Kafkas Univ Vet Fak Derg
23 (3): 459-465, 2017
DOI: 10.9775/kvfd.2016.17053

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Changes in Lipid Peroxidation, Glutathione and Fertility in Tuj Sheep After Combined Administration of Vitamin A and E and Passive Immunization with Testosterone Antibodies [1]

Nadide Nabil KAMİLOĞLU [1] Cihan KAÇAR [2] Aysel GÜVEN [3] Barış YILDIZ [1]
Mushap KURU [2] Semra KAYA [2] Hüseyin Avni EROĞLU [4] Evren KOÇ [5]

[1] *The study was financially supported by Kafkas University, Scientific Research Projects Coordination Unit (BAP Project number: 2010-VF-398)*

[1] Department of Physiology, Faculty of Veterinary Medicine, University of Kafkas, TR-36300 Kars - TURKEY; [2] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, University of Kafkas, TR-36300 Kars - TURKEY; [3] Department of Biochemistry, Faculty of Arts and Sciences, University of Kafkas, TR-36300 Kars - TURKEY; [4] Department of Physiology, Faculty of Medicine, University of Kafkas, TR-36300 Kars - TURKEY; [5] Department of Bioengineering, Faculty of Bioengineering and Architecture, University of Kafkas, TR-36300 Kars - TURKEY

Article Code: KVFD-2016-17053 Received: 08.11.2016 Accepted: 07.02.2017 Published Online: 10.02.2017

Citation of This Article

Kamiloğlu NN, Kaçar C, Güven A, Yıldız B, Kuru M, Kaya S, Eroğlu HA, Koç E: Changes in lipid peroxidation, glutathione and fertility in Tuj sheep after combined administration of vitamin A and E and passive immunization with testosterone antibodies. *Kafkas Univ Vet Fak Derg,* 23 (3): 459-465, 2017. DOI: 10.9775/kvfd.2016.17053

Abstract

This study investigated the effect of testosterone antibodies and a combination of vitamin A and E on reproductive performance and lipid peroxidation in Tuj sheep during the oestrus period. Two castrated Tuj rams were used to produce an ovine testosterone antibody. To perform the experiment, 30 clinically healthy adult Tuj sheep were divided into three groups, in each group had 10 sheep. The Control group were given a placebo, Group I was injected with the testosterone antibody alone and Group II was injected with testosterone antibody plus a combination of vitamins A and E in Freund's incomplete adjuvant. The testosterone antibody and vitamin combination were administered at synchronization and 1 week before synchronization. To synchronize the sheep, 2.5 ml GnRH was injected to sheep in Control, Group I and Group II. Control, Group I and II were subsequently given 600 IU PMSG with 2 ml $PGF_{2\alpha}$ at 5th day of synchronization. Progesterone levels were higher in the two treatment groups than in the control group as pregnancy progressed. Plasma malondialdehyde levels were higher during initial drug application and prior to mating but were lower in the experimental groups than in control during pregnancy and after parturition. Erythrocyte glutathione levels remained significantly higher in experimental groups than in Control during pregnancy. The number of offspring and the lambing rates in Group I and Group II was higher than the Control. There were no stillbirths in Group I. The number of non-pregnant sheep was lowest in Group II. In summary, injections of testosterone antibody and a combination of vitamins A and E led to an increased incidence of multiple pregnancies in sheep and a greater number of lambs were born. These data indicate that the immunoneutralization of testosterone combined with a reduction in free radicals via the antioxidant activities of vitamins led to increased rates of conception and twinning. Also, it is thought that to allow the growth of the herd in a shorter time, testosterone antibody and combination of vitamins A and E can be applied.

Keywords: *Testosterone antibody, Vitamin A, Vitamin E, Tuj sheep, MDA, GSH, Progesterone, Fertility*

Testosteron Antikoru ile Pasif İmmunizasyon ve A-E Vitamini Kombinasyonu Uygulanmış Tuj Koyunlarında Döl Verimi, Glutatyon ve Lipid Peroksidasyonda Meydana Gelen Değişikler

Özet

Bu çalışmada Tuj koyunlarına östrus döneminde testosteron antikoru ile yapılan pasif immunizasyonun ve A ve E vitamini kombinasyonu uygulamalarının üreme döneminde döl verimi ve oksidatif stres üzerine etkileri araştırıldı. Bu amaçla, 30 Tuj koyunu her grupta 10 koyun olmak üzere 3 gruba ayrıldı. İlk grup Kontrol grubu olarak değerlendirildi ve senkronizasyondan 7 gün önce plesebo uygulandı. Grup I'deki koyunlara testesteron antikoru (AnT), Grup II'deki koyunlara AnT ve Freund's adjuvant incomplate içinde A-E vitamini kombinasyonu uygulandı. Vitamin ve AnT uygulamaları senkronizasyon günü ve senkronizasyondan bir hafta önce yapıldı. Hayvanları senkronize etmek için, Kontrol, Grup I ve Grup II'deki koyunlara 2.5 ml GnRH enjekte edildi. Kontrol, Grup I ve Grup II'deki koyunlara senkronizasyonun 5. günü 600 IU PMSG ile 2 ml $PGF_{2\alpha}$ uygulandı. Deney gruplarının plazma progesteron düzeyleri gebelik süresince kontrol grubuna göre yüksek olarak belirlendi. Deney gruplarının Plazma malondialdehit düzeyleri ilk ilaç uygulamaları yapıldığında ve koç katımından önce yüksekken, gebelik süresince ve doğumdan sonra kontrol grubuna göre düşük olarak tespit edildi. Deney gruplarının, kontrol grubuna göre yüksek eritrosit glutatyon düzeylerini gebelik döneminde ve doğumdan sonra koruduğu gözlemlendi. I. ve II. Grupların bir batında doğan yavru sayılarının ve kuzulma oranının kontrol grubuna göre daha yüksek olduğu gözlendi. I. Grupta hiç ölü doğum olmazken, II. Grupta gebe kalmayan hayvan sayısı diğer gruplardan daha düşüktü. Sonuç olarak, testosteron antikorunun serbest testosteron düzeyini düşürmesi ve vitaminlerin antioksidan etkileri ile serbest radikal düzeylerinin azalmasının koyunlarda gebelik performansını ve bir batında doğan yavru sayısını arttırdığı tespit edilmiştir. Ayrıca, testosteron antikoru ve testosteron antikoru ile A ve E vitamin kombinasyonlarının büyük sürülerde uygulanması ile işletmelerde daha kısa zamanda sürülerin büyütülmesinin mümkün olabileceği düşünülmektedir.

Anahtar sözcükler: *Testosteron antikoru, Vitamin A, Vitamin E, Tuj koyunu, MDA, GSH, Progesteron, Fertilite*

İletişim (Correspondence)
+90 538 6370348
nkamiloglu@hotmail.com

INTRODUCTION

The number of offspring per parturition is the most important factor in terms of managing the productivity of sheep. To manipulate the number of offspring, sheep are selectively crossed with others, using techniques such as flashing and a variety of different synchronisation protocols. Earlier studies have described the development of effective ways to improve fecundity in sheep as well as the number of offspring. These include both active [1,2] and passive [3] immunization against a variety of steroid hormones. Immunization against specific steroids represents a useful management option to increase reproductive performance in sheep via a relatively simple treatment. Increased levels of fertility were reported by many authors following passive immunization against testosterone [4-6]. Immunization against testosterone leads to changes in the concentration of biologically active progesterone as a result of cross-reacting with antibodies. Antibodies are known to bind efficiently with their appropriate endogenous circulating steroid [2,3]. The effect of immunization upon receptivity and fertility is via reductions in the amount of unbound and biologically inactive hormones [7]. In addition, sheep that were passively immunized against testosterone showed induced changes in the secretion of gonadotrophins [3,8]. Furthermore, this procedure leads to increased rates of ovulation and lambing [1,5].

Recent research [9-16] has aimed, first, to develop reproductive technologies to produce high-yielding lambs in large numbers and, second, to create supplementation strategies to protect sheep and embryos from oxidative damage by free radicals. If the antioxidant system is impaired, reactive oxygen species (ROS) can initiate lipid peroxidation and DNA damage, leading to cell death [9]. Therefore, excessive oxidative stress during the mating and gestation periods of sheep can be controlled by the administration of antioxidants [10]. Within the prepartum period, the administration of vitamin A and E, scavengers of free radicals, can protect oocytes and embryos from oxidative damage during gestation [11-14]. Furthermore, levels of antioxidant enzymes, such as glutathione, glutathione peroxidase, superoxide dismutase, can be elevated via the combined administration of vitamin A and E, leading to reduced levels of lipid peroxidation and ROS generation in oviductal and follicular fluid [13,15,16].

Vitamin A and E play important roles in a variety of biological processes, including fertility, the regulation of embryonic growth and cell differentiation. In addition, vitamin A and E play key roles in the patterns of cellular differentiation occurring during embryonic and foetal development and are responsible for proximodistal patterning, limb development and regeneration, neural differentiation and axon outgrowth [15-17]. Micronutrient deficiencies have also been associated with major reproductive risks, ranging from foetal structural defects to infertility. The periconceptional period consists of a number of critical stages, including preconception, conception, implantation, placentation and embryo organogenesis. These phases are critical in determining successful foetal development and health and can be influenced by maternal nutrition, particularly imbalances in micronutrients [13]. Embryonic and foetal development, implantation and placentation are particularly vulnerable to maternal micronutrient levels. Micronutrient supplementation may also play a role in altering development of the placenta, a structure that is critical for nourishing the foetus throughout pregnancy. In addition, evidence indicates a role for micronutrient supplementation in preventing some pregnancy disorders [5]. In fact, despite initial normal growth cycles, foetuses may develop impaired growth during the second part of gestation as a result of nutrient deprivation occurring early in gestation. Furthermore, sheep in poor body condition are typically less productive and the supplemental injections of vitamin A and E have been shown to increase viability of embryos and lambs [18].

The present study investigated the influence of supplementary injections of testosterone antibody and a combination of vitamin A and E, upon reproductive performance, lipid peroxidation, glutathione and progesterone levels in Tuj sheep.

MATERIAL and METHODS

Animal Treatment

Thirty clinically healthy, weighing average 55±5 kg, 3-5 years of age Tuj sheep were randomly divided into three groups. Applications were initially made during estrus period of ewes. The first group was used as the Control (n=10) and were given a placebo, Group I (n=10) was injected with testosterone antibody alone, whereas Group II (n=10) was injected with testosterone antibody and a combination of 100.000 IU of vitamin A [31.58 mg all-trans retinol (Sigma®, R2500, USA) dissolved in 0.5 ml Freund's adjuvant incomplete (Sigma®, F5506, USA)] and vitamin E [18.22 mg DL-α-Tocopherol acetate (Sigma®, T3376, USA)] dissolved in 0.5 ml Freund's adjuvant incomplete (Sigma®, F5506, USA)]. Study design in experimental groups are shown in *Fig 1*. Testosterone antibodies and the vitamin combination were administered 1 week before synchronization (-7th day) and at the point of synchronization (premating). To synchronize the sheep, Control, Group I and Group II were given an IM injection of 2.5 ml GnRH (0.004 mg Bucerelin acetate, Receptal®, MSD, Turkey). Control, Group I and II were subsequently administered with 600 IU PMSG (Chronogest/PMSG, 6000 IU, MSD, Turkey) with 2 ml PGF 2α (5 mg Dinoprost, Dinolytic®, Zoetis, Turkey) at 5th day of synchronization. Rams were then added to the sheep enclosures and oestrus monitored in the sheep for 6 days *(Fig. 1)*. The rams used in the study were examined andrologically and macroscopic

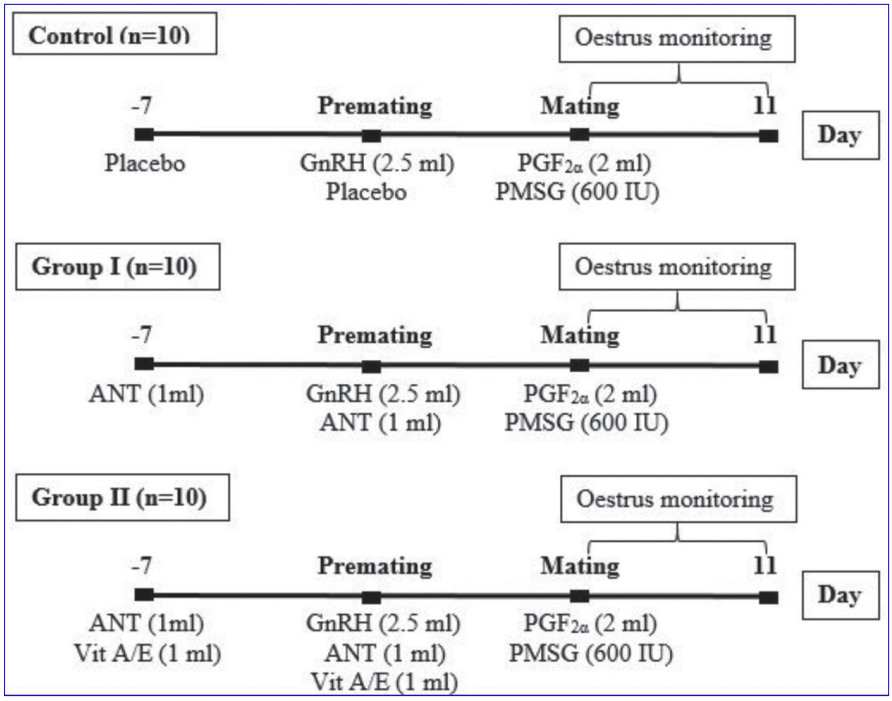


Fig 1. Study design in experimental groups

and microscopic sperm examinations were performed at the same time. Sheep were assessed for pregancy after 35 days by ultrasonography (Sonosite, Vet 180 Plus, USA).

Antibody Production

Two castrated healthy Tuj rams were used to produce ovine anti-testosterone antiserum. Rams were given five injections with an interval of 3 weeks between each injection. For the first injection, 5 mg of testosterone-3-carboxymethyloxime-bovine serum albumin (T-3-CMO-BSA, Sigma®, T3392, USA) conjugate in 2.5 ml of non-ulcerative complete Freund's Adjuvant (Sigma®, F5881, USA) was injected into different areas of dorsal skin in an intra-cutaneous manner. After 3 weeks, a booster dose of 3 mg of the batch of conjugate in incomplete Freund's adjuvant (Sigma®, F5506, USA) was injected via the same route and blood samples taken from the jugular vein 7 days later. Samples were taken from the rams every 2 weeks when antibody titres were appropriate. Testosterone antibody levels were determined by ELISA. Plasma was separated by centrifugation at 4°C and 3000 × *g* for 10 min and frozen at -20°C.

Sample Collection

To measure levels of progesterone and lipid peroxidation in the plasma, blood samples were obtained from the jugular vein either at synchronization or 1 week previously, before and after mating, and once a month during pregnancy and after giving birth. Blood was sampled using heparinized vacutainer tubes. Plasma was then separated by centrifugation (3000 × *g*, for 10 min at 4°C) and frozen (-20°C) to await further analysis.

Analytical Procedures

Lipid peroxidation contents were assessed by measuring thiobarbituric acid reacting substance (TBARS) in plasma according to the method of Placer et al.[19]. TBARS was determined in terms of malondialdehyde (MDA) content, which served as a standard of 1,1,3,3-tetraethoxy-propane (Sigma Chemical Company, T9889, USA). The values of MDA reactive material were expressed in terms of TBARS (nmol/ml plasma). Glutathione (GSH) levels of haemolysed red blood cells were measured spectrophotometrically using Ellman's reagent [20]. Haemoglobin concentration in lysed erythrocytes was also determined by the cyanmet haemoglobin method [21].

Progesterone Measurements

Progesterone levels in blood samples were determined by radioimmunoassay (RIA) using commercial kits (Immunotech®, France). Intra- and inter-assay coefficients for these kits were 6.5% and 7.2% respectively.

Statistical Analysis

Data were analysed by analysis of variance (ANOVA) using SPSS 16.0 software. Tukey's test was used to separate and compare mean data. Pregnancy and lambing rates were compared with the chi-square test. All results were expressed as the mean ± standard deviation (SD). P value <0.05 was considered to be statistically significant.

RESULTS

Plasma Progesterone Levels

Changes in plasma progesterone levels of the sheep are shown in *Fig. 2*. Progesterone levels began to increase after mating. Levels of progesterone were higher in treatment groups than the control group after this time (P<0.05) and increased as pregnancy progressed (P<0.001). In the first month of pregnancy, progesterone levels were highest in Group II (P<0.001), whereas those were higher in the Group I than in the other groups during the 3rd and 4th month of pregnancy (P<0.001).

Plasma MDA Levels

Changes in plasma MDA levels of the sheep are

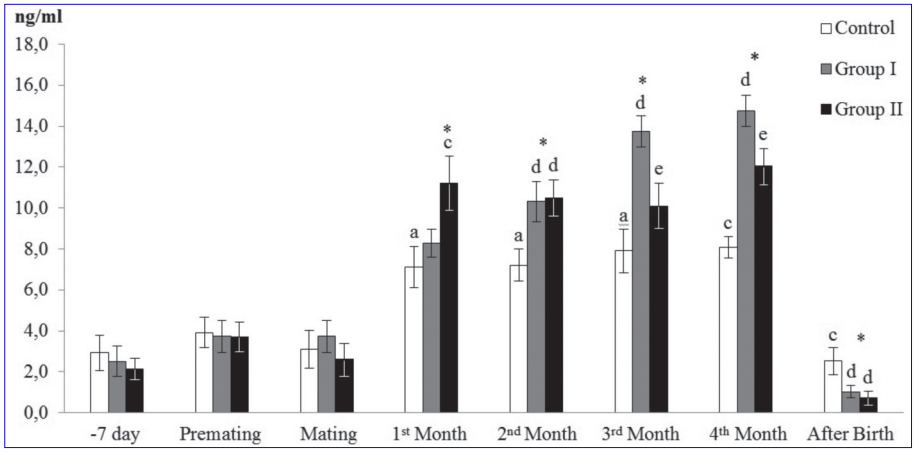
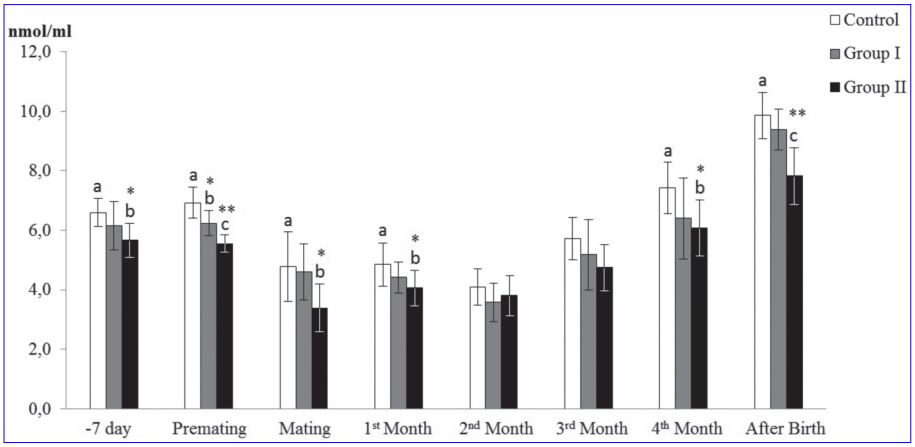


Fig 2. Levels of plasma progesterone (ng/ml) before and during pregnancy and after parturition in Tuj sheeps injected with Vitamin A-E combination and AnT. **Group I:** AnT alone, **Group II:** AnT + Vitamin A-E combination. a, b, c, d, e = Different letters indicate significant differences between groups, * P<0.001

Fig 3. Levels of Plasma MDA (nmol/ml) before and during pregnancy and after parturition in Tuj sheeps injected with Vitamin A-E combination and AnT. **Group I:** AnT alone, **Group II:** AnT + Vitamin A-E combination. a, b, c, d, e = Different letters indicate significant differences between groups, * P<0.05, ** P<0.01



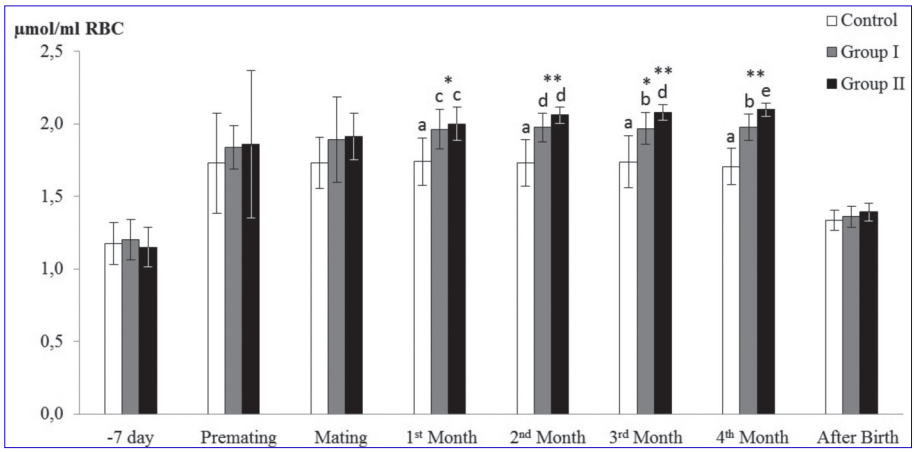


Fig 4. Levels of Plasma GSH (µmol/ml RBC) before and during pregnancy and after parturition in Tuj sheeps injected with Vitamin A-E combination and AnT. **Group I:** AnT alone, **Group II:** AnT + Vitamin A–E combination. a, b, c, d, e = Different letters indicate significant differences between groups, * P<0.01, ** P<0.001

shown in *Fig. 3*. Plasma MDA levels were high during drug application and prior to mating (P<0.01) but were lower in the experimental groups than in the control group during the 1st and 4th month of pregnancy (P<0.05) and after parturition (P<0.001). Although the levels of MDA in the plasma were higher in the control group than in the experimental groups, no significance was observed during the 2nd and 3rd month of pregnancy.

GSH Levels in Erythrocytes

Changes in the GSH levels of erythrocytes isolated from blood samples are shown in *Fig. 4*. Levels of erythrocyte GSH levels did not change following injections in the experimental groups, although a significant increase was observed during pregnancy (P<0.01). Erythrocyte GSH levels remained significantly higher in experimental groups than in the control group during pregnancy (P<0.001) and levels increased as pregnancy progressed.

Reproductive Performance

The effect of a combination of vitamins A and E and AnT injections on the reproductive performance of sheep

Table 1. *Effect of vitamin A-E combination and AnT injections on the reproductive performance of sheep*

Determined Measurements	Control (n=10)	Group I (n=10)	Group II (n=10)	P-value
Rate of Lambing (%)	66	125	100	-
Number of singlelambs	4	7	7	-
Number of tweens	-	-	1	-
Number of triplet	-	1	-	-
Number of offsprings	4	10	9	-
Number of stillbirths	2	-	1	-
Number of non-pregnant sheep	4	2	1	-
Ram joining - start of estrus (h)	67.20	64.44	62.29	-
First estrus response (%)	70^{b} (7/10)	90^{a} (9/10)	90^{a} (9/10)	a:b: 0.001
Pregnancy rate (%)	60^{b} (6/10)	80^{ac} (8/10)	90^{a} (9/10)	ac:b: 0.003; a:b: 0.001
Fecundity rate	0.7 (7/10)	1.2 (12/10)	1.0 (10/10)	

Oestrus rate *= number of sheep showing estrus × 100/total number of sheep;* ***Pregnancy rate*** *= number of pregnant sheep × 100/total number of sheep;* ***Lambing rate*** *= number of foetuses × 100/number of pregnant sheep;* ***Fecundity rate*** *= number of foetuses/total sheep number*

and formula for determining key fertility indices are given in *Table 1*. The number of offspring in Group I and Group II were higher than that in the Control group. In addition, there were no stillbirths recorded for Group I. The number of non-pregnant sheep was lowest in Group II. As shown in *Table 1*, the number of lambs and the rate of lambing in Group I and Group II were 125% and 100% higher, respectively, than those in the Control group. In addition, twins and triplets were seen in Group I and Group II. The time when the ram joined the sheep at the start of estrus, first estrus response and pregnancy rate were higher in Group II than in the other groups. However, fecundity rate was higher in Group I than in other groups.

DISCUSSION

Several authors have shown that gonadal hormones can be manipulated by both active and passive immunization methods and thus increase fertility in sheep [3,6]. However, the responses of sheep to active immunization against a variety of steroids have tended to be variable and have resulted in reduced rates of conception. Other studies have shown that passive immunization against testosterone leads to excessive ovarian stimulation and increased secretion of steroids into the ovarian vein and the follicullar fluid of ewes [7,22]. It is possible that the removal of biologically active local androgens using a testosterone antibody may result in an increased ovulation rate and improved fecundity in both sheep and cattle [3,23]. In the present study, we used passive immunization to testosterone, along with a combination of vitamin A and E to improve reproductive traits in sheep. Lambing rate and the number of live lambs were higher in experimental groups than in control groups. We also achieved a high conception rate and increased the proportion of twins and triplets, indicating that the use of testosterone antibody, with a vitamin combination, can markedly improve reproductive capacity in sheep.

Pregnancy involves anabolic states that are directed via hormones to produce nutrients in the maternal tissues and their transfer to the developing foetus via the placenta. Nevertheless, reproductive loss during pregnancy is the most significant problem in sheep breeding and it is known that progesterone plays a key role in the establishment and maintenance of pregnancy. As the hormone of pregnancy, progesterone stimulates maintenance of the early uterine environment and also development of the placenta that takes over progesterone production after week 5-8 of gestation and causes the smooth muscle of the uterus to relax. Studies of the application of vitamins A and E [24,25] and AnT [4,26] have previously shown an effect upon plasma levels of progesterone, embryonic viability and twinning rate [3,22]. However, to date, there has not been any research focussed upon the precise effects of their use together, or actually direct comparison of their actions. The present results suggest that the combination of vitamin A and E along with AnT significantly increased plasma progesterone level after mating until the 1st month of gestation. After the 2nd month of pregnancy, the application of AnT increased and maintained high progesterone levels. This may be due to the synergistic effect of antioxidants with AnT.

In pregnancy, the synthesis of hormones and changes in the partial pressure of oxygen in the placenta, leads to the formation of ROS in both the placenta and foetus. Furthermore, the multiplication and proliferation of cells, and their high rates of metabolism, cause the formation of ROS after electrons escape from mitochondria within the embryo and foetus [15]. Oxidative stress via the release of ROS, and lipid peroxidation, would be highly detrimental to the viability of both the mother and foetus. Stores of

vitamins and minerals in gestating females protect the mother and foetus from ROS fluxes and lipid peroxidation, which is essential to create an imbalance between ROS production and scavenging activity [11,27]. A study indicated that higher levels of antioxidants such as superoxide dismutase (SOD), catalase (CAT) activities or total antioxidant power (TAP) and lower levels of oxidative stress markers such as lipid peroxidation (LPO) in the endometrial secretions were associated with successful in vitro fertilization outcome [28]. During specific states of pregnancy, vitamin A and E deficiency can lead to a failure to express some genes and is also followed by increased release of MDA and of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (a marker of DNA peroxidation) and by a reduction in mitochondrial GSH content [29]. Also, Nawiota et al.[16] suggested that pregnancy constituted the most oxidative stress and lipid peroxidation facing the grazing and concentrated diet feed sheep and goats under arid and saline conditions. In this study, achieved increase in oxidative stress and decrease in GSH levels during mating and pregnancy reduced by application of the combination of vitamin A-E and ANT.

Vitamin A and vitamin E have a synergic effect upon ROS trapping and the placental transport of vitamins A and E between the mother and foetus is sufficient enough to protect them from the destructive affects of lipid peroxidation. Inhibition of lipid peroxidation and the trapping of ROS via the actions of vitamin A and E have been reported to protect the integrity of mitochondria in the placenta and thus prevent extensive oxidative degradation [30]. Reduction in nutrient intake and mineral and vitamin requirements, especially vitamin A and E, from 28 to 78 days of gestation are highly likely to reduce growth and development of the ovine foetus [9]. Reports have stated that it is therefore necessary to provide supplements during the mating period of sheep in Autumn months, when the quality of grass declines and the vitamin requirements of grazing sheep increases [24,25,31]. Thomas and Kott [31] also concluded that unsupplemented ewes on rangeland lost a significant amount of weight during early to mid-gestation and that even after supplementation during late gestation, the health of their lambs was compromised. In addition, a recent report indicate that daily supplementation of vitaminE during the last 6-7 weeks before lambing decreases the stillbirth rate of ewes [14]. Low levels of maternal vitamin A and E has been shown to be associated with intrauterine growth retardation of both the embryo and foetus [24,32]. In a similar fashion, Johansson et al.[33] showed that cows in organic dairy production can fulfill their requirements of vitamins A and E without any supplementation of synthetic vitamins, except at the time around calving, when the requirements are high. Collectively, these findings support the need for vitamin supplementation during mating and pregnancy to protect both the mother and offspring from the deleterious effects of lipid peroxidation and to maintain a healthy pregnancy. Moreover, researchers offer many programs for nutrition and reproduction support to enhance reproductive performance, on the basis that increased nutritional requirements can support foetal growth and development, as well as improve ROS trapping and antioxidant levels [3,11,22,30]. The present research investigated the application of vitamins and E alone, or in combination with AnT, upon lipid peroxidation and GSH activity, when the quality of the pasture deteriorated during times of short day length. Data showed reductions in both lipid peroxidation and MDA levels, along with increased activity of GSH, an enzyme associated with ROS trapping.

Consequently, our present data indicate that the application of AnT and a combination of vitamin A and E increased fertility by reducing stress generated by mating and pregnancy in sheep. The passive immunization procedure is already progressing to farm trials. The present study suggests that the combination of AnT and vitamins A and E may result in further improvements in reproductive performance. Further research should aim to establish optimized ways of applying such techniques under practical conditions.

REFERENCES

1. Cox RI, Wilson PA: Proceedings: Effects of immunizing sheep to steroid oestrogens and phyto-oestrogens. *J Reprod Fertil*, 46 (2): 524,1976.

2. Elsaesser F: Effects of active immunization against oestradiol-17β, testosterone or progesterone on receptivity in the female rabbit and evaluation of specificity. *J Reprod Fertil*, 58, 213-218, 1980. DOI: 10.1530/jrf.0.0580213

3. Land RB, Morris BA, Baxter G, Fordyce M, Forster J: Improvement of sheep fecundity by treatment with antisera to gonadal steroids. *J Reprod Fertil*, 66, 625-634, 1982. DOI: 10.1530/jrf.0.0660625

4. Alabart JL, Folch J, Cocero MJ, Cognie Y: Effect of immunization on reproductive performance, embryo quality and progesterone in rasa aragonesa ewes actively immunized against androstenedione or passively immunized against testosterone. *Theriogenology*, 35, 799-813**,** 1991. DOI: 10.1016/0093-691X(91)90421-9

5. Kaçar C, Kamiloğlu NN, Gürbulak K, Pancarcı ŞM, Güngör Ö, Güvenç K, Saban E: Üreme mevsimi dışındaki Tuj ırkı koyunlarda testosteron antikoru ile β-karoten ve E vitamini uygulamalarının çoğul gebelik ve MDA (Malondialdehit) üzerine etkisi. *Kafkas Univ Vet Fak Derg*, 14, 51-56, 2008. DOI: 10.9775/kvfd.2008.03-A

6. Lermite V, Delaby L, Thimonierx J, Dufour R, Terquil M: Effect of passive immunization against testosterone on reproductive hormone secretion and ovarian function in dairy cows and pubertal beef heifers. *Theriogenology*, 39, 507-526, 1993. DOI: 10.1016/0093-691X(93)90393-J

7. Scaramuzzi RJ, Baird DT, Martensz ND, Turnbull KE, Van PFA: Look ovarian function in the ewe after active immunization against testosterone. *J Reprod Fertil*, 61, 1-9, 1981. DOI: 10.1530/jrf.0.0610001

8. Pathiraja N, Carr WR, Fordyce M, Forster J, Land RB, Morris BA: Concentration of gonadotrophins in the plasma of sheep given gonadal steroid antisera to raise ovulation rate. *J Reprod Fertil*, 72, 93-100, 1984. DOI: 10.1530/jrf.0.0720093

9. Ashworth CJ, Antipatis C: Micronutrient programming of development throughout gestation. *Reproduction,* 122, 527- 535, 2001. DOI: 10.1530/rep.0.1220527

10. Kamiloğlu NN, Beytut E, Aksakal M: Alteration in antioxidant status and lipid peroxidation of sheep previously injected with vitamin A and

ß-Carotene during the periparturient period. *Bull Vet Inst Pulawy,* 50, 171-177, 2006.

11. Aurousseau B, Gruffat D, Durand D: Gestation linked radical oxygen species fluxes and vitamins and trace mineral deficiencies in the ruminant. *Reprod Nutr Dev*, 46, 601-620, 2006. DOI: 10.1051/rnd:2006045

12. Hirschi KK, Keen CL: Nutrition in embryonic and fetal development. *Nutrition*, 16, 495- 499, 2000. DOI: 10.1016/S0899-9007(00)00362-2

13. Pasqualotto EB, Agarwal A, Sharma RK, Izzo VM, Pinotti JA, Joshi NJ, Rose BI: Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. *Fertil Steril*, 81, 973-976, 2004. DOI: 10.1016/j.fertnstert.2003.11.021

14. Dønnema I, Randbya ÅT, Hektoenb L, Avdemc F, Melingd S, Vågeb ÅØ, Ådnøya T, Steinheima G, Waage S: Effect of vitamin E supplementation to ewes in late pregnancy on the rate of stillborn lambs. *Small Ruminant Res,* 125, 154-162, 2015. DOI: 10.1016/j.smallrumres.2015.02.012

15. Agarwal A, Gupta S, Sharma RK: Role of oxidative stress in female reproduction. *Reprod Biol Endoc*, 3, 1-21, 2005. DOI: 10.1186/1477-7827-3-28

16. Nawito MF, Abd El Hameed AR, Sosa ASA, Mahmoud KGM: Impact of pregnancy and nutrition on oxidant⁄antioxidant balance in sheep and goats reared in South Sinai, Egypt. *Vet World*, 9, 801-805, 2016. DOI: 10.14202/vetworld.2016.801-805

17. Maden M: Retinoic acid in the development, regeneration and maintenance of the nervous system. *Neuroscience*, 8, 755-765, 2007. DOI: 10.1038/nrn2212

18. Kamiloğlu NN, Beytut E, Güven A, Altınsaat Ç: Changes in the erythrocyte anti-oxidant system of offspring of dams treated with vitamin A and ß-carotene during gestation. *Small Ruminant Res,* 65, 142-148, 2006. DOI: 10.1016/j.smallrumres.2005.06.001

19. Placer ZA, Cushman LL, Johnson BC: Estimation of product of lipid peroxidation (malonyldialdehyde) in biochemical systems. *Anal Biochem*, 16, 359-364, 1966. DOI: 10.1016/0003-2697(66)90167-9

20. Sedlak J, Lindsay RH: Estimation of total protein-bound and non-protein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem*, 25, 192-205, 1968. DOI: 10.1016/0003-2697(68)90092-4

21. Drapkin DL: Spectrophotometric studies: XIV. The crystallographic and optical properties of the hemoglobin of man in comparison with those of other species. *J Biol Chem*, 164, 703-708,1946.

22. Cox RI, Wilson PA, Scaramuzzi RJ, Hoskinson RM, George JM, Bindon BM: The active immunization of sheep against oestrone, androstenedione or testosterone to increase twinning. *Anim Prod Aust*, 14, 511-514, 1982.

23. Clayton J, Rhind SM, Groves DJ, Morris BA: Pharmacokinetic studies of passively administered ovine anti-testosterone antibodies given to cattle by the subcutaneous and intravenous routes. *Immunology*, 69, 603-609, 1990.

24. Vonnahme KA, Hess BW, Hansen TR, McCormick RJ, Rule DC, Moss GE, Murdoch WJ, Nijland MJ, Skinner DC, Nathanielsz PW, Ford SP: Maternal undernutrition from early- to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep. *Biol Reprod*, 69, 133-140, 2003. DOI: 10.1095/biolreprod.102.012120

25. Raasch GA, Morrical DG, Youngs CR: Effect of parenteral vitamin e and/or a on reproductive performance of ewes mated on pasture or in drylot. *Sheep Res Report*, 1997, 10, 1998.

26. Rangel PL, Rodriguez A, Gutierrez CG: Testosterone directly induces progesterone productionand interacts with physiological concentrations of LH to increase granulosa cell progesterone production inlaying hens *(Gallus domesticus). Anim Reprod Sci*, 102, 56-65, 2007. DOI: 10.1016/j.anireprosci.2006.09.029

27. Rani V, Deep G, Singh RK, Palle K, Yadav UCS: Oxidative stress and metabolic disorders: Pathogenesis and therapeutic strategies. *Life Sci*, 148, 183-193, 2016. DOI: 10.1016/j.lfs.2016.02.002

28. Rahiminejad ME, Moaddab A, Ganji M, Eskandari N, Yepez M, Rabiee S, Wise M, Ruano R, Ranjbar A: Oxidative stress biomarkers in endometrial secretions: A comparison between successful and unsuccessful in vitro fertilization cycles. *J Reprod Immunol*, 116, 70-75, 2016. DOI: 10.1016/j.jri.2016.05.003

29. Barber T, Borras E, Torres L, Garcia C, Cabezuelo F, Lloret A, Pallardo FV, Vina JR: Vitamin A deficiency causes oxidative damage to liver mitochondria in rats. *Free Radic Biol Med*, 29, 1-7, 2000. DOI: 10.1016/S0891-5849(00)00283-5

30. Tesoriere L, Bongiorno A, Pintaudi AM, D'Anna R, D'Arpa D, Livrea MA: Synergystic interactions between vitamin A and vitamin E against lipid peroxidation in phosphatidylcholine liposomes. *Arch Biochem Biophys*, 326, 57-63, 1996. DOI: 10.1006/abbi.1996.0046

31. Thomas VM, Kott RW: A review of Montana winter range ewe nutrition research. *Sheep Goat Res J*, 11, 17-24, 1995.

32. Picciano MF: Pregnancy and lactation: physiological adjustments, nutritional requirements and the role of dietary supplements. *J Nutr*, 133, 1997-2002, 2003.

33. Johansson B, Waller KP, Jensen SK, Lindqvist H, Nadeau E: Status of vitamins E and A and β-carotene and health in organic dairy cows fed a diet without synthetic vitamins. *J Dairy Sci,* 97, 1682-1692, 2014. DOI: 10.3168/jds.2013-7388

Kafkas Univ Vet Fak Derg
23 (3): 467-471, 2017
DOI: 10.9775/kvfd.2016.17096

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Microsatellite Analysis for Parentage Verification and Genetic Characterization of the Turkmen Horse Population

Hamid Reza SEYEDABADI [1] Sima SAVAR SOFLA [1]

[1] Animal Science Research Institute of Iran (ASRI), Agricultural Research, Education and Extension Organization (AREEO), Karaj, IRAN

Article Code: KVFD-2016-17096 Received: 15.11.2016 Accepted: 22.01.2017 Published Online: 22.01.2017

Citation of This Article

Seyedabadi HRK, Savar Sofla S: Microsatellite analysis for parentage verification and genetic characterization of the Turkmen horse population. *Kafkas Univ Vet Fak Derg,* 23 (3): 467-471, 2017. DOI: 10.9775/kvfd.2016.17096

Abstract

Microsatellites are a class of genetic markers commonly used for parentage verification and population studies. This study determined the efficiency of microsatellite markers for identification and pedigree analysis in horses based on the example of Turkmen horse population. For this purpose, 748 Turkmen horse samples including 574 adults (92 stallions, 345 males and 137 mares) and 174 foals (98 colts and 76 fillies),were genotyped by using seventeen microsatellite markers recommended by ISAG. The number of allele per locus varied from 5 (HMS01 and HTG07) to 10 (HTG10) with an average value of 7.65. The observed heterozygosity and the expected heterozygosity ranged 0.365-0.953 (mean 0.703), from 0.617-0.884 (mean 0.792) respectively. PIC value ranged from 0.586 (HTG7) to 0.873 (HTG10) with average 0.767. The total exclusion probability of the 17 microsatellite loci was 0.9999. The pedigree study of the Turkmen horse using microsatellite markers was efficient in detecting mistakes during genealogical records. These results suggested that the DNA typing method had high potential for systematic control of the genealogical registrations and genetic resources to improve genetic aspects in Turkmen horses.

Keywords: *Parentage verification, Genetic characterization, Microsatellite markers, Turkmen horse*

Türkmen At Popülasyonunda Soy Tespiti amacıyla Mikrosatellit Analiz ve Genetik Karakterizasyon

Özet

Mikrosatellitler yaygın olarak soy tespiti amacıyla ve popülasyon çalışmalarında kullanılan bir sınıf genetik belirteçlerdir. Bu çalışma, Türkmen at popülasyonunda identifikasyon ve soy analizinde mikrosatellit belirteçlerin kullanılabilirliğini tespit etmek amacıyla yapılmıştır. Bu amaçla, 574 ergin (92 aygır, 345 beygir ve 137 kısrak) ve 174 tay (98 erkek tay ve 76 dişi tay) içeren toplam 748 Türkmen ata ait örnekler ISAG tarafından önerilen 17 mikrosatellit belirteç kullanılarak genotiplendirildi. Her bir lokusta allel sayısı 5 (HMS01 ve HTG07) ile 10 (HTG10) arasında olmak üzere ortalama 7.65 olarak tespit edildi. Gözlemlenen heterozigotluk ve beklenen heterozigotluk sırasıyla 0.365-0.953 (ortalama 0.703) ve 0.617-0.884 (ortalama 0.792) olarak belirlendi. Polimorfizm bilgi içeriği ortalama 0.767 olmak üzere 0.586 (HTG7) ile 0.873 (HTG10) arasında değişim gösterdi. 17 mikrosatellit bölgenin total dışlama olasılığı 0.9999 idi. Mikrosatellit belirteçler kullanılarak yapılan Türkmen atlarındaki soy araştırması soy kayıtlarındaki hataları tespit etmede etkiliydi. DNA tiplendirme metodu soy kayıtlarının sistemik kontrolünde yüksek potansiyele sahip olup Türkmen atlarının genetik kaynağını artırmada kullanılabilir.

Anahtar sözcükler: *Soy tespiti, Genetik karakterizasyon, Mikrosatellit belirteçler, Türkmen atı*

INTRODUCTION

Horses are belonging to Equidae family; the horse's influence on human history and civilization make it one of the most important animals [1]. Iran has a long history of horse domestication and breeding [2]. Iranian horse breeds may be classified into 4 main groups according to their origins and habitats: North alluvial plains such as Caspian breed, northeast fields such as Turkmen breed, and west highlands such as Kurd breed and southwest and central plateau such as Persian-Arab breed [2]. Turkmen horse is one of the oldest breeds in the world and always achieves high ranks in courses and jumping competitions [3]. Studbook data includes some errors in the registration of the Turkmen studbook. Those data important to the conservation of the breed and correct lines of ancestral might be essential for breeding of Turkmen horse. This information might be verified by a molecular data. It

İletişim (Correspondence)
+98 26 34430010
hseyedabady@gmail.com

is necessary, because the genetic characterization of a breed is the first step in the conservation of breeds, determination of future breeding strategies, and is important to protect breed integrity [4]. Identification of genetic variation among various horse breeds requires the development of genetic markers [5]. Microsatellite markers are useful to diversity studies in the animal. They are tandemly repeated sequences which can be genotyped by PCR techniques [6]. Even in the time of wide genome scanning with the use of SNP microarrays, microsatellites are still used in the construction of linkage maps, when narrowing down the regions of QTLs [7]. Microsatellite markers were first characterized in Swedish horse [8,9]. Commonly, in horses set of seventeen basic microsatellites loci are used (Equine Genetics and Parentage Analysis Workshop, 2012). This horse genotyping panel has been designed to provide high discrimination power (PD), with minimum time need to sample preparation and minimum use of reagents [10]. Those markers constitute a panel of loci recommended by International Society for Animal Genetics (ISAG) in horses parentage testing. In Iran, the polymorphism of these markers has been proved to be useful in Caspian horse [11]; Kurd [12] and Iranian-Arab horse [13]. Application of microsatellite markers in evaluation of the genetic structure in Turkmen horses has not been done yet and this is the first research for parentage verifications based on seventeen microsatellites loci recommended by ISAG's in this breed. The purpose of this study was to evaluate these microsatellite loci in Turkmen horse population and design a marker system for future low-cost genotyping, which will give high combined exclusion probabilities (EPs).

MATERIALS and METHODS

Animals and DNA Extraction

The animals were randomly chosen by their breeders who were able to document their pedigrees (parents, offspring). Blood samples were collected from 748 Turkmen horses, 574 adults (92 stallions, 345 males and 137 mares) and 174 foals (98 colts and 76 fillies). Genomic DNA was extracted from blood samples using the salting-out method [14].

Microsatellite Markers Genotyping

Seventeen microsatellites were selected for this study that had been reported by ISAG for individual identification and parentage verification of Turkmen horses. Microsatellite markers *(Table 1)* were combined in multiplex PCR reaction using fluorescently labeled primers and amplified in a total volume of 20 µl of the following mixture: 40 ng of genomic DNA, 2 mM $MgCl_2$ (Fermentas, Canada), 250 µM of each dNTP (Roche Applied Science, Germany), 0.03 µM of both primers (Metabion, Germany), 1X PCR buffer (Fermentas, Canada) and 0.5U Taq DNA polymerase (Fermentas, Canada). Amplifications were performed using the GeneAmp PCR 9700 (Applied Biosystems, USA). PCR amplification was as follows: the first step was performed by initial denaturation for 5 min at 95°C, followed by 35 cycles at 95°C for 30 sec, 58°C or 60°C for 30 sec, and 72°C for 1 min then extension step of 72°C. The set of proofreading activity and fluorescently labeled 17 primers specific for STRs was tested. PCR products were further sequenced using capillary electrophoresis system on the 3130×l Genetic Analyser (Applied Biosystems). The GeneScan-500 LIZ Size Standard was used in each sample run for an application of automated DNA fragments analysis with four fluorescent dyes. Analysis of DNA profiles for 17 STR loci was conducted in GeneMapper 4.0 software (Applied Biosystems).

Data Analysis

Number of alleles (Na), Allele's frequencies for each locus, observed heterozygosity (HO), expected heterozygosity (He), Polymorphic information content (PIC) and combined probability of exclusion (PE), were computed using CERVUS version 3 software [15]. Deviations from HWE and inbreeding coefficient (F_{is}) were estimated by GENEPOP version 4.4 program [16].

RESULTS

Microsatellite Polymorphism

A total of 130 alleles were observed among the 748 animals and demonstrated that they were highly polymorphic in Turkmen horse populations. A number of alleles, observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphic information content and exclusion probability (PE) in the Turkmen horse were shown in *Table 2*. The number of allele per locus varied from 5 (HMS01 and HTG07) to 10 (HTG10) with an average value of 7.65 in the Turkmen horse. The observed heterozygosity and the expected heterozygosity ranged 0.365-0.953 (mean 0.703), from 0.617-0.884 (mean 0.792) respectively. Microsatellite markers showing PIC values higher than 0.5 are commonly considered as informative in horse population [17]. All marker loci engaged in this study were informative since the average PIC value calculated at 0.767. The lowest PIC value was for HTG7 (0.586), while the highest value was for HTG10 (0.873). The within population inbreeding estimate (F_{is}) ranged between -0.246 and 0.482 with an average of 0.081. Thus, on an average, deficiency (8.1%) of heterozygote existed in the Turkmen horse population *(Table 2)*. Statistically significant deviation from Hardy-Weinberg equilibrium ($P<0.05$) was found at total loci, except (AHT5, ASB17, HTG4, LEX33and UCDEQ425) loci *(Table 2)*. The obtained PE for each polymorphic locus was ranged from 0.3098 for HTG07 to 0.803 for HTG10 with a combined average probability of exclusion of 0.99999 *(Table 2)*. The parentage testing

Table 1. *Characteristics of 17 Horse microsatellites DNA loci*

Loci	Primer Sequences 5'-3'	Dye	Size Range (bp)
AHT04	F: AACCGCCTGAGCAAGGAAGT R: CCCAGAGAGTTTACCCT	6-Fam	166 - 140
AHT05	F: ACGGACACATCCCTGCCTGC R: GCAGGCTAAGGGGGCTCAGC	VIC®	147 - 126
ASB02	F: CCTTCCGTAGTTTAAGCTTCTG R:CACAACTGAGTTCTCTGATAGG	VIC®	268 - 237
ASB17	F: GAGGGCGGTACCTTTGTACC R: ACCAGTCAGGATCTCCACCG	PET®	116 - 104
ASB23	F: GAGGTTTGTAATTGGAATG R: GAGAAGTCATTTTTAACACCT	VIC®	212 - 176
HMS01	F: CATCACTCTTCATGTCTGCTTGG R:TTGACATAAATGCTTATCCTATGGC	PET®	178 - 166
HMS02	F: ACGGTGGCAACTGCCAAGGAAG R:CTTGCAGTCGAATGTGTATTAAATG	NED™	236 - 215
HMS03	F: CCAACTCTTTGTCACATAACAAGA R: CCATCCTCACTTTTTCACTTTGTT	NED™	170 - 146
HMS06	F: GAAGCTGCCAGTATTCAACCATTG R: CTCCATCTTGTGAAGTGTAACTCA	VIC®	170 - 154
HMS07	F: CAGGAAACTCATGTTGATACCATC R:TGTTGTTGAAACATACCTTGACTGT	6-FAM™	187 - 167
HTG04	F: CTATCTCAGTCTTCATTGCAGGAC R: CTCCCTCCCTCCCTCTGTTCTC	6-FAM™	137 - 116
HTG06	F: CCTGCTTGGAGGCTGTGATAAGAT R: GTTCACTGAATGTCAAATTCTGCT	VIC®	103 - 74
HTG07	F: CCTGAAGCAGAACATCCCTCCTTG R: ATAAAGTGTCTGGGCAGAGCTGCT	NED™	128 - 114
HTG10	F: CAATTCCCGCCCCACCCCCGGCA R: TTTTTATTCTGATCTGTCACATTT	NED™	110 - 83
LEX33	F: TTTAATCAAAGGATTCAGTTG R: TTTCTCTTCAGGTGTCCTC	PET®	217 - 203
UCDEQ425	F: AGCTGCCTCGTTAATTCA R: CTCATGTCCGCTTGTCTC	PET®	247 - 224
VHL20	F: CAAGTCCTCTTACTTGAAGACTAG R: AACTCAGGGAGAATCTTCCTCAG	6-FAM™	102 - 83

of the 174 foals was verified by the compatibility of 17 microsatellite markers according to Mendelian laws and using likelihood based method. However, 26 foals did not inherit alleles from the registered sire and 9 foals did not inherit alleles from the registered dam.

DISCUSSION

The use of microsatellite markers for individual identification and parentage verification of horses is a routine method in several countries [18]. The present study describes the utility of seventeen microsatellite markers in parentage verification in Turkmen horse breeds. For a clear genetic differentiation between breeds, it has recommended a minimum of four alleles per locus by FAO [19]. Consequently, All 17 microsatellite markers applied in this study showed reliable polymorphism for evaluating genetic variation within the Turkmen horse population. The allele numbers and heterozygosity levels observed this study, indicate a presence of a reasonably high level of genetic variability in Turkmen horse population. The genetic structure of the Turkmen horse population revealed an increased allelic diversity for 17 microsatellites in relation to other studies. With the same set of microsatellite markers, Georgescu et al.[20], investigated the structure of indigenous Romanian Hucul horse breed. The observed and expected heterozygosity per breed ranged from 0.662 and 0.676, respectively. Genetic variation among four Italian horse breeds was assessed using a set of 11 microsatellites [21]. In the breed level, it was showed a high level of gene diversity (He) ranging from 0.71 in Sicilian Oriental Purebred to 0.81 in Sicilian Indigenous [21]. The Polymorphism Information Content (PIC) similar to heterozygosity and is calculated from allele frequencies. A high PIC value is indicative of a locus with high informativeness. In this study average PIC value was 0.767 which is moderate polymorphic. For linkage mapping, Dierks et al.[7], selected microsatellite markers with PIC values >0.5 as markers with values below this level are insufficient for parentage verification. In their study, the

Table 2. *Number of alleles (Na), observed heterozygosity (H_O), expected heterozygosity (H_E), Polymorphic information content (PIC), inbreeding coefficient (F_{is}), exclusion probabilities (PE) and Hardy Weinberg Equilibrium (HWE) of 17 microsatellites loci for Turkmen horse*

Loci	Na	H_O	H_E	PIC	F_{is}	PE	HWE
AHT04	9	0.755	0.825	0.826	0.092	0.701	**
AHT05	8	0.521	0.725	0.799	0.281	0.619	NS
ASB02	9	0.625	0.734	0.825	0.148	0.633	**
ASB17	8	0.452	0.758	0.810	0.403	0.629	NS
ASB23	8	0.852	0.725	0.815	-0.170	0.476	**
HMS01	5	0.425	0.821	0.664	0.482	0.476	**
HMS02	7	0.732	0.789	0.783	0.072	0.605	**
HMS03	9	0.786	0.725	0.829	-0.084	0.655	**
HMS06	7	0.724	0.821	0.730	0.118	0.532	*
HMS07	9	0.701	0.822	0.794	0.166	0.627	*
HTG04	7	0.671	0.727	0.593	0.077	0.310	NS
HTG06	6	0.809	0.712	0.703	-0.136	0.491	**
HTG07	5	0.733	0.726	0.586	-0.009	0.309	**
HTG10	10	0.753	0.604	0.873	-0.246	0.803	**
LEX33	8	0.609	0.793	0.836	0.232	0.701	NS
UCDEQ425	7	0.758	0.723	0.725	-0.048	0.555	NS
VHL20	8	0.794	0.801	0.832	0.008	0.713	*
Mean **± Sd**	7.65	0.690±0.18	0.731±0.19	0.767±0.15	0.081±0.03	0.99999	-

average PIC value was 0.596 with a maximum of 0.866 [7]. The inbreeding index Fis indicates moderate level of inbreeding in Turkmen horse population, but Fis for locus HMS1 and ASB2 was high in this population. The inbreeding detected in Turkmen horse population may be as a result of depauperate population size, small breeding areas and/or with an insufficient number of breeding males in the breeding region. However, high levels of heterozygousity, PIC and moderate level of inbreeding in Turkmen horse population reflect high genetic variability that can be exploited by horse breeders for planning breeding strategies and prioritizing the breed for its conservation. The International Stud Book Committee (ISBC) has required that the combined exclusion probability (CPE) value for paternity testing and an individual identification in a horse be higher than 0.9995 [22]. In this study, the CPE using 17 microsatellite markers was greater than the value required by the ISBC. Other studies reported similar values of total exclusion probability (0.999) in Thoroughbred and Arabian horse [23-25]. Ellegren et al.[8], proposed at least ten microsatellite loci should be used to gain maximum exclusion in horses. Marklund et al.[9], analyzed eight microsatellite loci in parentage testing to gain a combined exclusion probability of 0.96 to 0.99 in different breeds. At least five microsatellite loci with PE more than 97% should be used to obtain a high degree of excluding probability [26]. Seyedabadi et al.[11], also reported a total PE of 0.973 for seven microsatellite loci used in Caspian horse parentage control. These various results comparison with our results, shows that our selected microsatellites have greater power of exclusion. The prosperity of paternity testing is not only depends on the number of loci but on the level of informativeness that these markers provide. The level of informativeness of a microsatellite marker is specified by its values of heterozygosity, PIC, PE and genetic diversity and these values are dependent on the number and frequency of alleles in the population [27]. These values obtained for microsatellite markers used in our study indicated the high level of informativeness of these markers in Turkmen horse population. So, these microsatellite markers (ISAG), showed to be adequate to parentage verification and for individual identification in Turkmen horse. Our data showed decrepitude in the individual identification system and confirmed interest in using genetic markers in this system. Identification and parentage verification of the Turkmen horse population using a panel of microsatellite markers would be of great importance for the conservation program being applied to this breed.

Acknowledgments

This research was financially supported by Animal Science Research Institute of Iran (ASRI). We specially acknowledge Dr. Ali Javanrouh and Dr Amiriniai. We are beholden to all horse owners in Province of North Khorasan for their cooperation during sample collection.

REFERENCES

1. Mahrous KF, Hassanane M, Abdel Mordy M, Shafey HI, Hassan N: Genetic variations in horse using microsatellite markers. *J Genet Eng*

Biotech, 9, 103-9, 2011. DOI: 10.1016/j.jgeb.2011.11.001

2. Rafeie F, Amirinia C, Nejati Javaremi A, Mirhoseini SZ, Amirmozafari N: A study of patrilineal genetic diversity in Iranian indigenous horse breeds. *Afr J Biotechnol*, 10, 75, 17347-17352. 2011.

3. Golshan A: Introduction to the Turkmen horse in Iran. 37-71 Tehran, Pejvake Keyhan (in Persian), 2005.

4. Burocziova M, Riha J: Horse breed discrimination using machine learning methods. *J Appl Genet*, 50, 375-77. 2009. DOI: 10.1007/BF03195696

5. Rukavina D, Hasanba D, Pojski N, Rami A, Zahirovi A: Analysis of genetic diversity among certain horse breeds from Bosnia and Herzegovina. *Veterinaria*, 64, 25-28, 2015.

6. Chauhan M, Gupta AK, Dhillon S: Genetic characterization of Indian Spiti horses. *J Genet*, 83, 291-295, 2004.

7. Dierks C, Lohring K, Lampe V, Wittwer C, Drogemuller C, Distl O: Genome-wide searchfor markers associated with osteochondrosi in Hanoverian warm blood horses. *Mam Genome*, 18, 739-747, 2007. DOI: 10.1007/s00335-007-9058-9

8. Ellegren H, Johansson M, Sandberg K, Andersson L: Cloning of highly polymorphic microsatellites in the horse. *Anim Genet*, 23, 133-142, 1992. DOI: 10.1111/j.1365-2052.1992.tb00032.x

9. Marklund S, Ellegren, H, Eriksson S, Sandberg K, Andersson L: Parentage testing and linkage analysis in the horse using a set of highly polymorphic microsatellites. *Anim Genet*, 25, 19-23, 1994. DOI: 10.1111/j.1365-2052.1994.tb00050.x

10. Dimsoski P: Development of a 17-plex microsatellite polymerase chain reaction kit for genotyping horses. *Croat Med J*, 44, 332-335, 2003.

11. Seyedabadi HR, Amirinia C, Banabazi MH, Emrani H: Parentage verification of Iranian Caspian horse using microsatellites markers. *Iran J Biotechnol*, 4, 260-264, 2006.

12. Rafeie F: Genetic Relationships between Iranian Caspian Horses and Other Iranian Horse Breeds. *PhD Thesis,* SRBIAU, Tehran, Iran, 2011.

13. Moshkelani S: DNA fingerprinting of Iranian Arab horse using fourteen microsatellites marker. *Res J Biol Sci*, 6, 402-405, 2011.

14. Javanrouh A, Banabazi MH, Esmaeilkhanian S, Amirinia C, Seyedabadi HR, Emrani H: Optimization on salting out method for DNA extraction from animal and poultry blood cells. *The 57th Annual Meeting of the European Association for Animal Production.* 17 to 20 September, Antalya, Turkey, 2006.

15. Kalinowski ST, Taper ML, Marshall TC: Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol Ecol*, 16, 1099-1106, 2007. DOI: 10.1111/j.1365-294X.2007.03089.x

16. Rousset F: GENEPOP' 007: A complete re-implementation of the GENEPOP software for Windows and Linu. *Mol Ecol Resour*, 8, 103-106, 2008. DOI: 10.1111/j.1471-8286.2007.01931.x

17. Botstein D, White RL, Skolnick M, Davis RW: Construction of a genetic linkage map in man using restriction fragment length polymorphism. *Am J Hum Genet*, 32, 314-331, 1980.

18. Seo JH, Park KD, Lee HK, Kong HS: Genetic diversity of Halla horses using microsatellite markers**.** *J Anim Sci Technol*, 58, 1-5, 2016. DOI 10.1186/s40781-016-0120-6

19. Pandey AK, Sharma R, Singh Y: Evaluation of genetic variability in Kenkatha cattle by microsatellite markers. *J Anim Sci*, 12, 1685-1690, 2006. DOI: 10.5713/ajas.2006.1685

20. Georgescu SE, Manea MA, Costache M: The genetic structure of indigenous Romanian Hucul horse breed inferred from microsatellite data. *Roum Biotechnol Lett*, 13, 4030-4036, 2008.

21. Zuccaro A, Bordonaro S, Criscione A, Guastella AM, Perrotta G, Blasi M, D'Urso G, Marletta D: Genetic diversity and admixture analysis of Sanfratellano and three other Italian horse breeds assessed by microsatellite markers. *Animal*, 7, 991-998, 2008. DOI: 10.1017/S1751731108002255

22. Tozaki T, Kakoi H, Mashima S, Hirota KI, Hasegawa T, Ishida N, Miura N, Choi-Miura NH, Tomita M: Population study and validation of paternity testing for Thoroughbred horses by 15 microsatellite loci. *J Vet Med Sci*, 63, 1191-1197, 2001. DOI: 10.1292/jvms.63.1191

23. Lee SY, Cho GJ: Parentage testing of Thoroughbred horse in Korea using microsatellite DNA typing. *J Vet Sci*, 7, 63-67, 2006. DOI: 10.4142/jvs.2006.7.1.63

24. Khanshour AM, Conat EK, Juras R, Cothran EG: Microsatellite analysis for parentage testing of the Arabian horse breed from Syria. *Turk J Vet Anim Sci*, 37, 9-14, 2013. DOI: 10.3906/vet-1104-8

25. Cho GJ: Microsatellite DNA polymorphism of Thoroughbred horses in Korea. *Korean J Genet*, 24, 177-182, 2002. DOI: 10.4142/jvs.2006.7.1.63

26. Jakabova D, Trandzik J, Chrastina J: Effectiveness of six highly polymorphic microsatellite markers in resolving paternity cases in Thoroughbred horses in Slovakia. *Czech J Anim Sci*, 47, 497-501, 2002.

27. Curi RA, Lopes CR: Evaluation of nine microsatellite loci and misidentification paternity frequency in a population of Gyr breed bovines, Braz. *J Vet Res Anim Sci*, 39, 129-135, 2002. DOI: 10.1590/S1413-95962002000300004

Kafkas Univ Vet Fak Derg
23 (3): 473-479, 2017
DOI: 10.9775/kvfd.2016.17111

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Effects of Flavonoids from Mulberry Leaves and *Candida tropicalis* on Performance and Nutrient Digestibility in Calves

Le-Ying ZHANG [1] Pei-Bin QU [1,2] Yan TU [2] Chun-Tao YANG [2] Qi-Yu DIAO [2]

[1] College of Agriculture, Hebei University of Engineering, Handan 056021, CHINA
[2] Key Laboratory of Feed Biotechnology, Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing, 100081, CHINA

Article Code: KVFD-2016-17111 Received: 17.11.2016 Accepted: 12.01.2017 Published Online: 13.01.2017

Citation of This Article

Zhang LY, Qu PB, Tu Y, Yang CT, Diao QY: Effects of flavonoids from mulberry leaves and *Candida tropicalis* on performance and nutrient digestibility in calves. *Kafkas Univ Vet Fak Derg*, 23 (3): 473-479, 2017. DOI: 10.9775/kvfd.2016.17111

Abstract

Flavonoids from mulberry leave (FML) are natural flavonoids, and *Candida tropicalis* (CT) is yeast like microbe. In this study, forty-eight male calves were selected with born weight (40.5±0.7 kg) and 20±2 days age, divided into 4 groups randomly. FML was supplemented in a dairy calf starter at 2 g/d per calf before weaning, or 4 g/d per calf after weaning (FML group), while CT was added in a dairy calf starter at 1 g/d per calf (CT group). Our results showed that FML could be used to enhance body weight (BW) of calves through enhancing apparent digestibility of ether extract (EE) of the diet, and increased the levels of serum growth hormone (GH) and insulin-like growth factors 1 (IGF-1) in calves after the age of 56 days. CT enhanced the BW of calves before weaning through increasing the apparent digestibility of neutral detergent fiber (NDF) of the diet, and elevated the apparent digestibility of EE of the diet of calves after weaning through increasing the level of serum IGF-1 in calves. Furthermore, the mixture of FML and CT plays a synergistic role in enhancing growth, improving feed intake and nutriment digestibility. In conclusion, FML and CT could be used as additives to increase growth and nutrient digestibility in calves.

Keywords: *Calves, Flavonoids from mulberry leave, Candida tropicalis, Nutriment digestibility, Hormone level*

Dut Yaprağından Elde Edilen Flavonoidler İle *Candida tropicalis*'in Buzağılarda Performans ve Besin Sindirilebilirliği Üzerine Etkileri

Özet

Dut yaprağından elde edilen flavonoidler (FML) doğal flavonoidler olup *Candida tropicalis* (CT) de mantar benzeri mikroplardır. Bu çalışmada, doğumda 40.5±0.7 kg ağırlığa sahip 20±2 günlük kırk sekiz erkek buzağı kullanılarak rastgele 4 gruba ayrıldı. FML sütten kesme öncesi her bir buzağı için 2 g/d olarak buzağı başlangıç yemi içerisinde veya her bir buzağı için 4 g/d olarak sütten kesme sonrası (FML grup), CT ise her bir buzağı için 1 g/d olarak buzağı başlangıç yemi içerisinde (CT grup) verildi. Çalışma sonucunda FML'nin diyetin eter ekstraktının (EE) sindirilebilirliğini ve serum büyüme hormonu (GH) ve insülin benzeri büyüme faktörü 1 (IGF-1)'in seviyelerini belirgin bir şekilde arttırmak yoluyla 56 günlükten sonrasında buzağıların vücut ağırlığını (BW) geliştirmek amacıyla kullanılabileceği tespit edilmiştir. CT; buzağıların vücut ağırlığını sütten kesme öncesinde diyetteki nötr deterjan fiberin sindirilebilirliğini belirgin ölçüde geliştirdi ve serum IGF-1'in seviyesini yükseltmek suretiyle sütten kesme sonrasında diyetteki eter ekstraktının sindirilebilirliğini belirgin ölçüde artırdı. FML ve CT karışımı büyümeyi geliştirme, gıda tüketimini ve besin sindirilebilirliğini iyileştirmekte sinerjistik bir rol oynadı. Sonuç olarak, FML ve CT büyüme ve besin sindirilebilirliğini artırmak amacıyla buzağılarda bir katkı olarak kullanılabilir.

Anahtar sözcükler: *Buzağı, Dut Yaprağı Flavonoidleri, Candida tropicalis, Besin sindirilebilirliği, Hormon seviyesi*

INTRODUCTION

It is well documented that there are a number of potential risks for human health in using antibiotics in food-producing animals, including drug residues in meat products, increasing bacterial resistance and environmental contamination [1]. Flavonoids are found in berries, tea, cocoa, soybeans, grains, and plant leaves, are a class of organic polyphenolic compounds [2]. It is through various mechanisms including protection against oxidative stress, and preservation of epithelial barrier function and immuno-modulatory properties that flavonoids are used in acute or chronic intestinal inflammation [3]. Dietary flavonoids (quercetin and morin) have marked effects on the fatty

İletişim (Correspondence)
+86 10 82106055
diaoqiyu@caas.cn

acid composition of blood plasma, liver, or breast muscle lipids in vitamin E-deficient chicks [4]. Condensed tannins (a polyflavonoid) can mitigate methane emission by feeding *Leucaena leucocephala* in sheep [5]. It is effective in improving rumen fermentation and reducing the incidence of rumen acidosis through supplementation of natural flavonoids extract from bitter orange and grapefruit [6]. Sainfoin which contains rich condensed tannins can increase beneficial fatty acids and reduce skatole content in lamb meat [7]. Flavonoids from mulberry leaves (FML) have positive effects on the hypoglycaemic, antihypercholesterolemic and anti-oxidative potential in rats [8].

Probiotics are live microorganisms including bacteria and yeasts, which can improve intestinal health and immune response, prevent acute and antibiotic-associated diarrhea [9]. It is reported that GSY10 is the most promising oleaginous yeast for microbial lipid production from molasses, and it can be used as feed supplement for microbial lipid production in dairy cattle [10]. The yeast culture extract can activate natural killer (NK) cells and B lymphocytes *in vitro*, which plays a role in the anti-inflammatory effects [11]. Yeast-based immunogen (EpiCor) possesses conspicuous anti-inflammatory activity, and can directly induce activation of chemotactic awareness of lymphocyte subsets *in vitro* [12]. After experimental challenged with *Salmonella*, pre-weaned dairy cows fed with *Saccharomyces cerevisiae* fermentation products have fewer bouts of diarrhea and fever, more beneficial microbe in rumens and higher weight gain comparing with no-fed group [13]. *Lactic acid bacteria* or *Bacillus* species generally target the lower intestine to stabilize the gut microbiota, which decreases the risk of pathogen colonization in young ruminants [14]. As a fungal organism, *Candida tropicalis* (CT) can grow as yeast morphology [15]. It is known that the somatotropic axis primarily consists of growth hormone (GH), insulin-like growth factors (IGF), as well as their associated carrier proteins and receptors, which plays a key role in the control of the protein anabolism, fat deposition, and growth rate in animals [16]. It was hypothesized that supplementation of FML and CT in starter of calves might improve consumption of nutriment, accelerate animal growth, and feed intake. Thereby, the aim of current study was to determine the effects of supplementation FML and CT in the starter on growth, performance and the concentrations of GH and IGF-1 in plasma during the first 80 days of age in calves.

MATERIALS and METHODS

Materials

The extract of FML was purchased from Xi'an Feida Biotechnology Co. Ltd., and there were 50 mg of FML per g of extract, which was analyzed by the manufacturer. CT was from Beijing Vano Biological Engineering Co., Ltd., and the concentration of live bacteria was 5×10^9 CFU/g, which was provided by the manufacturer. The milk replacer is provided by Beijing Jingzhun Animal Nutrition Center, and the starter is from Beijing Sanyuan Luhe Feed Factory. The basal diet consists of milk replacer and starter with no antibiotics and microbial preparation. The ingredients and nutrient levels of basal diet are shown in *Table 1*.

Animals and Experimental Design

Holstein male calves were managed on the first farm of Western Suburbs, Beijing Sanyuan Luhe Cow Breeding Center. The experimental protocol was approved by the Chinese Academy of Agricultural Sciences Animal Ethical Committee, and humane animal care and handling procedures were followed throughout the experiment. Forty-eight male calves with 20±2 days old, and 40.5±0.7 kg birth weight were selected from cows with natural childbirth and between 3 and 5 years old. The calves were fed adequate colostrum during the first 3 days and then fed milk replacer until 80 days of age. Calves randomly were divided into 4 groups (n=12) based on parity and birth weight. The control group (Ctrl group) was fed with basal diet, while the other three groups were added with FML (FML group, 2 g/d per calf before weaning, or 4 g/d per calf after weaning), with CT (CT group, 1 g/d per calf) and with the above two additives (FML + CT group, FML

Table 1. *Nutrient composition and levels of basal diet (air-dry basis) %*

Items	Starter	Milk Replacer
Corn	20.0	-
Extrude corn	22.9	
Soybean meal	20.0	
Extruded soybean	18.0	
Whey powder	5.00	
Wheat bran	10.0	
$CaHPO_4$	0.800	
Limestone	1.80	
Salt	0.500	
Premix*	1.00	
Total	100	
Nutrient levels		
DM (Dry matter)	85.4	95.4
OM (Organic matter)	92.2	94.9
CP (Crude protein)	19.1	24.3
EE (Ether extract)	2.21	12.9
NDF (Neutral detergent fiber)	18.6	4.02
ADF (Acid detergent fiber)	10.7	2.11
Ca	1.09	1.07
P	0.473	0.482
GE (Gross energy) MJ/kg	15.5	19.9

** Premix supplemented with VA 15.000 IU, VD 5.000 IU, VE 50 mg, Fe 90 mg, Cu 12.5 mg, Mn 30 mg, Zn 90 mg, Se 0.3 mg, I 1.0 mg and Co 0.5 mg for per kg starter*

2 g/d + CT 1 g/d per calf before weaning, or FML 4 g/d + CT 1 g/d per calf after weaning). The experiment began at 21 days old and lasting 60 days. At the age of 55 days old, calves were weaned. The diet for calves contained milk replacer and starter. Milk replacer was offered daily at 10% of body weight (BW) (adjusted weekly) and starter was offered *ad libitum* throughout the 60 days trial period. Each calf was housed in an individual hutch during the whole experiment period except the metabolic study period during which each calf was raised in an individual metabolic cage. There are two metabolic trials in the whole experiment period. One trial began at the age of 43 days lasting 5 days (preweaning), the other began at the age of 60 days lasting 5 days too (postweaning). BW was measured at 21, 28, 42, 56 and 80 days old during the whole trial period, and starter intake was recorded daily.

Sample Collection and Analysis

Every three calves that were from the correspond group, and reached the average BW were selected for the metabolic trial. Urinary and fecal excretions of every calf (4 groups in all, one group having 3 calves) were entirely collected daily for analyzing the apparent digestibility of dry matter (DM), ether extract (EE), neutral detergent fiber (NDF) and acid detergent fiber (ADF). The mixed sample was from 10% of total amount of feces, and 10 g mixed fecal sample was treated with 10 mL of 10% dilute hydrochloric acid for nitrogen fixation. Then a 500-g feces sample was taken, dried at 103°C for 48 h, ground in a Cyclotec 1093 mill (Tecator, Sweden). The digestion rate was calculated as previously described [17]. The blood samples (jugular venipuncture) were collected before the morning feeding at 28, 42, 56 and 80 days old, respectively. Plasma samples were stored at -19°C after centrifugation (3000×g, for 15 min, at 25°C) for analysis of GH and IGF-1 by radioimmunoassay (RIA) as previously described [18]. IGF-1 antibody (sc-1422, Santa Cruz Biotech, CA) was used to analyze the IGF-1 concentration, and the GH concentration was determined using an antibody (sc-10365, Santa Cruz Biotech, CA).

Statistical Analysis

The experimental design was a randomized complete block design. Continuous variables were analyzed by ANOVA using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, 2003). The model included fixed effect and the random effect. Treatments, days (as a repeated effect), and their interaction were as the fixed effects, and the calf was as the random effects. Restricted Maximum Likelihood was used to estimate least square mean values. Where treatment effects were significant the means were analyzed using Tukey's procedure for multiple comparisons. The initial BWs were modeled as a Covariate to further control the experimental error. Differences were considered statistically significant at the 95% confidence level (P<0.05).

RESULTS

The Effect of FML on Performance and Plasma Hormone Level

It was shown in *Fig. 2* that the BW of FML group was significantly higher (P<0.05) than that of Ctrl group after weaning, but there was no significant effect of FML on BW before weaning. It was observed in *Fig. 1* that there was no significant effect of FML on starter intake throughout the experiment in calves (P<0.05). Meanwhile significant changes (P<0.05) were observed between FML and Ctrl group in the levels of plasma GH and IGF-1 at the age of 80 days *(Table 3)*, and the digestibility of EE in FML group was significantly higher (P<0.05) than that in Ctrl group after weaning *(Table 2)*.

The Effect of CT on Performance and Plasma Hormone Level

The diet supplementation with CT did not affect starter intake (P>0.05) throughout the experiment in calves *(Fig. 1)*, but the BW *(Fig. 2)* of calves of CT group was significantly higher than that of Ctrl group only at the age of 56 days (P<0.05), while the *apparent digestibility* of NDF

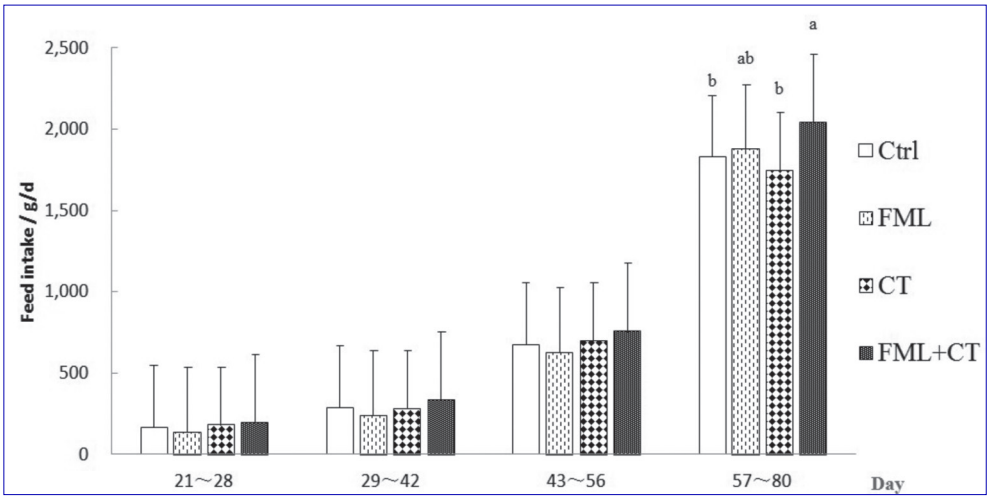


Fig 1. Effect of flavonoids from mulberry leaves and *Candida tropicalis* on feed intake. Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, *Candida tropicalis* (1 g/d per calf); FML+CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + *Candida tropicalis* (1 g/d per calf). Within the same row with different superscripts indicated significant differences (P<0.05)

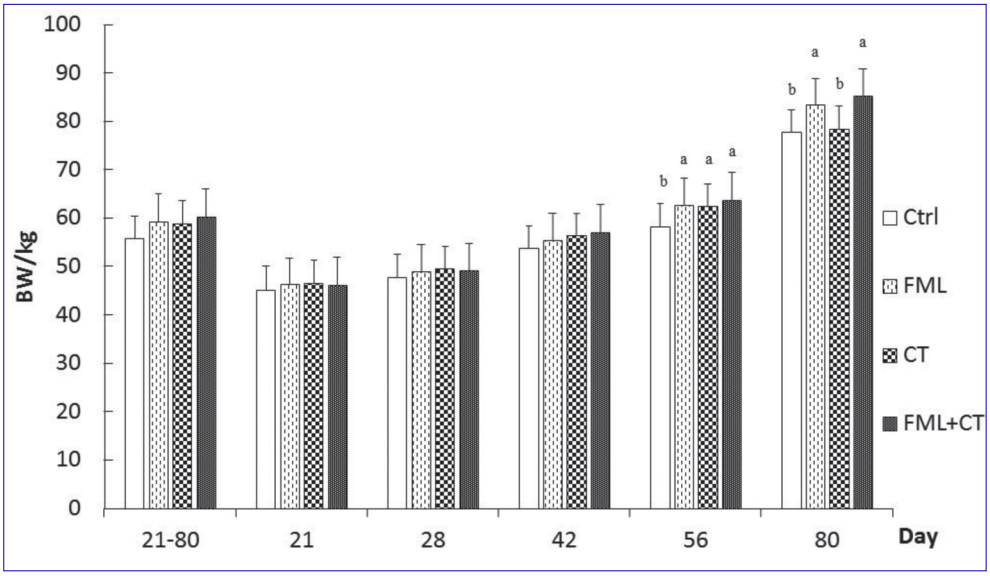


Fig 2. Effect of flavonoids from mulberry leaves and *Candida tropicalis* on body weight. Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, *Candida tropicalis* (1 g/d per calf); FML + CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + *Candida tropicalis* (1 g/d per calf). Within the same row with different superscripts indicated significant differences ($P<0.05$)

Table 2. *Effect of flavonoids from mulberry leaves and Candida tropicalis on nutrient digestibility*

Items	Treatment				SEM	*P*-value
	Ctrl	FML	CT	FML+CT		
Pre-weaning male calves						
Dry matter	79.3	79.2	79.0	80.1	1.09	0.976
Organic matter	81.3	80.5	80.6	81.5	1.07	0.883
Ether extract	79.3	75.8	79.1	69.5	2.55	0.905
Neutral detergent fiber	19.2^{b}	21.3^{b}	25.7^{a}	19.8^{b}	0.983	0.163
Acid detergent fiber	27.4	29.6	27.7	29. 4	1.62	0.0994
Post-weaning male calves						
Dry matter	79.3	84.7	81.6	84.9	1.52	0.342
Organic matter	81.3	86.4	82.2	86.9	1.34	0.328
Ether extract	33.7^{b}	53.5^{a}	46.3^{a}	62.7^{a}	3.16	0.0317
Neutral detergent fiber	49.3^{b}	57.5^{ab}	48.3^{b}	60.7^{a}	2.03	0.0287
Acid detergent fiber	63.7^{b}	66.3^{ab}	65.4^{b}	72.5^{a}	1.36	0.0322

Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, Candida tropicalis (1 g/d per calf); FML+CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + Candida tropicalis (1 g/d per calf). Within the same row with different superscripts indicated significant differences (P<0.05)

of CT group was significantly higher than that of Ctrl group before weaning ($P<0.05$). The digestibility of EE in CT group was significantly higher ($P<0.05$) than that in Ctrl group after weaning *(Table 2)*, while the level of plasma IGF-1 in CT group was obviously higher ($P<0.05$) than that in Ctrl group post weaning *(Table 3)*.

The Effect of FML + CT on Performance and Plasma Hormone Level

The results of current study *(Fig. 1, Fig. 2)* indicated that BW and starter feed in FML + CT group were significantly higher ($P<0.05$) than that in Ctrl group at the age of 57-80 days. There was no significant change in BW between FML + CT and Ctrl group at the age of 21-42 days ($P>0.05$). The levels of plasma GH and IGF-1 in FML + CT group were significantly higher ($P<0.05$) than that in Ctrl group at the age of 80 days *(Table 3)*. The diet supplementation with FML+CT simultaneously enhanced digestibility of EE, NDF and *ADF* ($P<0.05$) significantly compared with that no supplementation after weaning in calves *(Table 2)*.

DISCUSSION

The use of antibiotics for growth promotion has been totally banned in many countries, owing to drug residues in meat products and increasing bacterial resistance by use and misuse of in-feed antibiotics in food-producing animals [19], so utilization of phytogenic compounds in feed for food animal production has good potential [20]. It is fed flavonoids extracted from propolis that calves have

Table 3. *Effect of flavonoids from mulberry leaves and Candida tropicalis on GH and IGF-1*

Items	Treatment				SEM	P-value		
	Ctrl	FML	CT	FML+CT		Treatment	age	Treatment × Age
GH (ng/mL)								
28-80	3.21	3.31	3.33	3.39	0.0526	0.613	0.00157	0.169
28	3.28	2.95	3.02	3.09	0.0618	0.645	-	-
42	3.23	3.18	3.25	3.16	0.0578	0.463		
56	3.25	3.43	3.67	3.71	0.293	0.0681		
80	3.23^{b}	3.72^{a}	3.31^{ab}	3.75^{a}	0.181	0.0357		
IGF-1 (ng/mL)								
28-80	162	225	211	221	9.35	0.0452	0.0132	0.246
28	173	171	206	189	13.2	0.383	-	-
42	167	195	194	158	13.5	0.336		
56	159	215	209	214	19. 5	0.0543		
80	177^{b}	295^{a}	277^{a}	294^{a}	20. 60	0.00219		

Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, Candida tropicalis (1 g/d per calf); FML+CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + Candida tropicalis (1 g/d per calf). Within the same row with different superscripts indicated significant differences (P<0.05)

higher BW than those fed no flavonoids until 120 days of age [21]. Results of our present study revealed that calves supplement with FML in the diet have higher BW than those fed no flavonoids after the age of 56 days, without significant difference in feed intake comparing to those fed no flavonoids, which is consistent with the previous reports. Flavonoids have beneficial effects on urinary tract infections, cognitive function and age-related cognitive decline, cancer and cardiovascular disease in human [22], and flavonoids and their metabolites modulated the expression and activity of several metabolic key enzymes, and are involved in regulation of lipid and carbohydrate metabolism [23]. Therefore, the higher BW in FML group may be due to its beneficial effects on several metabolic key enzymes.

Our results showed that the *apparent digestibility* of ADF and NDF was not affected by FML. It was also reported that feeding quebracho tannin extract, a diverse group of polymeric flavonoids, had no effect on ADF and NDF digestibility in Angus heifers [24], which was consistent with our results. However, there was significant effect on the EE of the diet of calves by supplementation with FML comparing Ctrl group after weaning in this study. It was reported that condensed tannins altered ruminal biohydrogenation process of unsaturated fatty acids [25], and the greater digestibility of EE in the FML group than that in Ctrl group may be due to the antioxidant capacities of FML after weaning.

Our results also indicated that there were higher levels of serum GH and IGF-1 in FML group comparing with that in Ctrl group at the age of 80 days. The binding of Genistein to estrogen receptors in the hypothalamus influences the production of GH and growth factors (GF), which lead to increasing the uterine weight, uterine wall thickness and ovarian weight in Sprague Dawley rats [26]. It is reported that flavanone 8-prenylnaringenin, as a phytoestrogen, increases serum GH, but decreases serum IGF-1 levels in rats [27], and IGF-1 has negative effect on GH gene expression in somatotroph cell line [28]. However, GH can strongly stimulate production of IGF-1 *in vivo*. Many tissues and cells can produce IGF-1, and IGF-1 is mainly secreted by the liver under the control of GH, meanwhile have effects on growth and development mediated partly by the effects of GH [29]. Our results may suggest that FML promotes the growth and development of calves by increasing the levels of serum GH and IGF-1. Therefore, FML enhanced apparent digestibility of EE of the diet, and increased the levels of serum GH and IGF-1 in calves, which led to the increased BW after the age of 56 days by supplemented with FML.

It is reported that yeast culture can enhance crude protein and cell wall digestibility, ruminal molar proportion of propionate and plasma glucose concentration in Baluchi lambs [30], and Jersey calves feed live yeast product have greater final BW at 63 days than calves fed none [31]. Our results indicated that there was significant effect on BW of calves supplemented with CT comparing with that no CT only at the age of 56 days, with no significantly difference in the feed intake during total experimental stage. Meanwhile the apparent digestibility of NDF of CT group was significantly higher than that of Ctrl group before weaning. Lesmeister *et al.*[32] reported that Holstein calves fed 2% yeast culture had greater BW at 42 days of age than calves with receiving no yeast. There are improvements in grain intake, BW gain, and blood parameters of calves when fed live yeast only during the pre-weaning period [33]. The calves fed *Saccharomyces cerevisiae* have greater BW during the

pre-weaning period, because yeast can improve growth and activity of fiber-degrading bacteria and fungi, stabilize rumen pH, prevent lactate accumulation, improve ruminal microbial colonization, and set up fermentative processes [34], which is consistent with our study. Our study suggested that CT enhanced the BW of calves before weaning through manipulating rumen fermentation and increasing the apparent digestibility of NDF of the diet.

Furthermore, the results of present study showed that the apparent digestibility of EE in CT group was significantly higher than that in Ctrl group after weaning, and CT have significant effect on the level of serum IGF-1 comparing with Ctrl group in post-weaning male calves. It is supplemented with *Lactobacillus plantarum* that serum IGF-1 can return to pre-challenge values by day 13 post-challenge orally with *Salmonella* in pigs [35]. It is treated with *Lactobacillus rhamnosus* that Zebrafish exhibits a high gene expression level for IGF-1 comparing to untreated group at 6 days post fertilization [36]. Therefore, CT enhanced the apparent digestibility of EE of the diet in calves after weaning through increasing the level of IGF-1 and manipulating rumen fermentation.

Results of the current experiment showed that mixture of FML and CT plays a synergistic role in enhancing growth, improving feed intake and nutriment *digestibility*. It achieved the best effect among four treatments through simultaneously supplementation the two additives (FML + CT) to the starter of calves. BW, starter feed and the levels of plasma GH and IGF-1 were observed significantly higher ($P<0.05$) in FML + CT group compared with Ctrl group at the age of 80 days. Furthermore, the *digestibility of* EE, NDF and ADF in FML + CT group was significantly higher ($P<0.05$) than that in Ctrl group after weaning.

In conclusion, FML could be used to enhance BW of calves through enhancing apparent digestibility of EE of the diet, and increased the level of serum GH and IGF-1 in calves after the age of 56 days. CT enhanced the BW of calves before weaning through increasing the apparent digestibility of NDF of the diet, and elevated the apparent digestibility of EE of the diet of calves after weaning through increasing the level of serum IGF-1 in calves. Furthermore, the mixture of FML and CT plays a synergistic role in enhancing growth, improving feed intake and nutriment *digestibility*.

Acknowledgments

This work was supported by National Dairy Industry Technology System-Beijing Innovation Team, National Science and Technology Support Program, China (2012BAD12B06), and Science and Technology R&D Project of Handan City, China (1522101042-2).

REFERENCES

1. Redding LE, Cubas-Delgado F, Sammel MD, Smith G, Galligan DT, Levy MZ, Hennessy S: The use of antibiotics on small dairy farms in rural Peru. *Prev Vet Med,* 113, 88-95, 2014. DOI: 10.1016/j.prevetmed.2013.10.012

2. Bell L, Lamport DJ, Butler LT, Williams CM: A review of the cognitive effects observed in humans following acute supplementation with flavonoids, and their associated mechanisms of action. *Nutrients,* 7, 10290-10306, 2015. DOI: 10.3390/nu7125538

3. Vezza T, Rodríguez-Nogales A, Algieri F, Utrilla MP, Rodriguez-Cabezas ME, Galvez J: Flavonoids in inflammatory bowel disease: A review. *Nutrients,* 8, 211, 2016. DOI: 10.3390/nu8040211

4. Jenkins KJ, Atwal AS: Flavonoids increase tissue essential fatty acids in vitamin E-deficient chicks. *J Nutr Biochem,* 6, 97-103, 1995. DOI: 10.1016/0955-2863(94)00018-H

5. Soltan YA, Morsy AS, Sallam SM, Lucas RC, Louvandini H, Kreuzer M, Abdalla AL: Contribution of condensed tannins and mimosine to the methane mitigation caused by feeding *Leucaena leucocephala*. *Arch Anim Nutr,* 67, 169-184, 2013. *Arch Anim Nutr,* 2013. DOI: 10.1080/1745039X.2013.801139

6. Balcells J, Aris A, Serrano A, Seradj AR, Crespo J, Devant M: Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets. *J Anim Sci,* 90, 4975-4984, 2012. DOI: 10.2527/jas.2011-4955

7. Girard M, Dohme-Meier F, Silacci P, Ampuero Kragten S, Kreuzer M, Bee G: Forage legumes rich in condensed tannins may increase n-3 fatty acid levels and sensory quality of lamb meat. *J Sci Food Agric,* 96,1923-1933. 2016. DOI: 10.1002/jsfa.7298

8. El-Sayyad HI, El-Sherbiny MA, Sobh MA, Abou-El-Naga AM, Ibrahim MA, Mousa SA: Protective effects of *Morus alba* leaves extract on ocular functions of pups from diabetic and hypercholesterolemic mother rats. *Int J Biol Sci,* 7, 715-728, 2011. DOI: 10.7150/ijbs.7.715

9. Wong A, Ngu DY, Dan LA, Ooi A, Lim RL: Detection of antibiotic resistance in probiotics of dietary supplements. *Nutr J,* 14, 95, 2015. DOI: 10.1186/s12937-015-0084-2

10. Paserakung A, Pattarajinda V, Vichitphan K, Froetschel MA: Selection and identification of oleaginous yeast isolated from soil, animal feed and ruminal fluid for use as feed supplement in dairy cattle. *Lett Appl Microbiol,* 61, 325-332, 2015. DOI: 10.1111/lam.12475

11. Jensen GS, Patterson KM, Yoon I. Yeast culture has anti-inflammatory effects and specifically activates NK cells. *Comp Immunol Microbiol Infect Dis,* 31, 487-500, 2008. DOI: 10.1016/j.cimid.2007.08.005

12. Jensen GS, Hart AN, Schauss AG: An antiinflammatory immunogen from yeast culture induces activation and alters chemokine receptor expression on human natural killer cells and B lymphocytes *in vitro*. *Nutr Res,* 27, 327-335, 2007. DOI: 10.1016/j.nutres.2007.04.008

13. Brewer MT, Anderson KL, Yoon I, Scott MF, Carlson SA: Amelioration of salmonellosis in pre-weaned dairy calves fed *Saccharomyces cerevisiae* fermentation products in feed and milk replacer. *Vet Microbiol,* 172, 248-255, 2014. DOI: 10.1016/j.vetmic.2014.05.026

14. Uyeno Y, Shigemori S, Shimosato T: Effect of probiotics/prebiotics on cattle health and productivity. *Microbes Environ,* 30, 126-132, 2015. DOI: 10.1264/jsme2.ME14176

15. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J: *Candida glabrata, Candida parapsilosis* and *Candida tropicalis*: Biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol Rev,* 36, 288-305, 2012. DOI: 10.1111/j.1574-6976.2011.00278.x

16. Renaville R, Hammadi M, Portetelle D: Role of the somatotropic axis in the mammalian metabolism. *Domest Anim Endocrinol,* 23, 351-360, 2002. DOI: 10.1016/S0739-7240(02)00170-4

17. Kammes KL, Allen MS: Nutrient demand interacts with grass particle length to affect digestion responses and chewing activity in dairy cows. *J Dairy Sci,* 95, 807-823, 2012. DOI: 10.3168/jds.2011-4588

18. Mejía M, Gonzalez-Iglesias A, Díaz-Torga GS, Villafañe P, Formía N, Libertun C, Becú-Villalobos D, Lacau-Mengido IM: Effects of continuous ivermectin treatment from birth to puberty on growth and reproduction in dairy heifers. *J Anim Sci,* 77, 1329-1334, 1999. DOI: 10.2527/1999.7761329x

19. Casewell M, Friis C, Marco E, McMullin P, Phillips I: The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J Antimicrob Chemother,* 52, 159-161, 2003. DOI: 10.1093/jac/dkg313

20. Yang C, Chowdhury MA, Huo Y, Gong J: Phytogenic compounds as alternatives to in-feed antibiotics: Potentials and challenges in application. *Pathogens* 4, 137-56, 2015. DOI: 10.3390/pathogens4010137

21. Yaghoubi SM, Ghorbani GR, Rahmani HR, Nikkhah A: Growth, weaning performance and blood indicators of humoral immunity in Holstein calves fed supplemental flavonoids. *J Anim Physiol Anim Nutr (Berl),* 92, 456-462, 2008. DOI: 10.1111/j.1439-0396.2007.00734.x

22. Rodriguez-Mateos A, Vauzour D, Krueger CG, Shanmuganayagam D, Reed J, Calani L, Mena P, Del Rio D, Crozier A: Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: An update. *Arch Toxicol,* 88, 1803-1853, 2014. DOI: 10.1007/s00204-014-1330-7

23. Kobayashi Y, Miyazawa M, Kamei A, Abe K, Kojima T: Ameliorative effects of mulberry *(Morus alba* L.) leaves on hyperlipidemia in rats fed a high-fat diet: Induction of fatty acid oxidation, inhibition of lipogenesis, and suppression of oxidative stress. *Biosci Biotechnol Biochem,* 74, 2385-2395, 2010. DOI: 10.1271/bbb.100392

24. Beauchemin KA, McGinn SM, Martinez TF, McAllister TA: Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. *J Anim Sci,* 85, 1990-1996, 2007. DOI: 10.2527/jas.2006-686

25. Benchaar C, Chouinard PY: Short communication: Assessment of the potential of cinnamaldehyde, condensed tannins, and saponins to modify milk fatty acid composition of dairy cows. *J Dairy Sci,* 92, 3392-3396. 2009. DOI: 10.3168/jds.2009-2111

26. Zin SR, Omar SZ, Khan NL, Musameh NI, Das S, Kassim NM: Effects of the phytoestrogen genistein on the development of the reproductive system of Sprague Dawley rats. *Clinics (Sao Paulo),* 68, 253-262, 2013. DOI: 10.6061/clinics/2013(02)OA21

27. Böttner M, Christoffel J, Wuttke W: Effects of long-term treatment with 8-prenylnaringenin and oral estradiol on the GH-IGF-1 axis and lipid metabolism in rats. *J Endocrinol,* 198, 395-401, 2008. DOI: 10.1677/JOE08-0127

28. Romero CJ, Pine-Twaddell E, Sima DI, Miller RS, He L, Wondisford F, Radovick S: Insulin-like growth factor 1 mediates negative feedback to somatotroph GH expression via POU1F1/CREB binding protein interactions. *Mol Cell Biol,* 32, 4258-4269, 2012. DOI: 10.1128/MCB.00171-12

29. Zhou Y, Capuco AV, Jiang H: Involvement of connective tissue growth factor (CTGF) in insulin-like growth factor-I (IGF1) stimulation of proliferation of a bovine mammary epithelial cell line. *Domest Anim Endocrinol,* 35, 180-189, 2008. DOI: 10.1016/j.domaniend.2008.05.003

30. Malekkhahi M, Tahmasbi AM, Naserian AA, Danesh Mesgaran M, Kleen JL, Parand AA: Effects of essential oils, yeast culture and malate on rumen fermentation, blood metabolites, growth performance and nutrient digestibility of Baluchi lambs fed high-concentrate diets. *J Anim Physiol Anim Nutr (Berl),* 99, 221-229, 2015. DOI: 10.1111/jpn.12230

31. Hill SR, Hopkins BA, Davidson S, Bolt SM, Diaz DE, Brownie C, Brown T, Huntington GB, Whitlow LW: The addition of cottonseed hulls to the starter and supplementation of live yeast or mannanoligosaccharide in the milk for young calves. *J Dairy Sci,* 92, 790-798, 2009. DOI: 10.3168/jds.2008-1320

32. Lesmeister KE, Heinrichs AJ, Gabler MT: Effects of supplemental yeast *(Saccharomyces cerevisiae)* culture on rumen development, growth characteristics, and blood parameters in neonatal dairy calves. *J Dairy Sci,* 87, 1832-1839, 2004. DOI: 10.3168/jds.S0022-0302(04)73340-8

33. Galvão KN, Santos JE, Coscioni A, Villaseñor M, Sischo WM, Berge AC: Effect of feeding live yeast products to calves with failure of passive transfer on performance and patterns of antibiotic resistance in fecal *Escherichia coli. Reprod Nutr Dev,* 45, 427-440, 2005. DOI: 10.1051/rnd:2005040

34. Fonty G, Chaucheyras-Durand F: Effects and modes of action of live yeasts in the rumen. *Biologia,* 61, 741-750, 2006. DOI: 10.2478/s11756-006-0151-4

35. Gebru E, Lee JS, Son JC, Yang SY, Shin SA, Kim B, Kim MK, Park SC: Effect of probiotic-, bacteriophage-, or organic acid-supplemented feeds or fermented soybean meal on the growth performance, acute-phase response, and bacterial shedding of grower pigs challenged with *Salmonella enterica* serotype Typhimurium. *J Anim Sci,* 88, 3880-3886, 2010. DOI: 10.2527/jas.2010-2939

36. Avella MA, Place A, Du SJ, Williams E, Silvi S, Zohar Y, Carnevali O: *Lactobacillus rhamnosus* accelerates zebrafish backbone calcification and gonadal differentiation through effects on the GnRH and IGF systems. *PlosOne,* 7, e45572, 2012. DOI: 10.1371/journal.pone.0045572

Kafkas Univ Vet Fak Derg
23 (3): 481-485, 2017
DOI: 10.9775/kvfd.2016.17183

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Investigation of Acute Phase Reactants and Antioxidant Capacity in Calves Infected with *Cryptosporidium parvum* [1]

Metin ÇENESİZ [1] Aliye SAĞKAN ÖZTÜRK [2] Duygu DALĞIN [3] Gül Fatma YARIM [4]
Gülay ÇİFTÇİ [4] Ramazan ÖZDEMİR [2] Murat GÜZEL [3] Filiz KAZAK [4] Sena ÇENESİZ [4]

[1] *This study is presented in Vet İstanbul Group Congress, 7-9 April 2015 St. Petersburg - RUSSIA*

[1] Ondokuz Mayıs Üniversitesi Veteriner Fakültesi, Fizyoloji Anabilim Dalı, TR-55139 Samsun - TÜRKİYE
[2] Mustafa Kemal Üniversitesi Veteriner Fakültesi, İç Hastalıkları Anabilim Dalı, TR-31040 Hatay - TÜRKİYE
[3] Ondokuz Mayıs Üniversitesi Veteriner Fakültesi, İç Hastalıkları Anabilim Dalı, TR-55139 Samsun - TÜRKİYE
[4] Ondokuz Mayıs Üniversitesi Veteriner Fakültesi Veteriner Fakültesi, Biyokimya Anabilim Dalı, TR-55139 Samsun - TÜRKİYE

Article Code: KVFD-2016-17183 Received: 02.12.2016 Accepted: 07.02.2017 Published Online: 07.02.2017

Citation of This Article

Çenesiz M, Sağkan Öztürk A, Dalğın D, Yarım GF, Çiftçi G, Özdemir R, Güzel M, Kazak F, Çenesiz S: Investigation of acute phase reactants and antioxidant capacity in calves infected with *Cryptosporidium parvum*. *Kafkas Univ Vet Fak Derg*, 23 (3): 481-485, 2017. DOI: 10.9775/kvfd.2016.17183

Abstract

Cryptosporidiosis is a zoonotic infection contaminating via fecal-oral route. *Cryptosporidium parvum* has a wide host prevalance, but is more epidemic in calves. This disease courses with high morbidity and mortality resulting considerable economic losses. In this study, halofuginon (100 µg /kg/day for 7 days) was applied to calves infected with *C. parvum* and the effect of this treatment on acute phase proteins and antioxidant capacity were investigated. Study group was comprised of sera of 10 Holstein calves aged 1-3 weeks, infected with *C. parvum*. Blood samples were obtained from the animals before and after treatment of 7 days and serum amyloid A (SAA), haptoglobin (Hp), C-reactive protein (CRP), ceruloplasmin (CP), malondialdehyde (MDA) levels and superoxide dismutase (SOD) and adenosine deaminase (ADA) activities were measured in sera. Obtained data showed that there was no statistical difference between pre and post treatment SAA, CRP and MDA levels, but a decrease was determined in post treatment Hp ($P<0.001$) and CP ($P<0.05$) levels, with ADA ($P<0.05$) and SOD ($P<0.001$) activities. Eventually, it was determined that ADA and SOD activities and Hp and CP levels decreases by treatment in calves infected with *C. parvum*.

Keywords: *Acute phase reactants, Antioxidant capacity, Cryptosporidium parvum*

Cryptosporidium parvum ile Enfekte Buzağılarda Akut Faz Reaktanları ve Antioksidant Kapasitenin Araştırılması

Özet

Kriptosporidiozis, fekal-oral yolla bulaşan bir zoonoz enfeksiyondur. *Cryptosporidium parvum* yaygın prevalans göstermekle birlikte, buzağılarda daha epidemik olarak seyretmektedir. Hastalık yüksek morbidite ve mortalitesine bağlı olarak, ciddi ekonomik kayıplara neden olur. Bu çalışmada, *C. parvum* ile enfekte buzağılara halofuginon (100 µg /kg/gün-7 gün) tedavisi uygulanmış ve bu tedavinin akut faz proteinleri ile antioksidant kapasite üzerindeki etkileri araştırılmıştır. Çalışma grubu, *C. parvum* ile enfekte, 1-3 haftalık 10 Holstein buzağıdan oluşturulmuştur. Tedavi öncesi ve sonrası alınan kan numunelerinde, serum amiloid A (SAA), haptoglobulin (Hp), C-reaktif protein (CRP), seruloplazmin (CP), malondialdehid (MDA) seviyeleri ile süperoksid dismutaz (SOD) ve adenozin deaminaz (ADA) aktiviteleri tespit edilmiştir. Elde edilen veriler, tedavi öncesi ve sonrası SAA, CRP ve MDA seviyelerinde istatistiksel bir farklılık olmadığını, fakat tedavi sonrası Hp ($P<0.001$) ve CP ($P<0.05$) seviyeleri ile ADA ($P<0.05$) ve SOD ($P<0.001$) aktivitelerinde istatistiksel olarak anlamlı bir düşüş meydana geldiğini ortaya koymuştur. Sonuç olarak, *C. parvum* ile enfekte buzağılarda tedavi ile ADA ve SOD aktiviteleri ile Hp ve CP seviyelerinde düşüş sağlandığı tespit edilmiştir.

Anahtar sözcükler: *Akut faz reaktanları, Antioksidant kapasite, Cryptosporidium parvum*

INTRODUCTION

C. parvum is the most common enteral pathogen of neonatal calves and mortal cause of neonatal calf diarrhoea worldwide [1,2]. Nearby leading major economic losses in breeding, the agent is zoonotic and has a potential of public health concern. Humans gain the infection by direct contact with infected individuals or animals and ingestion

İletişim (Correspondence)
+90 506 7872719
duyguc@omu.edu.tr

of *Cryptosporidium* oocysts via contaminated food or water [3].

C*ryptosporidium* spp. directly affects the intestines by multiplying at the microvillus borders of the enteric epithelium, giving serious damage to the villi thereby reduction of the absorptive surface and maldigestion and malabsorption followed by diarrhoea [4]. Occasionally, the parasite may affect other tissues such as the respiratory and renal epithelia in all the species of its spectrum [5,6].

Majority of cattle infections are due to *C. parvum, C. bovis,* and *C. Andersoni*, but in pre-weaned calves *C. parvum* is the dominant species [2,7].

The sporulated oocysts spread by the faeces which has long survival capacity with high resistence to environmental conditions and this is the form frequently used in the diagnosis in practice [6]. Studies comparing the diagnostic methods for *Cryptosporidium parvum* in calf faeces revealed high sensitivity and specificity for the rapid tests [8].

There is no definite effective treatment assigned currently for the therapy and prevention in bovine cryptosporidiosis, but conventional therapy includes halofuginone lactate together with providing good husbandry. Studies revealed that halofuginone lactate is satisfactory both in the clinical and prophylactic aspects reducing the environmental contamination with Cryptosporidium oocysts [9].

Acute phase proteins are involved in the restoration of homeostasis and preventing bacterial growth before the acquired immunity development [10]. Haptoglobin, ceruloplasmin and α-1 acidglycoprotein are some of the acute phase proteins triggered by infection. It is concluded that, oxidative stress and reactive oxygen species (ROS) due to tissue damage has a crucial role in the enteric damage pathogenesis of farm animals [11]. Acute phase proteins are an alternative path for monitoring clinical course and may be useful for providing information in the severity and prognosis [12].

The aim of this study is to investigate the effects of conventional halofuginon (100 µg/kg/day for 7 days) treatment on acute phase proteins and antioxidant capacity by determining serum amyloid A (SAA), haptoglobin (Hp), C-reactive protein (CRP), ceruloplasmin (CP), malondi-aldehyde (MDA) levels and superoxide dismutase (SOD) and adenosine deaminase (ADA) activities in calves infected with *C. parvum*.

MATERIAL and METHODS

Animals

The study group was comprised of 10 Holstein calves aged 1-3 weeks, infected with *C. parvum*. The calves were housed in a 200 cow farm in which yellow, nasty smelling and watery diarrhoea was observed in the neonates. Stool specimen obtained directly from the rectum were analysed for cryptosporidium oocyts with rapid kit, BİO K 155 (1x10 strips- Bio X Diagnostics) according to the manufacturers instructions. All specimen were also analysed with carbol fuchsin method. Blood specimen were obtained from the calves before and after the treatment. Conventionel treatment included Halocur® (MSD), Baytril® (Bayer) nearby fluid therapy and supplemental vitamins.

Carbol Fuchsin Staining Method

Stool specimen obtained directly from rectum were transferred to the laboratory in sterile plastic containers in cold chain. All specimen were analysed with Heine's carbol fuchsin staining method. For this purpose, 50 µl of homogenised stool specimen were placed on slides cleaned with ether-alcohol mixture. Same amount of carbol fuchsin was added and a thin specimen smear was prepared. After drying, a drop of immersion oil was added and the slide cover was placed. Smears were examined at X40 aggrandizement at microscope for *Cryptosporidium* oocysts *(Fig. 1)*.

SAA, CRP, Hp Analyses

CRP, SAA and haptoglobuline values were analysed with ELISA, using phase bovine (Tridelta Development Limited, Ireland) kits. Tests were performed according to the standards and guidelines provided by the manufacturer. All samples were calculated with a spectrophotometer (Digital and Analogue Systems S.R.L.) at 450 nm.

Ceruloplasmin Analyses

Serum ceruloplasmin analyses were performed with spectrophotometrically revised modified Ravin method based on the oxidation of colorless phenilen diamine to a blue-purple color product [13].

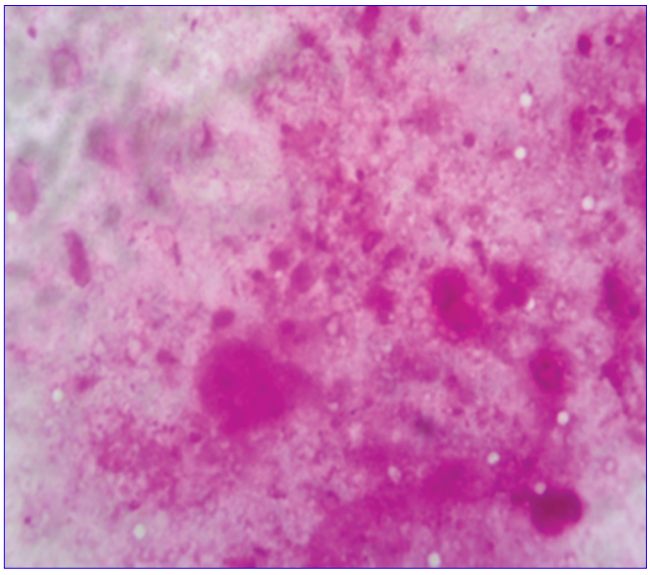


Fig 1. Cryptosporidium oocyts with carbol fuschin staining

MDA Analyses

MDA levels were determined according to the method of Yoshioko and Kawada [14], based on thiobarbuturic acid (TBA) reactivity. According to this method, a stabile red matter giving absorbans at 535 nm was formed by warming lipid content in low pH and thiobarbituric acid (TBA) containing medium resulting in unition of MDA and two TBA molecules was spectophotometrically determined. 1.1.3.3-tetraetoxypropan dissolved in 2.5-5-10 and 20 µmol/L concentration ethyl alcohol was used for calibration. MDA concentration was measured as an indirect marker of oxidative stress in terms of TBARS (thiobarbituric acid reactive substances), spectrophotometrically.

ADA Analyses

ADA in sera was determined at 37°C according to the method of Giusti and Galanti [15], based on the Bertholet reaction, formation of coloured indophenol complex from ammonia liberated from adenosine, and quantified colorimetrically with spectrophotometer (Thermo Scientific, Genesys 10S UV-Vis, USA). One unit of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia/min from adenosine at standard assay condition. Results were expressed as international unit of enzyme activity.

SOD Analyses

SOD analyses were performed according to the method of Podczasy and Wei [16]. The method is based on the principle of reduction of nitroblue tetrazolium (NBT) by xantine-xantinoxidase system which is a superoxide producer. Reducted NBT transforms to blue colored fomazon and measured spectrophotometrically at 560 nm (Thermo Scientific, Genesys 10S UV-Vis, USA). SOD activity was expressed as unit/g protein (U/g).

RESULTS

Obtained data revealed that there was no statistical difference between pre and post treatment SAA, CRP and MDA levels, but a decrease was determined in post treatment Hp ($P<0.001$) and CP ($P<0.05$) levels, with ADA ($P<0.05$) and SOD ($P<0.001$) activities *(Table. 1, Fig. 2)*. Eventually, it was determined that ADA and SOD activities and Hp and CP levels decreases by treatment in calves infected with *C. parvum*

Table 1. *Pre and posttreatment acute phase proteins and antioxidant capacity parameters*

Parameter	Pretreatment (n=10)	Posttreatment (n=10)
SOD	51.8±16.4	138.6±14.4
MDA	0.34±0.06	0.44±0.08
ADA	42.33±5.21	16.84±4.22
CP	20.65±1.25	18.41±0.68
SAA	426.3±52.7	339.4±26.6
HAPTO	1.26±0.13	0.54±0.07
CRP	0.29±0.03	0.22±0.04

DISCUSSION

Enteritis and diarrhoea are major causes of neonatal calf mortality and *C. parvum* is the most common enteral pathogen of neonatal calves [2] also having a zoonotic potential [17]. The disease is spread by the oocyts shed with stool and once ingested by the host, the endogenous phase starts with the invasion of target cells. Following biological steps are schizogony, gametogony, fertilization, and sporogony [6].

$CD4^+$ T cells has a crucial defense action in the immune response against *C. parvum* infection [18]. One other component of protective immunity is interferon (IFN)-γ action and Th2 cytokines [18]. Interleukin (IL)-12 is another part of defens against *C.parvum* [19]. The involvement of IL-12 and (IFN)-γ in host defense shows that a Th1 cell-mediated response is important [20]. IFN-c is also a significant element in the immune response against *C. Parvum* [21].

The acute phase response is a nonspecific reaction to tissue damage as the result of infection, inflammation, neoplasia and immunological disease [10]. It triggers the production of acute-phase proteins (such as a-1 acidglycoprotein, haptoglobin and ceruloplasmin) and involves local and systemic effects and acute phase proteins are also the components of innate immunity mediated by cytokines [10]. In the present study, elevation of Hp and ceruloplasmin in clinically ill calves infected with *C. parvum* and statistically significant decrease following recovery by the therapy is concordant with literature.

Oxidative stress is created as the result of insufficient antioxidant enzyme asset or overproduction of free radicals in the body. Free radicals and lipid peroxidation has detrimental effects to the cell [22]. MDA a product of lipid peroxidation is an important indicator of oxidative damage of cell membrane as it is the most abundant aldehyde formed [23]. Our findings was surprising in this aspect because MDA was the only parameter elevated following therapy. This may be attributed to the very abundant nature of the enzyme as the posttreatment specimen obtaining was just after the therapy and the animals were newly recovered from a devastating disease condition.

Superoxide dismutase (SOD) is a component of the compensatory reflex of the metabolism to oxidative damage targeting to neutralize the free radicals [24]. Our findings supported the literature as the high levels of SOD regressed by the treatment.

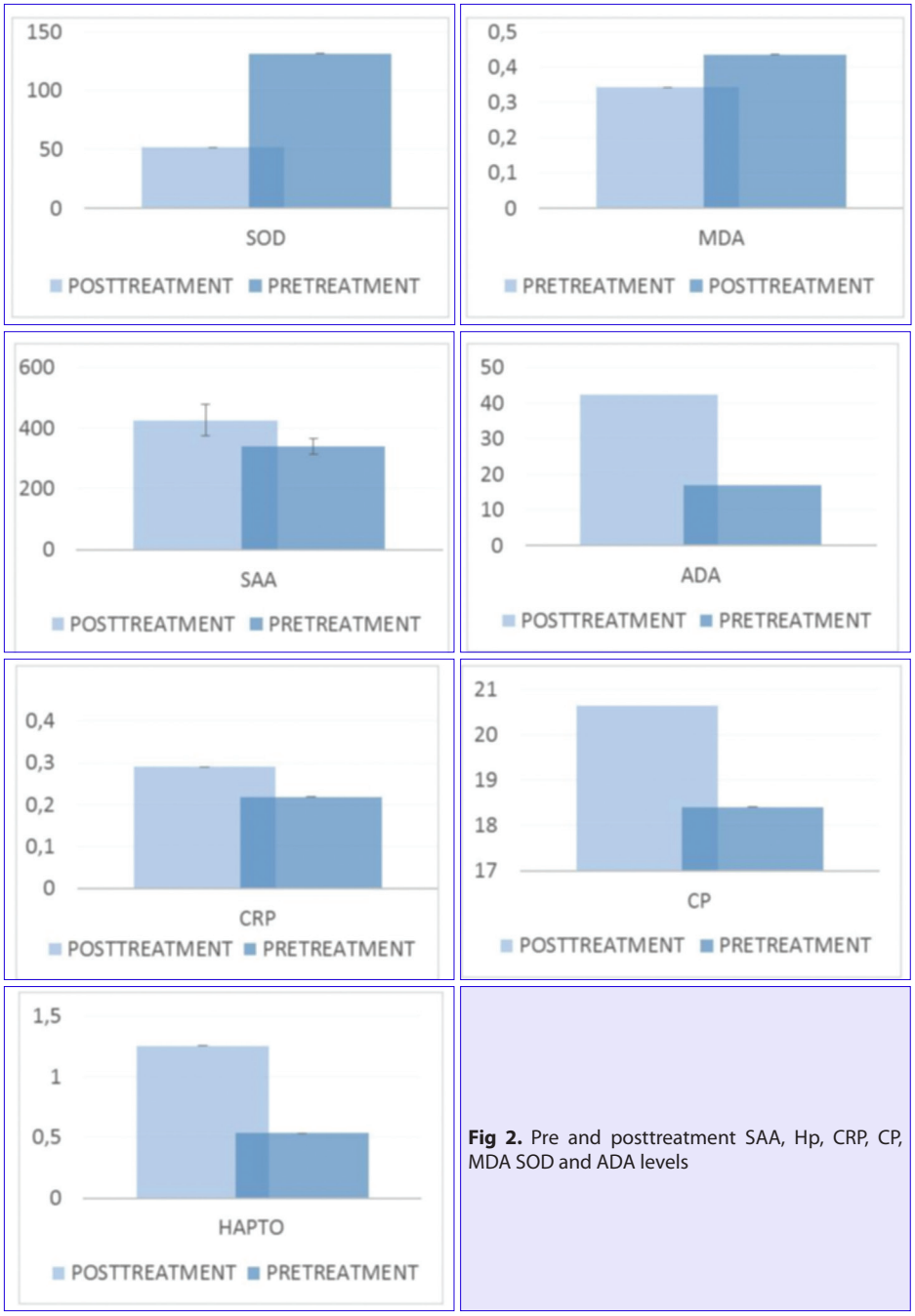


Fig 2. Pre and posttreatment SAA, Hp, CRP, CP, MDA SOD and ADA levels

ADA is important in cellular immunity and its major site of action is in the formation and differentiation of lymphocytes in lymphoid cells [25]. ADA binds cell surface receptors and promps T cells and ADA activity is directly related to the immune resonse [26], therefore the high ADA activity observed pretreatment in the present study reveals the potent immune response against *C. Parvum* infection and though statistically insignificant, a slight decrease in ADA level following therapy shows the regression of the infection.

The acute phase response SAA was characterised by a large individual variation [27], SAA is reported as one of the major acute phase proteins that increases significantly in diarrhoeic calves up to four weeks of age and could be used as a reliable indicator of clinical severity [28]. Concordantly, high SAA levels were determined in clinically infected neonatal calves in the present study, but decrease in SAA level after therapy was not statistically significant.

CRP increase during acute phase response like other acute phase proteins [29]. Our data showed high CRP levels in clinically ill calves whereas not a significant decrease was determined following therapy. This may be related to the regression period.

As the existence of oxidant activity is proven in *C. parvum* infection of calves, some authors reports that antioxidant

supplementation with standard treatment will promise a better therapeutic response [29,30], where on the other side some other researchers administration of antioxidants will exacerbate *C. parvum* infection [31,32]. Although a therapy attempt with antioxidants and evaluation of the effects was not aforementioned in the present study, obtained data on the acute phase response and antioxidant capacity in *C. parvum* infection of calves makes us think that the effects of antioxidant supplementation additional to the conventional therapy must be enlightened with the further studies.

In conclusion, obtained data of the present study showed that serum Hp and CP levels with ADA and SOD activities significantly decreased after treatment in calves clinically infected with *C. parvum* and screening for these values, though not sufficient for establishing a specific diagnosis, may be alternative indicators of the severity and the prognosis of the disease.

REFERENCES

1. Nydam DV, Susan EW, Stephanie LS, Hussni OM: Number of *Cryptosporidium parvum* oocysts or *Giardia* spp cysts shed by dairy calves after natural infection. *Am J Vet Res*, 62, 1612-1615, 2001. DOI: 10.2460/ajvr.2001.62.1612

2. Santin M, Trout JM, Fayer R: A longitudinal study of cryptosporidiosis in dairy cattle from birth to 2 years of age. *Vet Parasitol,* 155, 15-23, 2008. DOI: 10.1016/j.vetpar.2008.04.018

3. Okhuysen PC, Chappell CL: Cryptosporidium virulence determinants-Are we there yet? *Int J Parasitol*, 32 (5): 517-25, 2002. DOI: 10.1016/S0020-7519(01)00356-3

4. Gookin JL, Chiang S, Allen J, Armstrong MU, Stauffer SH, Finnegan C, Murtaugh MP: NF-kappaB-mediated expression of iNOS promotes epithelial defense against infection by *Cryptosporidium parvum* in neonatal piglets. *Am J Physiol Gastrointest Liver Physiol*, 290, G164-G173, 2006. DOI: 10.1152/ajpgi.00460.2004

5. Mercado R, Buck G, Manque P, Ozaki L: *Cryptosporidium hominis* infection of the human respiratory tract. *Emerg Infect Dis,* 1, 462-464, 2007. DOI: 10.3201/eid1303.060394

6. Fayer R: Taxonomy and species delimitation in *Cryptosporidium. Exp Parasitol,* 124, 90-97, 2010. DOI: 10.1016/j.exppara.2009.03.005

7. Feng Y, Ortega Y, He G, Das P, Xu M, Zhang X, Fayer R, Gatei W, Cama VLX: Wide geographic distribution of *Cryptosporidium bovis* and the deer-like genotype in bovines. *Vet Parasitol,* 144, 1-9, 2007. DOI: 10.1016/j.vetpar.2006.10.001

8. Klein D, Kern A, Lapan G, Benetka V, Möstl K, Hassl A, Baumgartner W: Evaluation of rapid assays for the detection of bovine coronavirus, rotavirus A and *Cryptosporidium parvum* in faecal samples of calves. *Vet J,* 182, 484-486, 2009. DOI: 10.1016/j.tvjl.2008.07.016

9. Waelea VD, Speybroeckc N, Berkvensc D, Mulcahyb G, Murphya TM: Control of cryptosporidiosis in neonatal calves: Use of halofuginone lactate in two different calf rearing systems. *Prevent Med,* 96, 143-151, 2010. DOI: 10.1016/j.prevetmed.2010.06.017

10. Petersen HH, Nielsen JP, Heegard PMH: Application of acute phase protein measurements in veterinary clinical chemistry. *Vet Res,* 35,163-187, 2004. DOI: 10.1051/vetres:2004002

11. Kim YJ, Kim EH, Hahm KB: Oxidative stress in inflammation-based gastrointestinal tract diseases: Challenges and opportunities. *J Gastroenterol Hepatol,* 27, 1004-1010, 2012. DOI: 10.1111/j.1440-1746.2012.07108.x

12. Gånheim C, Hultén C, Carlsson U, Kindahl H, Niskanen R, Waller KP: The acute phase response in calves experimentally infected with bovine viral diarrhoea virus and/or Mannheimia haemolytica. *J Vet Med B Infect Dis Vet Public Health,* 50 (4): 183-190, 2003. DOI: 10.1046/j.1439-0450.2003.00658.x

13. Ravin HA: An improved colometric enzymatic ceruloplasmin assay. *J Lab Clin Med,* 85, 161-168, 1961.

14. Yoshioka T, Kawada K, Shimada T, Mori M: Lipid peroxidation in maternal and cord blood and protective mechanism aganist actived-oxygen toxicity in the blood. *Am J Obstet Gynecol*, 135, 372-376, 1979.

15. Giusti B, Galanti B: Adenosine Deaminase: Colorimetric Method. **In,** Bergmeyer HU (Ed): Methods of Enzymatic Analysis Weinheim: Verlac Chemie, 1984.

16. Özçelik S, Poyraz Ö, Kalkan K, Malatyalı E, Değerli S: The investigation of Cryptosporidium spp. prevalence in cattle and farmers by ELISA. *Kafkas Univ Vet Fak Derg*, 18 (Suppl-A): A61-A64, 2012. DOI: 10.9775/kvfd.2011.5981

17. Podczasy JJ, Wei R: Reduction of iodonitrotetrazolium violet by superoxide radicals. *Biochem Biophys Res Comm,* 150, 1294-1301,1988. DOI: 10.1016/0006-291X(88)90770-X

18. Schmidt W, Wahnschaffe U, Schafer M, Zippel T, Arvand M, Meyerhans A, Riecken EO, Ullrich R: Rapid increase of mucosal CD4 T cells followed by clearance of intestinal cryptosporidiosis in an AIDS patient receiving highly active antiretroviral therapy. *Gastroenterology*, 120, 984-997, 2001. DOI: 10.1053/gast.2001.22557

19. Hayward AR, Cosyns M, Jones M, Ponnuraj EM: Marrow-derived CD40-positive cells are required for mice to clear *Cryptosporidium parvum* infection. *Infect Immun,* 69, 1630-1634, 2001. DOI: doi: 10.1128/IAI.69.3.1630-1634.2001

20. McDonald SAC, Gardy JE, Elliott MB, Notley CA, Alexander J, Brombacher F, McDonald V: Protection against the early acute phase of *Cryptosporidium parvum* infection conferred by interleukin-4-induced expression of T helper 1 cytokines. *JID,* 190, 1019-1025, 2004. DOI: 10.1086/422761

21. Okazawa A, Kanai T, Nakamaru K, Sato T, Inoue N, Ogata H, Iwao Y, Ikeda M, Kawamura T, Makita S, Uraushihara K, Okamoto R, Yamazaki M, Kurimoto M, Ishii H, Watanabe M, Hibi T: Human intestinal epithelial cell derived interleukin (IL)-18, along with IL-2, IL-7 and IL-15, is a potent synergistic factor for the proliferation of intraepithelial lymphocytes. *Clin Exp Immunol,* 136, 269-276, 2004. DOI: 10.1111/j.1365-2249.2004.02431.x

22. Lobo V, Patil A, Phatak A and Chandra N: Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev,* 4, 118-126, 2010. DOI: 10.4103/0973-7847.70902

23. Petry F, Jakobi V, Tessema TS: Host immune response to *Cryptosporidium parvum* infection. *Exp Parasitol*, 126, 304-309, 2010. DOI: 10.1016/j.exppara.2010.05.022

24. Daudelin JF, Lessard M, Beaudoin F, Nadeau É, Bissonnette N, Boutin Y, Brousseau JP, Lauzon K, Fairbrother JM: Administration of probiotics influences F4 (K88)-positive enterotoxigenic *Escherichia coli* attachment and intestinal cytokine expression in weaned pigs. *Vet Res,* 42, 69, 2011. DOI: 10.1186/1297-9716-42-69

25. Olson ME, Handley RM, Ralston BJ, McAllister TA, Thompson RC: Update on *Cryptosporidium* and *Giardia* infections in cattle. *Trends Parasitol,* 20, 185-191, 2004. DOI: 10.1016/j.pt.2004.01.015

26. Zintl A, Mulcahy G, Waal T, Waele V, Byrne C, Clyne M, Holden N, Fanning S: An Irish perspective on *Cryptosporidium*. Part 2. *Irish Vet J,* 59, 495-500, 2006. DOI: 10.1186/2046-0481-59-9-495

27. Pourjafar M, Badiei K, Nazifi S, Naghib SM: Acute phase response in Holstein dairy calves affected with diarrhoea. *BJVM,* 14 (3): 142-149, 2011.

28. Murata H, Shimada N, Yoshioka M: Current research on acute phase proteins in veterinary diagnosis: An overview. *Vet J,* 168, 28-40, 2004. DOI: 10.1016/S1090-0233(03)00119-9

29. Nathan C, Shiloh MU: Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *PNAS,* 97, 8841-8848, 2000. DOI: 10.1073/pnas.97.16.8841

30. De UK, Mukherjee R, Nandi S, Bhushan B, Dimri U, Verma AK: Disturbance of oxidant/antioxidant balance, acute phase response and high mobility group box-1 protein in acute undifferentiated diarrhea in crossbred piglets. *Indian J Anim Sci,* 84 (8): 860-863, 2014.

31. Huang K, Yang S: Inhibitory effect of selenium on *Cryptosporidium parvum* infection *in vitro* and *in vivo. Biol Trace Elem Res*, 90, 261-272, 2002. DOI: 10.1385/BTER:90:1-3:261

32. Yassien NA, Khalifa EA, el-Nouby KA: Role of anti-oxidant preparation (oxi-guard) on *Cryptosporidium parvum* infection in albino mice. *J Egyp Soc Parasitol*, 31, 95-106. 2001.

Kafkas Univ Vet Fak Derg
23 (3): 487-490, 2017
DOI: 10.9775/kvfd.2016.16562

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Short Communication

Comparison of Ultrasonographic Images Retrieved using Two Different Probes (Mechanical Sector and Linear Ones) and Macroscopic Features of Bovine Reproductive Organs: Biometric Studies [1]

Katarzyna Karolina PIOTROWSKA-TOMALA [1] Agnieszka Walentyna JONCZYK [1]
Mamadou Moussa BAH [1] Przemyslaw WARMOWSKI [1] Dariusz Jan SKARZYNSKI [1]

[1] *This paper was supported by the Grant in Aid from Polish National Sciences Center (DEC-2011/03/B/NZ9/01634)*

[1] Department of Reproductive Immunology and Pathology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, 10-747 Olsztyn, POLAND

Article Code: KVFD-2016-16562 Received: 22.07.2016 Accepted: 07.02.2017 Published Online: 09.02.2017

Citation of This Article

Piotrowska-Tomala KK, Jonczyk AW, Bah MM, Warmowski P, Skarzynski DJ: Comparison of ultrasonographic images retrieved using two different probes (mechanical sector and linear ones) and macroscopic features of bovine reproductive organs: Biometric studies. *Kafkas Univ Vet Fak Derg,* 23 (3): 487-490, 2017. DOI: 10.9775/kvfd.2016.16562

Abstract

Objective of the study was to assess whether there are some differences in biometric measurements of the reproductive organs using mechanical sector or linear array ultrasound probe in comparison to the macroscopic measurements. The results revealed no significant differences between ultrasonographic (USG) images in comparison to macroscopic features. High correlations between post - mortem biometric measurements of examined structures and monitored via USG in conscious animals using both probes were found (P<0.001). In conclusion, both USG systems can be effectively used as clinical and research tools in the field of examination of bovine reproductive tract status.

Keywords: *USG, Linear probe, Mechanical sector probe, Cow*

İki Farklı Prob (Mekanik Sektör ve Doğrusal Olanlar) Kullanarak Alınan Ultrasonografik Görüntülerin Karşılaştırılması ve Sığır Üreme Organlarının Makroskobik Özellikleri: Biyometrik Çalışmalar

Özet

Bu çalışmanın amacı, ineklerde üreme organlarının mekanik sektör veya linear ultrasonografi probu kullanılarak yapılan makroskopik biyometrik ölçümleri arasında bir farkın olup olmadığını karşılaştırmaktır. Çalışmadan elde edilen sonuçlar, makroskopik özellikler açısından ultrasonografik (USG) görüntüler arasında bir fark olmadığını göstermiştir. Post-mortem muayeneler ile canlı hayvanlarda yapılan muayeneler arasında, her iki USG probu ile incelenen yapılarda yüksek korelasyon bulundu (P<0.001). Sonuç olarak, ineklerde reprodüktif organların klinik muayenesinde ve bilimsel araştırmalarında her iki USG sistemi de etkin bir şekilde kullanılabilir.

Anahtar sözcükler: *USG, Lineer prob, Mekanik sektör prob, İnek*

INTRODUCTION

In veterinary practice, ultrasonography (USG) is the most profound technological advance to study changes in the ovarian morphology, including the characterization of bovine follicular waves and corpus luteum (CL) development during the estrous cycle and pregnancy [1]. The ultrasonographic examination is useful in the diagnosis of ovarian cysts and ovarian tumors in cattle [2]. Moreover, in the area of pregnancy diagnosis [3,4], fetal sex determination [4], characteristic of reproductive system disorders in cows (endometritis, pyometra), transvaginal oocyte retrieval (ovum pick up) [5], USG has proved to be particularly important technique [6,7]. Recent applications of USG in bovine reproduction includes color Doppler USG [8] and mammary gland USG [9]. Most ultrasound scanners routinely used in bovine reproduction are B-mode (brightness modality) real-time scanners, equipped with

İletişim (Correspondence)
+48 895393130
d.skarzynski@pan.olsztyn.pl

probes of varying frequencies. The commonly used frequencies in bovine reproduction are 3.5, 5.0 and 7.5 MHz, depending on the type of scanner [1]. The higher frequency probes create better USG images [6]. There are two types of probes used commonly in veterinary reproductive practice: linear (frequencies of 5-7.5 MHz) and the sector probe (frequencies of 3.5-7 MHz) [1]. The data comparing mechanical sector probe to the linear array probe are very limited [5,10].

In our study we assume that there are no differences between ultrasonographic features of bovine reproductive track structures and their macroscopic features. The purpose of present study was to compare biometric measurements of the reproductive organs using sector or linear probes against macroscopic measurements (post-mortem) in cows. The practical purpose of this study was to show the advantages or possible defects of both ultrasound scanners.

MATERIAL and METHODS

Animals

All animal procedures were approved by the Local Animal Care and Use Committee, University of Warmia and Mazury in Olsztyn, Poland (85/2012). This study was conducted in Pomerania, northern Poland during April 2014 to May 2015. Target population was consisted of Polish Holstein - Friesian cows (free of IBR/IPV, BVD/BM, EBL), which were under registration of the dairy herd improvement program, by Polish Federation of Cattle Breeders and Dairy Farmers. In the studied herd, the animals had non-seasonal reproductive programs and were bred routinely by artificial insemination. The farm had veterinary and nutrition consultants. Experimental cows (3 and more lactation) were culled from the farm because of the low milk production. The animals (n = 24) were housed in an intensive indoor barn system, milked twice a day and fed with a total mixed ration ad libitum to meet nutritional requirements of lactating cows (20-25 L per day), BCS (Body Condition Score) = 3.5.

Experimental Procedures

Comparison of ultrasonographic images retrieved using two different probes (mechanical sector and linear ones) and macroscopic features of bovine reproductive organs: biometric studies. For transrectal USG examinations two types of probes Draminski Animal Profi Scanner (Draminski Electronics in Agriculture, Poland) were used: (i) mechanical sector (3.5/5.0/7.0 MHz; 180°) and (ii) linear probe (7.5 MHz). All examined structures were imaged before animals slaughter in local abattoir (Zakłady Mięsne "Warmia" Biskupiec). Then not later than 1-h after ultrasonographic examination the genitalia were collected from slaughtered cows and transported to the laboratory within 40 min after collection. Ovaries were cut with the knife and observed in cross-section. Measurements of separated uteri were done in cross-section of the cranial tip of active uterine horn.

Statistical analysis

Data were analyzed using correlations analyses (GraphPad Prism, version 5.00; GraphPad Software). $P<0.05$ was considered significant.

RESULTS

In the experiment a representative USG images and macroscopic findings in a cross-section of the follicle, CL or uterine horn are present on *Fig. 1, Fig. 2* and *Fig. 3*. Corpora lutea (CL) were imaged in 16 ovaries, follicles (in various size) in 13 ovaries. Cysts were found in 6 ovaries, which were excluded for further correlation analyses. Uteri (n = 17) shown no pathological changes in their structures and were included for further correlation analyses. Additionally, we diagnosed endometritis (n = 5), pyometra (n = 1) and pregnancy (8-10 week; n = 1). These results were excluded for further correlation analyses. In respect to follicles *(Fig. 1),* high correlations between USG measurements of follicular diameter and assessed post-mortem were found (r = 0.88 and r = 0. 87, respectively; P<0.001). Similarly, we demonstrated correlations between USG and macroscopic measurements of the CL *(Fig. 2)* using sector or linear ultrasound probe compared to post-mortem findings (r = 0.94 and r = 0.91, respectively; P<0.001). Moreover, we found correlations between post-mortem biometric measurements and ultrasonographic images of uteri *(Fig. 3)* using sector or linear ultrasound probes (r = 0.96, r = 0.90; respectively; P<0.001).

DISSCUSION

Practical application of USG by veterinarians for reproductive organs examination is the most important method in livestock industry. Thus, the clarification of the ultrasonographic images is necessary to obtain a precision in the diagnosis of physiological and pathological ovarian structures and conditions of bovine uterus [11]. The USG examination of the ovaries and uterus in cows has been described in detail [11-13]. Moreover, previous studies have already compared the ultrasonographic features with macroscopic findings of examined structures [14]. However, in this study we compared ultrasonographic images obtained from both sector and linear probes. Therefore, practical purpose of our study was to show the advantages and similarity or possible defects of both probes.

In our experiment we found that the images of reproductive organs discernible by USG corresponded to their macroscopic features. Moreover, we determined the significant correlations between the size of examined structures measured by USG using both probes and related measurements assessed post-mortem. Similar results were

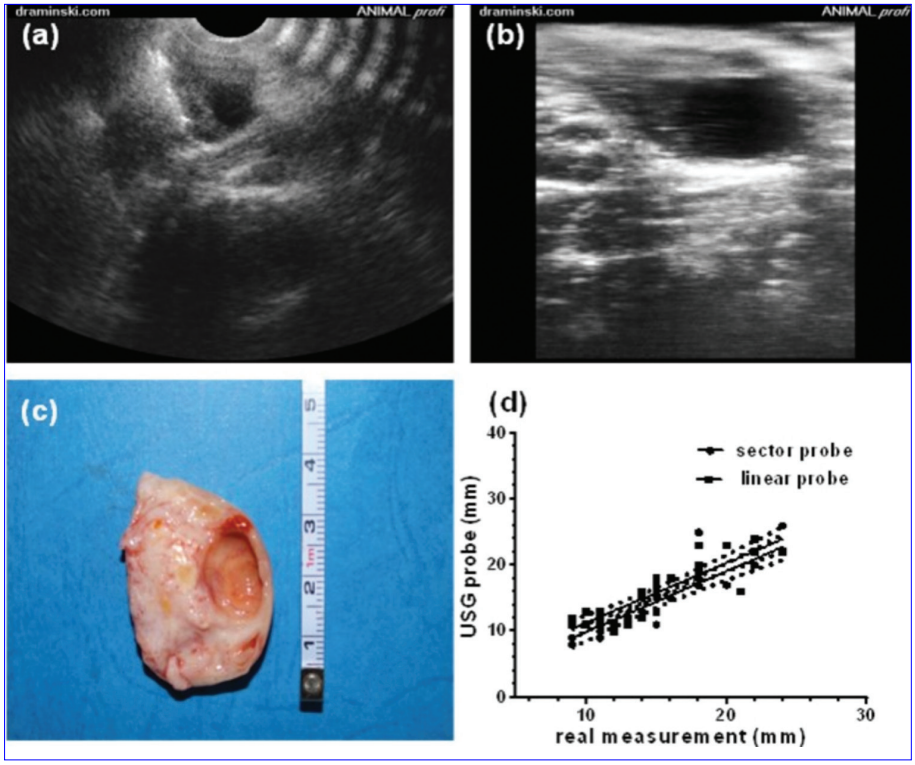


Fig 1. A representative ultrasonographic image and macroscopic finding in a cross-section of the follicle: **(a)** generated using a sector probe; **(b)** generated using a linear probe; **(c)** macroscopic finding of the follicle; **(d)** Correlation between diameter (mm) of the follicles evaluated using sector and linear probe in comparison to the real measurements (mm)

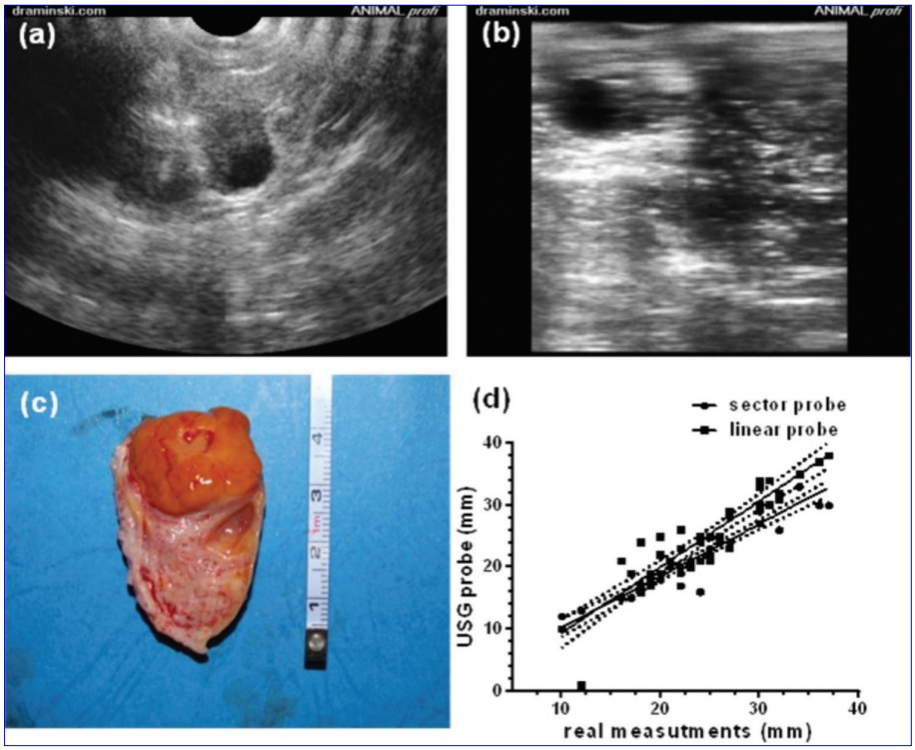


Fig 2. A representative ultrasonographic image and macroscopic finding in a cross-section of the CL: **(a)** generated using a sector probe; **(b)** generated using linear probe; **(c)** Macroscopic finding of the CL; **(d)** Correlation between diameter (mm) of the CL evaluated using sector and linear probe in comparison to real measurements (mm)

obtained by other authors who have found relationship between USG (using 5 MHz probe) and macroscopic measurements of follicles [13]. Moreover, Pierson and Ginther [13] have also reported high correlations between *in vivo* USG and post-mortem features of examined CL. In respect to the uteri similar findings were confirmed by Saito et al.[14].

In the practical purpose, both systems can be used for imagining of bovine reproductive organs. Our results showed that there were no differences between ultrasonographic features of reproductive organs achieved using both probes and their macroscopic features. Mechanical sector scanners offer multi-frequency capability, making them multifunctional and universal scanners. For early pregnancy diagnosis a 5 MHz or 7.5 MHz probe tends to provide more reliable results [3]. The linear probes using offer more detailed imaging of examined structures, which predisposes these probes for use in clinical trials (diagnosis of reproductive tract disorders). Moreover, Ribadu and Nakao [1] suggested that in routine bovine reproductive ultrasonography a 5 MHz linear rectal probe is the most effective.

In conclusion, the results of our experiment revealed no significant differences between ultrasonographic images retrieved by both probes in comparison to macroscopic features. Moreover, high correlations between post-mortem biometric measurements of examined structures and monitored via USG in conscious animals using both probes were found.

Acknowlegments

The article processing charge was covered by the grant of KNOW Consortium "Healthy Animal - Safe Food", MS&HE Decision No. 05-1/ KNOW2/2015

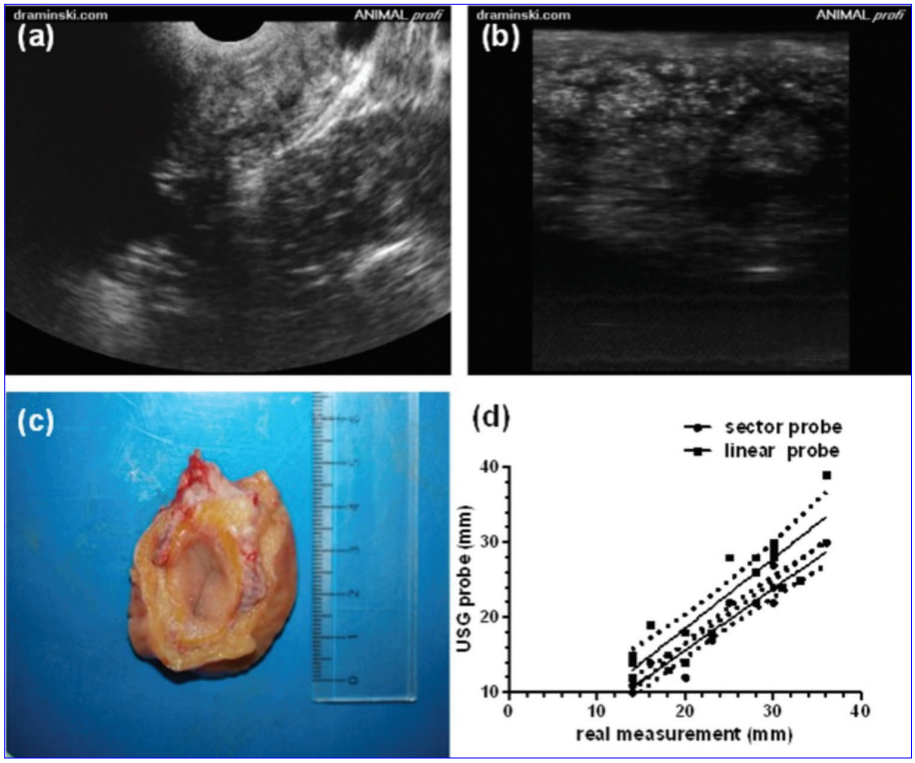


Fig 3. A representative ultrasonographic image and macroscopic finding in a cross - section of uterine horn: **(a)** generated using a sector probe; **(b)** generated using a linear probe; **(c)** Macroscopic finding of uterine horn; **(d)** Correlation between diameter (mm) of uterine horn generated using sector and linear probe in comparison to real measurements (mm)

The authors thank Maciej Baurycza, director of the animal farm in Cieszymowo, Poland for his excellent cooperation and permission to use the animals for the experiments. We also want to thank Draminski Ultrasound Scanners (DRAMINSKI S.A., Owocowa-st 17, Olsztyn, POLAND) for their excellent cooperation, encouraging us in the work and providing USG apparatus for this study.

Conflicts of Interest

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

REFERENCES

1. Ribadu AY, Nakao T: Bovine reproductive ultrasonography: A review. *J Reprod Dev*, 45, 13-28, 1999. DOI: 10.1262/jrd.45.13

2. Farin PW, Youngquist RS, Parfet JR, Gaverick HA: Diagnosis of luteal and follicular ovarian cysts in dairy cows by sector scan ultrasonography. *Theriogenology,* 34, 633-642, 1990. DOI: 10.1016/0093-691X(90)90019-P

3. Rantanen NW: General considerations for ultrasound examinations. *Vet Clin North Am: Equine Pract,* 2, 29-32, 1986.

4. Kamimura S, Kondoh S, Enomoto S, Sameshima H, Goto K, Hamama K: Ultrasonic diagnosis of bovine fetal sex by the relative location of the genital tubercle in early pregnancy. *J Reprod Dev.* 40, 4, 1994. DOI: 10.1262/jrd.40.343

5. Bols PEJ, Leroy JLMR, Vanholder T, Van Soom A: A comparison of mechanical sector and a linear array transducer for ultrasound-guided transvaginal oocyte retrieval (OPU) in the cow. *Theriogenology,* 62, 906-914, 2004. DOI: 10.1016/j.theriogenology.2003.12.016

6. Reeves JJ, Rantanen NW, Hauser M: Transrectal real-time ultrasound scanning of the cow reproductive tract. *Theriogenology,* 21, 485-494, 1984. DOI: 10.1016/0093-691X(84)90410-2

7. Ginther OJ: How ultrasound technologies have expanded and revolutionized research in reproduction in large animals. *Theriogenology,* 81, 112-125, 2014. DOI: 10.1016/j.theriogenology.2013.09.007

8. Lüttgenau J, Bollwein H: Evaluation of bovine luteal blood flow by using color doppler ultrasonography. *Reprod Biol,* 14, 103-109, 2014. DOI: 10.1016/j.repbio.2014.03.003

9. Franz S, Floek M, Hofmann-Parisot M: Ultrasonography of the bovine udder and teat. *Vet Clin North Am: Food Anim Pract,* 25, 669-685, 2009. DOI: 10.1016/j.cvfa.2009.07.007

10. Seneda MM, Esper CR, Garcia JM, Andrade ER, Binelli M, Oliveira JA, Nascimento AB: Efficacy of linear and convex transducers for ultrasound-guided transvaginal follicle aspiration. *Theriogenology,* 59, 1435-1440, 2003. DOI: 10.1016/S0093-691X(02)01188-3

11. Hanzen Ch, Pieterse M, Scenczi O, Drost M: Relative accuracy of the identification of ovarian structures in the cow by ultrasonography and palpation per rectum. *Vet J,* 159, 161-170, 2000. DOI: 10.1053/tvjl.1999.0398

12. Pierson RA, Ginther OJ: Reliability of diagnostic ultrasonography for identification and measurement of follicles and detection of the corpus luteum in heifers. *Theriogenology,* 28, 929-936, 1987. DOI: 10.1016/0093-691X(87)90043-4

13. Ribadu AY, Ward WR, Dobson H: Comparative evaluation of ovarian structures in cattle by palpation per rectum, ultrasonography and plasma progesterone concentration. *Vet Rec,* 135, 452-457, 1994. DOI: 10.1136/vr.135.19.452

14. Saito Y, Kamomae H, Tanaka T, Machida N, Kaneda Y: Correlations between ultrasonic imagines and macro-and microscopic features in the uterine horn of dairy cows. *J Reprod Dev,* 47, 1-17, 2001.

Kafkas Univ Vet Fak Derg
23 (3): 491-495, 2017
DOI: 10.9775/kvfd.2016.16706

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Short Communication

Research on the Report of Professor Rostafinski as a Sample of Scientific Cooperation in Animal Breeding in the First Years of the Republic of Turkey [1]

Ayşe MENTEŞ GÜRLER [1] Ali YİĞİT [2] Şule SANAL [3]

[1] *This study was presented as oral presentations at the 5th Congress of the Balkan Medical History and Ethics, 11-15 October 2011, Cerrahpasa Faculty of Medicine, İstanbul, Turkey*

[1] Department of Veterinary History and Deontology, Faculty of Veterinary Medicine Harran University, TR-63200 Şanlıurfa - TURKEY
[2] Department of Veterinary History and Deontology, Faculty of Veterinary Medicine Kafkas University, TR-36040 Kars - TURKEY
[3] Department of Veterinary History and Deontology, Faculty of Veterinary Medicine, Ondokuz Mayis University, TR-55139 Samsun - TURKEY

Article Code: KVFD-2016-16706 Received: 01.09.2016 Accepted: 10.12.2016 Published Online: 10.12.2016

Citation of This Article

Menteş Gürler A, Yiğit A, Sanal Ş: Research on the report of Professor Rostafinski as a sample of scientific cooperation in animal breeding in the first years of the Republic of Turkey. *Kafkas Univ Vet Fak Derg,* 23 (3): 491-495, 2017. DOI: 10.9775/kvfd.2016.16706

Abstract

Reconstructing the veterinary services was added to the agenda at the (National) Economy Congress in Izmir in 1923. Scientists from abroad were invited for their advice on animal diseases and animal breeding. Polish Professor Rostafinski was invited by Şükrü Kaya, the Minister of Agriculture during that period. Rostafinski came to Turkey in November 1924. After his researches on husbandry in Western Anatolia, he presented his opinions in a 31- page report to the Ministry of Agriculture. He advised that pedigree records of horses should be tracked, horse raising should be adopted as a government policy. For cattle, it is important to determine the needs of the peasants. This study aims to identify the husbandry conditions in that period concerning the report on the husbandry in Western Anatolia.

Keywords: *Rostafinski, Specialist report, Animal breeding, Husbandry in the Turkish Republic period, Veterinary history*

Türkiye Cumhuriyeti'nin İlk Yıllarında Hayvan Islahı Alanında Bilimsel İşbirliği Örneği Olarak Profesör Rostafinski'nin Raporu Üzerine Bir İnceleme

Özet

Cumhuriyetin ilânından sonra, İzmir'de 1923 yılında toplanan İktisat Kongresinde veteriner hekimlik hizmetlerinin de yeniden düzenlenmesi gündeme gelmiştir. Hayvan hastalıkları ve hayvan ıslahı konularına ilişkin görüşleri için yurtdışından bilim adamları davet edilmiştir. Dönemin Ziraat Vekili Şükrü Kaya tarafından davet edilen Polonyalı Profesör Rostafinski, 1924 yılı Kasım ayında Türkiye'ye gelerek Batı Anadolu'daki hayvancılık üzerinde incelemelerde bulunmuş ve görüşlerini Ziraat Vekâletine 31 sayfalık bir raporla sunmuştur. Raporda, atlar için pedigrilerinin tutulmasının, at yetiştirmenin devlet politikası olması, sığır içinse önce köylünün ihtiyacının belirlenmesi gerekliliğini gibi önerilerde bulunmuştur. Bu çalışma ile Batı Anadolu hayvancılığına ilişkin rapor temel alınarak dönemim hayvancılık alanındaki durumu belirlemek amaçlanmıştır.

Anahtar sözcükler: *Rostafinski, Mütehassıs raporu, Hayvan ıslahı, Türkiye Cumhuriyeti döneminde hayvancılık, Veteriner hekimliği tarihi*

INTRODUCTION

Between the late Ottoman and the early Turkish Republic period, husbandry, zootechnics and veterinary institutions activities became impossible under adverse circumstances because of the war [1,2]. Under these circumstances, in order to designate the economical restrictions and development procedures and principles of the newly established Turkish Republic, husbandry development and breeding subjects were evaluated at the 1st Economy Congress held in 1924. At the congress, it was emphasized on *'… takes pains over its animals as well as correcting their strains and augments*

İletişim (Correspondence)
+90 362 3121919/3362
suleo@omu.edu.tr

their quantity' under the act 8, National Economy Principles [3,4]. In addition, a five-year plan was formulated for the purpose of reconstructing the veterinary services, and it was put in practice in 1925 [1,5].

Animal breeding was detected in the curriculum of veterinary education during the Ottoman period [6,7]. During this period, it was seen that campaign activities on animal breeding and epidemic subjects were practiced. It is known that some studies were performed to build up a stud farm in Thessaloniki, a stallion warehouse in Monastery and in Kosovo in 1907, by Department of Veterinary Affairs and Animal Breeding (Islah-ı Hayvânat ve Umûru Baytâriye Şubesi), which was founded on 29th October, 1892. Along with the Proclamation of Constitutional Monarchy, animal breeding gained importance. The number of warehouses in Rumelia augmented to five, and 12 warehouses were established in Anatolia [5,8]. After the Proclamation of Republic, scientists from abroad were invited to study animal species in Turkey along with their breeding methods, and to advise on animal diseases. Within the scope of the advice of the scientists, laws and orders were imposed, whilst various studies were performed to develop animal husbandry in Turkey [5,9,10].

Prof. Jan Rostafinski (1882-1966) was one of the specialists who was invited to Turkey. He was a Polish scientist who studied animal husbandry and animal breeding and development. He was kept in captivity in Jewish prison camp [11].

In this study, it is aimed to present animal husbandry and veterinary conditions in Western Anatolia Dourineg that specific period related with the report of Professor Rostafinski.

MATERIAL and METHODS

Thirty-one pages of the report of Ministry of Agriculture Expertise Reports, Veterinary Part (Ziraat Vekâleti Mütehassıs Raporları, Baytar Kısmı) published, in 1927 forms the first chapter of the research material *(Fig. 1)*. The text was summarized whilst being translated from Ottoman Turkish to Modern Turkish, and it was evaluated through related surveyed documents and literature.

RESULTS

The report (31 pages) that Professor Rostafinski *(Fig. 2)* presented to the Ministry of Agriculture, which was written in Ottoman Turkish (240 pages) forms the first chapter of 'Ministry of Agriculture Expertise Reports, Veterinary Part'. The report begins with the clause 'A copy of the report presented by Professor Rostafinski' and states that upon the invitation received from Şükrü Kaya, term Minister of Agriculture, Rostafinski traveled to Western Anatolia for the purpose of studying and evaluating the animals in Western

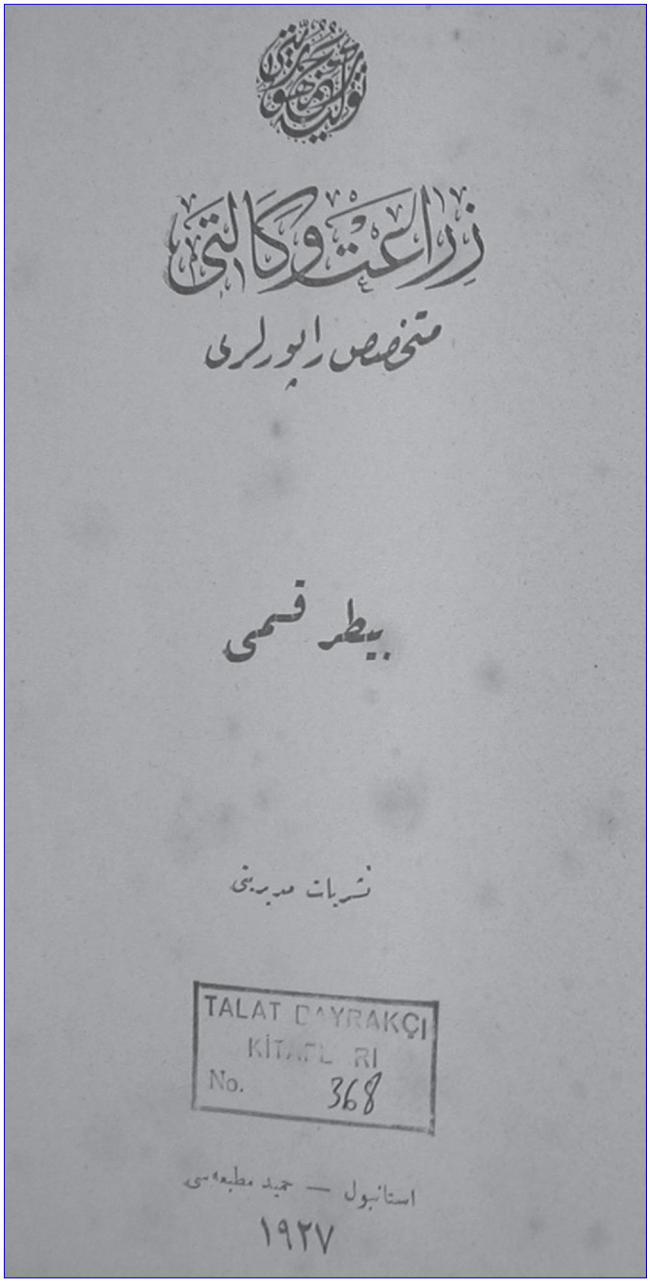


Fig 1. The first chapter of the research material

Anatolia with the help of Şefik (Kolayli), bacteriologist at Ministry of Agriculture and Mr. Yaşar, the Administrator of Agriculture in Izmir. Apart from Ankara, the journey includes stud horse warehouse in Eskisehir-Cifteler Farm, stud farm in Bursa-Karacabey, Buca-Izmir (draught animals, Izmir Agriculture School), Selcuk, Soke, Aydin, Omurlu, Denizli (stud horse warehouse), Dinar, Sandikli, Afyonkarahisar. Professor Rostafinski expressed his opinion in detail about the strains of horses, cattle and sheep. Even though he remarked that he added an animal breeding project to his report, that part does not appear in the book. In his report, Rostafinski gave wide publicity to Karacabey Farm, which was handed over to Ministry of Agriculture upon his arrival. The professor stated in his report that

Fig 2. Professor Rostafinski

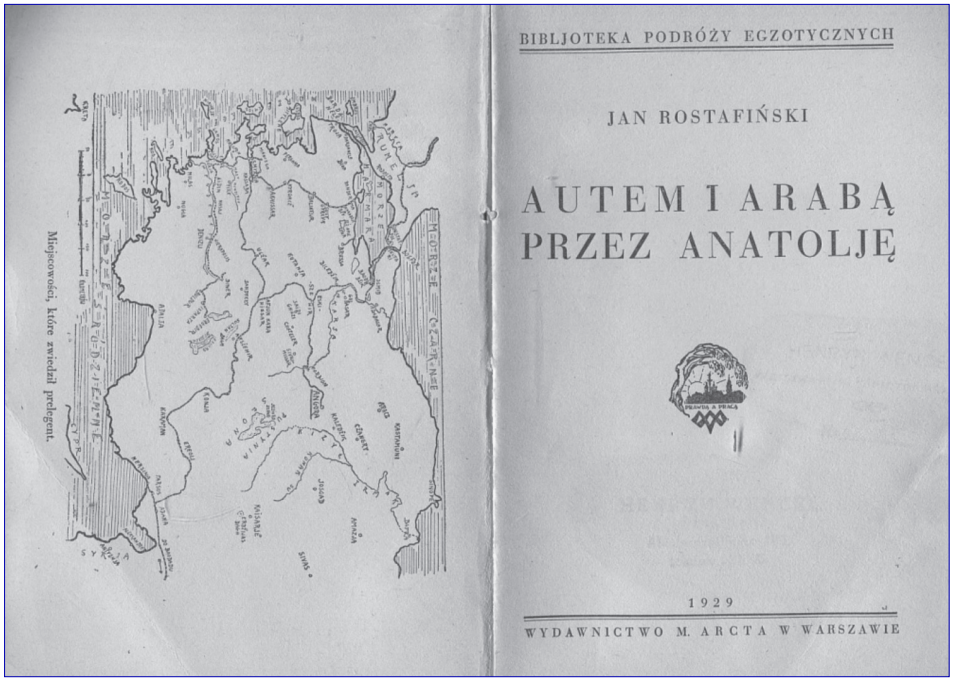


Fig 3. The book on Rostafinski's Anatolian observations

none of the animals in the Cifteler Stud Horse Warehouse had any pedigree and despite the misimplementation in the stud farm, it had a positive effect on the environment. Karacabey: Apart from the draught animals, it was stated that there were 12 studhorses, 28 mares in the warehouse, together with 7 mares, 23 studhorses and 70 foals, which were transferred from Cifteler warehouse. Since the most appropriate strain for Turkey is 'Karabag', he suggested that this strain should be bred and protected in Turkey unless it is hybridized with '*Nonius*'. He noted that the biggest problem of animal breeding was the reason that racing associations in Izmir, Istanbul and Karacabey were not subsidized. He specified that the field structure of Karacabey studfarm was suitable to be transform into a race track; and he suggested that the race horses should be bred in that place. He emphasized that if they work fast enough, Turkey could be a significant worldwide "east animal (probably Arabian horse) resource. He indicated that horse breeding should be adopted as a government policy and animal owners should be encouraged.

Suggestions of Professor for Western Anatolia region horse breeding follows as: *between 1.45 and 1.50 meters of pedigreed 'English-Arabian', 'Arabian-Karabag' or hybridized from amble stallions should be used in the coastal region. In the highlands without a pasture, Arabian or 'Arabian-Karabag' hybrids, mares or overweight stallions, with height of 1.48 should be used. Even if it is a 'Nonius' strain, it should not be imported from countries with severe climate conditions. Horse racing should be organized in the studfarms for Arabian, English and Karabag horse strains, even for short-mountain horses in Anatolia. Russian and Mongolian horses which were bred with tropical animals and have lost their pure race should not be used for breeding. Hybrids whose pedigree are not defined shall not be used for breeding. For an appropriate breeding programme, the pedigree must be determined and tracked decently.*' The qualities of broods in Çifteler Warehouse, Karacabey Studfarm and Denizli Warehouse were described one by one, with appendixes in the following part of the report about the horses. He suggested that the markers on animals should be changed so that the animals in Karacabey shall not be mixed with the animals in Çifteler farm.

He stated that the purpose was to distinguish the animals one from another. He said '*a horseshoe, with the endspointing down with a nail in the middle, which I present its drawing below can be considered as a marking. Acceptance of such marking method is important for state studhorses*'. He highlighted that the most important issue was that the stud farm had a budget and the amount determined directly from Ministry of Agriculture. He indicated that it was important that the stud farm should remain

interconnected to Ministry of Agriculture, which would help to gather the mandatory tools and materials quickly, this would prevent loss of money and time.

He argued that when it comes to cattle breeding, it is important to determine the needs of the peasants first. He stated that animal breeding in Turkey should be studied urgently on the animals at the coastal regions of Turkey first, and later on, it should be practiced on the grey and black cattle in the Central Anatolia.

In conclusion of this part of the report, he asserted that for cattle breeding and raising, Polona strain shall neither be raised nor used anymore, and this practice should stop immediately. In addition, in order to increase the population of red animals (cattle imported from Germany), it was advised to import 2-3 breeding cattle and that it is important to study and examine in Karacabey, which strain is viable for the coastal regions and Turkey's condition. He also mentioned that it is essential to study breeding the grey and black animals with 'Algav' and 'Svitch' hybrids. Moreover, it is essential to teach the peasants how to build and use a granary. He also added that the stud farms should be reformed.

He began his statement in sheep breeding chapter by expressing how sheep breeding is as important as horse breeding for Turkish economy to protect the borders. He asserted that the sheep stock in the country consists of curly-fleeced sheep, fat-tailed sheep and Karaman sheep which were imported from Rumelia. He indicated that all those strains are important for their milk, meat and fur. He remarked that there had been no considerable research for animal husbandry in Turkey; and animal husbandry studies should be carried on by the Ministry of Agriculture.

According to research, all his documents and photographs were destroyed during a bombardment in the war period [1]. However, one of his books about Turkey was published in Warsaw in 1929, which was named *'Autem i araba przez Anatolie' (Fig. 3)*.

DISCUSSION

In the evaluation section of the institution, previously called Çifteler Studhorse but later evacuated because of the incidence of Glanders and Dourine disease [12], and named Çifteler Stallion Ware House, he stated that despite the misimplementation of the stud management, the horses of villagers were in good condition. After 10 years, when invited for a related subject, the expert Professor Welleman emphasized in his report to the Ministry of Agriculture that Çifteler Studhorse made a positive impact on Turkish horse-breeding in 1934 [2,13].

In the assessment report of a 10-year study on animal husbandry in the Republican period [14], the other experts who were invited previously and Professor Rostafinksi were not mentioned; however, the subject about identification of races, which was also mentioned in his report, had been referred to. Professor made vital recommendations on how to improve presence of animals in future of Turkey. It can be said that in the experts' reports which were considered Dourineg the implementation of the Republic development policy [10], and in the above-mentioned report [13] conflicts with these recommendations.

His indication that the breeders should be encouraged through exhibitions and competitions in animal breeding, which highlights the impact of rewarding in good and quality animal husbandry by referring to the current situation can be accepted as an important guidance to be effective in animal breeding studies. In addition, considering an active participation of breeders on animal breeding, his suggestion on supporting the relevant non-governmental organizations - only if they are inspected - can be considered as an another dimension.

Professor Rostafinski offered to use a special marker for Karacabey Stud animals which was not used in European studs and stated that accepting such kind of marker is very important for a state stud. Professor indicated that he suggests a figure for this marker in his report However, it is not determined either in his report or in his studies.

In conclusion, before Professor Rostafinski's report, any scientific or actual study on the subject is not detected. It can be said that the report is important from the perspective of providing information about the origins and phenotypes of the animals in the related period. Also, when it is reviewed as the quality and quantity of Western Anatolia animals, it can be expressed as an important historical source.

[1] *E-mail correspondence with the grandson of Prof. Rostafinski in 2012*

REFERENCES

1. Dinçer F: Türk veteriner hekimliğinin 139. yılında Atatürk dönemi tarımsal politikası. *Ankara Üniv Vet Fak Derg (Atatürk'ün 100. Doğum yılı Özel Sayısı),* 28 (1-4): 307-310, 1981.

2. Yaşar A: Osmanlı Döneminde ve Cumhuriyet Tarihinde Haralar. *Doktora Tezi,* Ankara Üniv. Sağlık Bil. Enst., 1996.

3. İnan A: İzmir İktisat Kongresi. 17 Şubat-4 Mart 1923. Atatürk Kültür, Dil ve Tarih Yüksek Kurumu Türk Tarih Kurumu Yayınları XVI. Dizi, 2. Baskı, Türk Tarih Kurumu Basımevi, Ankara, 1989.

4. Ökçün G: Türkiye Kongresi 1923- İzmir Haberler- Belgeler- Yorumlar. A.Ü. Siyasal Bilgiler Fakültesi Yayın No: 262, 2. Baskı, Sevinç Matbaası, Ankara, 1971.

5. Ali Rıza: Beş Senelik Umur-u Baytariye Programı. Okut Matbaası, Ankara, 38 s. 1924.

6. Erk N, Dinçer F: Türkiye Veteriner Hekimlik Öğretimi ve Ankara Üniversitesi Veteriner Fakültesi Tarihi. Ankara Üniversitesi Veteriner Fakültesi Yayınları: 259, Ankara, 1970.

7. Godlewsky und Sommer: Türkiye'de Veteriner Hekimlik (19'uncu Yüzyıl Ortalarında). (Çev: N. Erk) Ankara Üniversitesi Veteriner Fakültesi Yayınları: 281, Ankara Üniversitesi Basımevi, Ankara, 1972.

8. Dinçer F: Türkiye'de Bilimsel veteriner hekimliğin başlaması, evreleri ve ilim tarihimizdeki yeri. *Ankara Üniv Elazığ Vet Fak Derg*, 2 (1): 81-91, 1975.

9. Bekman M: Veteriner Tarihi. Ankara Basım ve Cildevi, Ankara, 1940.

10. Osmanağaoğlu Ş, Melikoğlu B: Hayvancılık ve veteriner hekimlik alanlarında Türkiye ve Macaristan arasındaki ilişkilere bir bakış. *Türk Vet Hek Bir Derg*, 12, 3-4, 2012.

11. Rolniczy Magazyn Elektroniczny: Jan Rostafiński - profesorski ród. http://www.cbr.edu.pl/rme-archiwum/2006/rme15/rme.php?strona=48; *Accessed:* 05.04.2016.

12. Batu S: Türkiye'de Yetiştirme Çalışmaları ve Yetiştirme Kurumları. Ankara Üniversitesi Veteriner Fakültesi Yayınları, No: 74, Ankara, 1955.

13. Menteş Gürler A, Osmanağaoğlu Ş: Cumhuriyet'in İlk 10 yılının Veteriner Hekimlik Açısından Değerlendirilmesi: II. Kurumlar. *II. Ulusal Veteriner Hekimliği Tarihi ve Mesleki Etik Sempozyum Kitabı, 24-26 Nisan,* Konya, Türkiye, 2008.

14. T.C. Başbakanlık Cumhuriyet Arşivi: Cumhuriyet döneminde yapılan haralar, ahırlar ve hayvan hastalıkları ile mücadele merkezleri hakkında hazırlanan raporlar, grafikler ve fotoğraflar. Belge No: 030.1 0.187.285.1, 110 s. 1946.