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Effects of vitamin B_1, vitamin E and selenium on pregnancy and blood metabolites profile during non-breeding season and early prediction of pregnancy by thermographic monitoring in Merino ewes

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ABSTRACT

The objective of this study was to evaluate the effects of injectable selenium, vitamin E and vitamin B_1 compounds on serum metabolic profile and pregnancy rates (PR) during the non-breeding season in Merino ewes that were synchronized with progesterone and equine chorionic gonadotrophin (eCG). Thermographic monitoring of ewes on the last day of synchronization was successful in the early selection of ewes to be used in artificial insemination and its relationship with pregnancy outcomes. Cyclic and non-pregnant ewes (n=75) were randomly assigned to treatment (n=30) and control (n=45) groups. All animals were synchronized using $PGF_2\alpha$ at day 0, progesterone impregnated intravaginal sponges for 14 days, eCG 48 h before sponge withdrawal and artificial insemination were carried out 43–45 h after sponge removal. Animals of treatment group (n=30) were administered (intramuscularly) with 1 mg sodium selenite, 60 mg vitamin E and 40 mg vitamin B_1 on two occasions, at the beginning of sponge insertion and following sponge removal. Blood samples were collected at sponge insertion and removal. Perianal and perivulvar areas were considered to evaluate the thermal pattern at day 0 and 14 using a thermo camera. Overall, 90.6% of ewes exhibited estrus in response to the synchronization protocol. Ewes of treatment group showed greater PR as compared to that of control group (70.3% vs 56%). Perivulvar temperature was influenced by time only. At the time of sponge removal, perivulvar temperatures were significantly lower in pregnant ewes than in non-pregnant animals. In conclusion, injectable selenium, vitamin E and vitamin B_1 compounds increased PR after synchronization protocol and thermographic evaluation that was performed on the last day of synchronization could be used for very early prediction of pregnancy.

Keywords: Ewe, Synchronization, Thermography

The aim of sheep breeding is lambing three times in two years (Benoit *et al.* 2019). Thus, inducing ovulation out of breeding season would provide the opportunity to achieve the goal. To increase productivity, one of the successful and practical assisted reproductive techniques is induced ovulation out of breeding season (Martinez *et al.* 2015). Commonly used oestrus synchronization method is the administration of intravaginal sponges containing synthetic progesterone for 11 to 14 days using equine chorionic gonadotrophin (eCG) at the removal of sponge. However, the results of this protocol can be varied (Martinez-Ros and Gonzalez-Bulnes 2019).

Trace elements and vitamins are essential in reproduction and have a role from steroidogenesis to metabolism (Awawdeh *et al*. 2015). Trace mineral and vitamin deficiencies in diet or in the uptake impairs health status and declines ovulation rate especially in the case of sheep fed on the pasture (Abdollahi *et al*. 2015). It is known that intravaginal sponges are the sources of oxidative stress (Sonmez *et al.* 2009). Thus, using antioxidants during synchronization with intravaginal sponges may prevent oxidative stress and increase fertility (Kuru *et al.* 2017).

Thermography has been used in animal reproduction for evaluation of breeding soundness, udder soundness, determination of pregnancy, estrus detection or deciding of the artificial insemination time in cows, mares, ewes, goats, bitches, and pigs, for a decade (Sykes *et al.* 2012, Talukder *et al.* 2014).

Considering all this information, we hypothesized that use of trace elements and vitamin supplementation during non-breeding season may increase the response to synchronization treatment as well as increase fertility without affecting metabolism negatively. In this research, we aimed to evaluate the effects of injectable selenium, vitamin E and vitamin B_1 compounds on serum metabolic profile and pregnancy rates during the non-breeding season

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in Merino ewes that were synchronized with progesterone and eCG. Besides, we tried to find out whether the thermographic monitoring of ewes on the last day of synchronization was successful in the early selection of ewes to be used in artificial insemination and its relationship with pregnancy outcomes.

MATERIALS AND METHODS

Animals and housing: The present study was conducted in Ankara, Turkey, using a flock of 850 Merino ewes during April-May 2018. Seventy-five adult, cyclic and non-pregnant Merino ewes, aged between 2 and 4 years, were selected from the flock. The animals had *ad lib.* access to drinking water and fed on the pasture during the study period. The animals were housed in a well-ventilated shed in east-west orientation at nights. The roof of the shed was made up of galvanizing sheet, with all the sides of the shed kept open with wooden fence and the floor was made with clay sand. Vaccinations were carried out as prescribed against sheep diseases, viz. foot and mouth disease, sheep pox, enterotoxaemia; and preventive measures were adopted for endo-and ecto-parasitic infestations. All animals were in a healthy condition throughout the study. The experimental animals were kept isolated from other animals of the flock including rams.

Experimental design: Ewes were randomly assigned to treatment (n=30) and control (n=45) groups. Randomization was completed in Excel (Microsoft, Redmond, WA) using random number function, data imported from the farm. Estrus synchronization protocol was applied to animals of both groups, which consisted of intramuscular injection of 125 µg $PGF_2\alpha$, which was followed by insertion of intra-vaginal sponge containing 20 mg progesterone on day 0 and it was placed for 14 days. At day 12, 400 IU eCG was administered intramuscularly. Ewes were inseminated artificially between 43 and 45 h after the sponge removal. Animals of treatment group (n=30) were administered (intramuscularly) with 1 mg sodium selenite, 60 mg vitamin E and 40 mg vitamin B_1 on two occasions, at the beginning of sponge insertion and following sponge removal. Control ewes did not receive any trace element or vitamin treatment.

Blood samples were collected twice, at sponge insertion and removal with venipuncture from the jugular vein into vacutainer tubes with anticoagulant lithium-heparin. Samples were placed on ice until processing. One hour after collection, blood samples were centrifuged at 13,000 g at 4ºC for 10 min. Then plasma was separated and collected into 2 ml tubes and frozen at –20ºC until analysis. Plasma samples were analyzed for the determination of the concentration of calcium, total protein, urea, glucose, cholesterol by using commercial kits following manufacturer instructions with an autoanalyzer (ERBA XL 600®) in Faculty of Veterinary Medicine Diagnostic Laboratory, Ankara University. All analyses were calibrated by using ERBA XL Multical and calibration verified using two control serum (ERBA Norm® and ERBA PATH®).

Thermographic monitoring was performed at day 0 and 14, before sponge insertion and removing process by the thermographic camera (FLIR®, E60, USA). Thermographic images were taken twice for each ewe, as both perianal and perivulvar regions were in the same frame, at a distance of 1–1.5 m away from the regions. The perceived temperature of the thermographic camera was set in the range of 15–45°C. Images were analyzed by software (FLIR®, ResearchIR v4.20, USA) to detect average perianal and perivulvar temperatures. Differences in the averages between regions were calculated as ΔT.

On the day of artificial inseminations, semen was collected from three rams by the electro-ejaculation method and kept in a water bath (37°C) until semen analyses were completed. Semen was evaluated in terms of volume, pH, and colour, macroscopically; mass motility, progressive motility, and concentration, microscopically. Immediately after analyses, semen samples with desired characteristics were pooled and extended with TRIS/glucose/citric acid extender to obtain a final concentration of 500×10^6 spermatozoa/ml. One ml of extended semen was used for artificial insemination between 43 and 45 h after the sponge removal in each ewe. Deep vaginal artificial inseminations were performed with the help of vaginal speculum, rigid catheter, and syringe.

Fertility: Each ewe that showed clinical sign of oestrus was inseminated. Thirty days after inseminations, ewes were examined by transrectal ultrasonography to identify the presence of an embryonic heartbeat. The transrectal ultrasonographic examination was performed using a linear probe with a frequency of 6.5–9 MHz (SIUI®, CTS 800, China). All pregnant ewes were re-examined 60 days after the last insemination to determine the pregnancy loss.

Statistical analyses: Before performing the statistical analysis, data were examined with Shapiro-Wilk test for normality and Levene test for homogeneity of variances as parametric test assumptions. Descriptive statistics for each variable were calculated and presented as mean±SEM. All data were analyzed using the MIXED procedure of SPSS (V22.0; SPSS Inc., Chicago, IL, USA). The effect of group, sampling time and their interaction on calcium, total protein, urea, glucose, and cholesterol concentrations as well as perianal, perivulvar temperatures and DT were analyzed using the following model with repeated measures:

$$Y_{ijk} = \mu + G_i + D_j + (G\times P)_{ij} + e_{ijk}$$

where Y_{ijk} was the dependent variable, μ was the overall mean, G_i was the effect of the group (i, treatment ewes and control ewes), D_j was the effect of the sampling time (j, the day of sponge insertion and removal), $(G\times D)_{ij}$ represented interaction between group *i* and sampling time j, and e_{ijk} was the residual error.

Another model was evaluated for perianal, perivulvar temperatures and ΔT to analyze the effect of difference within the temperatures before and after the synchronization in ewes that subsequently pregnant and their interaction.

$$Y_{ijk} = \mu + G_i + P_j + (G\times P)_{ij} + e_{ijk}$$

where Y_{ijk}, is a dependent variable, μ is the overall mean, G_i is the effect of the group (i, pregnant and non-pregnant ewes), P_j measures the effect of period (j, the day of sponge insertion and removal), $(G \times P)_{ij}$ is the interaction between group and period, and e_{ijk} is used for residual error.

Animals within group were assessed as a random effect, while group, period, or day of sampling and their interaction were assessed as a fixed effect. $P<0.05$ was considered as significant in all analyses. When a significant difference was revealed, any significant terms were compared by simple effect analysis with Bonferroni adjustment.

RESULTS AND DISCUSSION

Most of the sheep breeds are seasonally polyestrous animals. Hormone treatments are used to induce estrus as well as ovulation between two breeding seasons. Many factors have a role in this process; such as applied methods, type and doses of hormones. But flock level factors are determining the success of the synchronization process. Metabolic factors as energy, protein and mineral metabolism, also oxidative stress and immune function directly alter the fertility parameters of synchronized sheep (Kuru *et al.* 2017).

Overall, 90.6% of ewes exhibited estrus in response to the synchronization protocol. The interval from device removal and estrus signs were 21±0.7 h and 20.8±1 h for treatment and control groups, respectively. There was significant difference in pregnancy rates, which was recorded as 70.3% and 56% for treatment and control ewes, respectively. Induction of estrus was similar among groups, as reported by Kuru *et al.* (2017). However, pregnancy outcomes were completely different in the present study. Ewes that were supplemented with trace elements and vitamin combinations showed greater pregnancy rate in comparison to other studies (Gabryszuk and Klewiec 2002, Kose *et al.* 2013, Kuru *et al.* 2017). The significant difference in pregnancy rates between treatment and control group in the present study might be attributed to the role of trace elements and vitamins on the ovarian level. Parenteral injections of trace elements and vitamin before mating increase possibility of multiple lambing (Mudd and Mackie 1973) probably by affecting the follicle development and embryonic growth. Antioxidants inhibit free radical formation, and thus, interrupting the propagation of free radicals by different mechanisms (Brewer 2011). Possibly, these mechanisms prevented cellular damage by free radicals during stressful conditions in the present study.

The results of present study support the findings of Koyuncu *et al.* (2006) who reported that nutritional factors such as protein, energy and calcium intake might influence the pregnancy rate. Serum calcium concentrations were in the physiological range in ewes during the sampling period. Group and time was influenced by calcium concentrations. Also, group × time interaction was noted for calcium levels. Ewes that were supplemented with trace mineral and vitamins at sponge insertion had greater calcium concentration at sponge removal. There was no significant interaction noted for total protein and urea concentrations in both groups. Cholesterol and glucose concentration was influenced by time only. There was no significant group × time interaction for cholesterol and glucose concentrations (Table 1). There are different information about glucose concentration in ewes. In the present study, glucose concentration increased; however, there was no significant difference among groups. The reason for underlying this increase is probably a result of the induction of estrus. The glucose concentration increases during follicular phase compared to the luteal phase after intra-vaginal sponge application in ewes (Wani *et al.* 2018). Cholesterol metabolism also changed over time in both groups. Murthy and Shipp (1981) reported that cholesterol concentration was non-significantly higher during estrus. Cholesterol is a key component of steroid hormones. During the estrus phase the production of estrogen increases which depended on cholesterol level and results in the manifestation of heat (Singh *et al.* 1983).

Body temperature, determined via perianal thermographic monitoring, is noticed by almost 1.4°C above from vulvar temperature in the ovarian inactivity period (Table 2). Moreover, on the last day of the synchronization

Table 1. Effects of injectable vitamin B_1, vitamin E and selenium on serum calcium, cholesterol and glucose concentrations before and after synchronisation (Mean±SEM)

Parameter	Group	Time		Est. Marginal	P value		
		Sponge insertion	Sponge removal	Means	Group	Time	G×T
Calcium	Control	8.534 ± 0.216^{aB}	7.003 ± 0.216^{b}	7.769±0.153	0.006	<0.001	0.003
	Treatment	9.962 ± 0.267^{aA}	6.938 ± 0.267^{b}	8.45±0.188			
Est. Marginal Means		9.248±0.172	6.971±0.172				
Cholesterol	Control	22.688±3.073	44.806±3.073	33.747±2.331	0.811	<0.001	0.908
	Treatment	21.429±3.794	44.286±3.794	32.857±2.877			
Est. Marginal Means		22.058 ± 2.441^{a}	44.546 ± 2.441^{b}				
Glucose	Control	32.622±3.468	54.163±3.468	43.392±2.686	0.149	<0.001	0.230
	Treatment	22.143±4.281	52.148±4.281	37.145±3.316			
Est. Marginal Means		27.382 ± 2.754^{a}	53.155 ± 2.754^{b}				

Means within a row (a-b) and column (A-B) with different superscript letters differ significantly (P<0.05).

Table 2. Effects of injectable vitamin B_1, vitamin E and selenium on the perianal, perivulvar temperature and Delta before and after synchronisation (Mean±SEM)

Parameter	Group	Time		Est. Marginal	P-value		
		Sponge insertion	Sponge removal	Means	Group	Time	G×T
Perianal Temp.	Control	38.768 ± 0.083^{bA}	39.080 ± 0.085^{a}	38.924±0.072	0.024	<0.001	0.006
	Treatment	38.320 ± 0.103^{bB}	39.014 ± 0.100^{a}	38.667±0.087			
Est. Marginal Means		38.544±0.067	39.047±0.066				
Perivulvar Temp.	Control	37.269±0.118	36.820±0.120	37.044±0.107	0.432	<0.001	0.673
	Treatment	37.104±0.145	36.724±0.141	36.914±0.129			
Est. Marginal Means		37.186 ± 0.094^{a}	36.772 ± 0.093^{b}				
ΔT	Control	1.504 ± 0.086^{bA}	2.264 ± 0.088^{a}	1.884±0.076	0.243	<0.001	0.019
	Treatment	1.207 ± 0.107^{bB}	2.283 ± 0.103^{a}	1.745±0.092			
Est. Marginal Means		1.356±0.069	2.274±0.068				

Means within a row (a-b) and column (A-B) with different superscript letters differ significantly (P<0.05).

Table 3. Perianal, perivulvar temperature and ΔT before and after synchronisation in pregnant and non-pregnant ewes (Mean±SEM)

Parameter	Group	Time		Est. Marginal	P value		
		Sponge insertion	Sponge removal	Means	Group	Time	G×T
Perianal Temp.	Non-pregnant	38.639±0.098	39.115±0.099	38.877±0.084	0.388	<0.001	0.840
	Pregnant	38.554±0.091	39.002±0.090	38.778±0.077			
Est. Marginal Means		38.596 ± 0.067^{B}	39.059 ± 0.067^{A}				
Perivulvar Temp.	Non-pregnant	37.272 ± 0.134^{a}	37.005 ± 0.134^{bA}	37.139±0.121	0.106	<0.001	0.039
	Pregnant	37.147 ± 0.124^{a}	36.593 ± 0.123^{bB}	36.870±0.111			
Est. Marginal Means		37.209±0.091	36.799±0.091				
ΔT	Non-pregnant	1.364 ± 0.099^{a}	2.110 ± 0.099^{bB}	1.737±0.087	0.150	<0.001	0.047
	Pregnant	1.407 ± 0.091^{a}	2.409 ± 0.091^{bA}	1.908±0.079			
Est. Marginal Means		1.386±0.067	2.259±0.067				

Means within a row (a-b) and column (A-B) with different superscript letters differ significantly (P<0.05).

that might be accepted as the beginning of the oestrus cycle, temperature differences between body and vulvar heat increased up to 2.4°C average (P<0.05). Decrease in the vulvar heat could be explained as the preparation process for oestrus such as differentiation of the vascularization in genitalia, alteration of blood flow, vulvar edema, etc. Besides this, mainly and statistically important finding of the study is that the ewes with pregnancy had 0.3–0.4°C lower temperatures than ewes without pregnancy on the last day of the oestrus (P<0.05; Table 3). These results indicate that it is possible to select suitable ewes for insemination or mating, two or three days before the processing time. Previous works of literature have shown pregnancy prediction by thermal monitoring is made in oestrus, post-oestrus and insemination or mating period (Simoes *et al.* 2014, Talukder *et al.* 2014, Stelletta *et al.* 2017). Although there are many works on artificial insemination timing or estrus detection with the thermographic imaging method, there is paucity of information on early selection of suitable or unsuitable animals for breeding. This study has indicated that thermographic evaluation that is performed on the last day of synchronization could be used for very early pregnancy prediction. Early detection and elimination of unsuitable ewes would prevent workers and owners from loss of time, cost and labour.

In conclusion, injectable vitamin B_1, vitamin E and selenium composition increased pregnancy rate during the non-breeding season in Merino ewes that were synchronized with progesterone and eCG; and thermographic evaluation on the last day of synchronization could be useful tool for very early pregnancy prediction.

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