater for pigs from sows fed tallow diets than for pigs from control sows. Pigs from tallow-fed sows had greater carcass fat weight and fat percentages and lower water and protein percentages. The diet containing 8% corn oil starting seven days before farrowing until weaning significantly increased the contents of serum-lipid-related indexes in the sows [51]. Although the triglyceride content did not change, the C18:2n-6 content was higher in the colostrum and in the *longissimus thoracis* muscle of offspring pigs at both investigated stages. The total n-6 content and the ratio of n-6 to n-3 generally increased. These results demonstrated that maternal dietary fat during lactation affected the FAs' composition of the *longissimus thoracis* muscle of progeny at weaning and had persistent effects in later life.

Growing-Finishing Pigs Nutrition: Fatty acids' composition in the pig body is related to their composition in the diet. The deposition of n-3 PUFAs in the body depended on the source of fat in the diet and the increase of n-3 PUFAs intake increased their deposition in the pig body. Sobol et al.[52] reported that loin and shoulder of pigs (with high intramuscular fat content) fed a diet enriched with the mixture of linseed, rapeseed, and FO meet the European Union recommendations for human nutrition for products considered as either PUFAs n-3 sources or products with high PUFAs n-3 content. Alvarez-Rodriguez et al.[53] found out that total n-3 PUFAs content (mainly α-linolenic acid, ALA) was greater in organic than in conventional pork, probably due to ALA content from dietary vegetable oils. The source of n-3 PUFAs and dietary n-6/n-3 ratios allow for the favourable manipulation of the fatty acid composition

of pork, indicating that fatty acids composition in the pig body was related to their composition in the diet and the deposition of n-3 PUFAs in the body depended on the source of fat in the diet. However, increasing ALA and EPA + DHA intake enhances their deposition in the body, their net DE decreases [54].

Boar Nutrition

Pig spermatozoa contains a significant amount of DHA. Intake of different types and sources of PUFA has been shown to change the fatty acid composition of animal sperm and affect sperm quality [55]. FO (rich in n-3 PUFAs) has been shown to alter sperm structure and penetration resistance, and to increase sperm number and antioxidant capacity [56]. In addition, semen quality was significantly affected by the dietary n-6/n-3 PUFAs ratio. It has previously been reported that diets with a n-6/n-3 FA ratio of 1.6:1 could increase the proportion of intact acrosome of the boar's spermatozoa. Oils that are rich in n-3 PUFAs also significantly increased sperm density and sperm number [57]. Lin et al.[58] determined the effects of different dietary n-6/n-3 ratios on the reproductive performance of breeding boars and found that proper n-6/n-3 PUFAs ratio in the diet of breeding boars enhanced the development of testis and accessory sex gland function, and improved sperm quality, which may be related to favorable hormone metabolism and antioxidant capacity.

Application in Ruminant Diets

Reproductive Performance: Different types of fats have been utilized in an attempt to improve reproductive function in ruminant animals. FAs derived from plants and oil seeds have exerted a major impact on reproductive performance, some of the most common sources include sunflower, linseed, cottonseed, rapeseed and soybean. Animal fat (tallow) and calcium salts of SFAs may escape in a significant percentage rumen hydrogenation to be incorporated into adipose tissue and milk. Fish by-products contain a high proportion of PUFAs and pass without being altered in the rumen exerting no effects on rumen fermentation. There is strong evidence linking consumption of diets high in n-3 PUFAs with reduced circulating peripheral inflammatory markers such as PGF2α. Supplementation of n-3 PUFA enriched diet improves the pregnancy rate in the cow and buffalo [59], which is explained by a reduction in the uterine PGF2α secretion and/or decrease in the sensitivity of the CL to PGF2α during critical stage of embryonic development, preventing the onset of luteolysis and facilitating the establishment of pregnancy. Inflammatory eicosanoids including PGF2α, in particular, can significantly affect reproduction outcomes such as the onset of oestrus, embryo survival and parturition. Supplementation of EPA and DHA rich FO for about 10 weeks around the time of mating improved the number of POF and ovulation rate. The twinning and kidding were also enhanced in FO supplemented goats possibly by lowering the E2 and PGFM concentration during the window of pregnancy recognition [60].

Polyunsaturated fatty acids are critical nutrients which play an important role in maintaining the physical properties of the sperm membrane fluidity. In general, PUFAs are major component of the sperm cell membranes and thus the major mechanism that PUFAs can affect the sperm quality is associated with membrane physiological characteristics. Te incorporation of PUFAs into diet is able to cause alteration of fatty acid profile of sperm plasma membrane and results in improved sperm quality. Dietary supplementation with PUFAs can alter fluidity/permeability of sperm membrane and enhance reproductive performance in male ruminants thought improving testis development, spermatogenesis, motility and viability of sperm before and post freezing [61]. There have been a lot of studies carried out in various ruminants species reports have been published *(Table 2)*.

Growth Performance: Ponnampalam et al.[71] investigated the effect of diets containing n-3 PUFAs on muscle long-chain n-3 FAs content in lambs fed low and medium-quality roughage diets. Fish meal (80 g DM) can increased the muscle long-chain n-3 PUFAs content and decreased the ratio of n-6/n-3 in lamb meat. Feeding soy meal (75g DM modestly increased both the long-chain n-3 and n-6 PUFAs content of meat, resulting in no difference in the n-6/n-3 ratio of meat. The protected canola seed (6% DM) diet did not have a major effect on muscle n-3 FA but resulted in an increase in n-6 and the n-6/n-3 ratio of meat. Demirel et al.[72] reported that the lambs supplemented with FO plus linseed oil had greater concentrations of C14:0 in the polar lipid fraction of lamb musculus semimembranosus than lambs supplemented with a Ca salt of palm oil, but this was not the case for the neutral lipid fraction. Kim et al.[73] determined the effect of modifying the n-6:n-3 PUFAs ratio in a concentrate-based diet on feed intake, apparent nutrient digestibility, plasma hormones, and long chain FA composition of the ruminal contents, liver, and muscle of lambs. The results indicated that increasing the n-3 PUFAs in the diet with select oil sources decreased the n-6 to n-3 ratio in ruminal digesta, liver, and fore-shank muscle of growing lambs fed high concentrate diets. This change would likely improve the suitability of lamb meat as a healthful food.

Lactation: Ruminal biohydrogenation, combined with mammary lipogenic and Δ-9 desaturation pathways, considerably modifies the profile of dietary FA and thus milk composition [74]. Ruminal biohydrogenation of dietary unsaturated FAs are relatively constant, whereas secretion of these in milk is more variable absorbed. Dietary lipids are extensively hydrogenated by rumen micro-organisms, and the extent of this biohydrogenation is a major determinant of long-chain fatty acid profiles of animal products (milk, meat). Numerous studies demonstrated that marine oil supplementation could affect rumen lipid metabolism by altering the activity of specific ruminal bacteria involved in

Table 2. *Effect of dietary PUFAs enriched fat sources on semen quality*

Reference	Species	Fat Source	Percent Inclusion	Effect
Samadian et al.[62]	Ram	Fish oil	3%	Increased the proportion of DHA in sperm fatty acid composition, improved sperm concentration and motility
Esmaeili et al.[57]	Ram	Palm oil, sunflower oil, fish oil	35 g/d	Improved prefreezing semen characteristics after thawing, 35 days after the removal of fatty acid source, the percentage of C22:6 was highest in the fish oil treated group
Fair et al.[63]	Ram	Protected fish oil	2%	Increased semen concentration but no effect on other semen quality parameters including semen volume, wave motion, and progressive linear motion
Radmanesh et al.[64]	Ram	Calcium salts of soybean oil	4%	Improved the volume of semen and total sperm count in ejaculate
Dolatpanah et al.[65]	Goat	Fish oil and Vit. E	2.5% and 0.3g/kg DM	Improved testes development enhanced the quality and quantity of goat semen
Adeel et al.[66]	Buffalo	Sunflower oil, sunflower seed	1%	Improved the quality of sperm including motility and hypo-osmotic swelling of post-thawed sperm
Santos et al.[67]	Buffalo	Palm kernel cake	1%	Improvement of sperm quality, with higher sperm motility and higher levels of spermatozoa with plasma membrane integrity
Brinsko et al.[68]	Stallion	Commercially available nutriceutical formulated	250 g	Improved the motion characteristics of cool-stored stallion semen and the freezability of semen
Moallem et al.[69]	Bull	Flaxseed oil, fish oil	450 g/d (84.2 g/d C18:3 n-3)	Changed in the characteristics of both fresh and frozen-thawed semen, increased motility and progressive motility of sperm
Khoshvaght et al.[70]	Bull	Fish oil	1.2% DM	Improved fresh and post-thaw semen quality in Holstein bulls via alteration of sperm fatty acid composition

bio-hydrogenation and isomerization of dietary PUFAs [75]. With greater interest in increasing the n-3 PUFAs content of human diets, interest has also developed to increase these FAs in animal products. FO can modify ruminal or systemic functions, stimulating increased conversion of linoleic acid into transvaccenic and conjugated linoleic acids [76]. Supplementing the diet of partially grazing cows with FO and sunflower oil increased the milk cis-9, trans-11 CLA content, and that increase remained relatively constant after one week of oil supplementation [77]. Zhao et al.[78] investigated the relationship of FAs composition with specific bacteria involved in hydrogenation of 18-carbon UFA in response to dietary oil sources. These results demonstrated that unprotected FO and sunflower oil affected ruminal fermentation and produced series of bio-hydrogenation intermediates. Alterations in ruminal bio-hydrogenation were associated with changes in the abundance of *B. proteoclasticus*, but *B. proteoclasticus* was not the dominant bacterium in producing C18:0.

CONCLUSIONS AND FUTURE PROSPECTS

Polyunsaturated fatty acids are critical nutrients for normal growth and development and play an important role in the composition of all cell membranes where they maintain homeostasis for correct membrane protein function and influence membrane fluidity, thus regulating cell signaling processes, cellular functions and gene expression. Dietary supplementation with PUFAs can alter fluidity/permeability of sperm membrane and enhance reproductive performance in ruminants thought improving testis development, spermatogenesis, motility and viability of sperm before and post freezing. More important, n-3 PUFAs can significantly affect circulating peripheral inflammatory markers such as PGF2α as well as a number of hormones and cytokines and these peripheral markers can have a significant effect on reproduction outcomes. In addition, the structure of fat tissue of monogastric animals (pigs, poultry) is very similar to the fat structure of the feedstuffs on which the animals are fed, which means that source and type of fat in an animal feed can greatly influence the composition of fatty tissue and deposits in the resultant carcasses. Since PUFAs give rise to a variety of biologically active compounds which all have important roles in pathological and physiological processes, a proper understanding is needed regarding the contribution these active compounds have on the coinciding increases in inflammatory diseases seen with the disruption of the balance in the ratio of n-6 to n-3 PUFAs. So, it is necessary to conduct further researches to make clear about the effect of optimum n-6 to n-3 PUFAs ratio on various animals and their different stages, thereby contribute to modifying their diets in practice and achieve maximum effect on reproduction.

Disclosure Statement

No potential conflict of interest was reported by the authors.

Acknowledgments

This study was partly funded by the Innovation Program of Modern Agricultural Industry System of Shandong (SDAIT-21-15) and Agricultural Scientific and Technological Innovation Project of Shandong Academy of Agricultural Sciences (No. CXGC2017B02).

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