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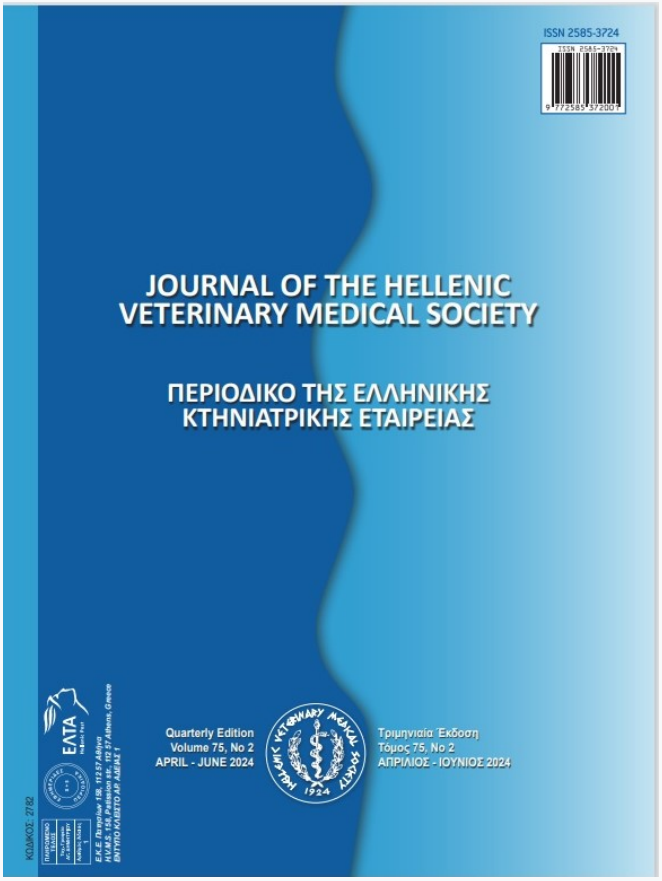

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Research article
Ερευνητικό άρθρο

Concentrations of plasma estradiol, progesterone, melatonin, serotonin and nitric oxide in Tuj ewes after estrus syncronization in and out of breeding season

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ABSTRACT: This study aimed to determine and compare the concentrations of progesterone, estradiol, melatonin, serotonin and nitric oxide in the blood plasma after induced estrus in and out of the natural breeding season in Tuj breed ewes. In addition, seasonal alteration in serotonin levels after induced estrus in ewes were examined for the first time. A total of 20 Tuj ewes were used in the study. The ewes were synchronized in the breeding (n=10) or non-breeding season (n=10) by using an intravaginal progestin-containing sponge (60 mg medroxy-progesterone acetate) for 12 days. Prostaglandin F2α (PGF2α, 2,5 ml, i.m.) plus equine chorionic gonadotropin (eCG 500 IU, i.m.) were injected at sponge withdrawal. The first blood samples were collected immediately after removal of the intravaginal sponge (at 0 hour) and the rest was collected at 4-hour intervals between 24 and 64 hours. Throughout both seasons, all animals displayed estrus. However, estrus appeared earlier in the breeding season than in the non-breeding season. Plasma progesterone, estradiol, nitric oxide and melatonin concentrations were higher ($P<0.05$) in the breeding season compared to the non-breeding season. A positive correlation was recorded between estradiol and progesterone (r=0.685; $P<0.05$) or nitric oxide (r=0.535; $P<0.05$). However, the plasma serotonin concentration was higher ($P<0.05$) in the non-breeding compared to breeding season and showed a positive correlation with melatonin (r=0.671; $P<0.05$). In addition, significant differences were noted between day and night concentration of melatonin and serotonin hormones in both seasons ($P<0.05$). In conclusion, the results show seasonal differences in the secretion of the ovarian steroid hormones, nitric oxide, melatonin and serotonin, after induced estrus, which suggests a different reproduction capacity in the two seasons. Seasonal differences in the profile of nitric oxide release during estrus may be related to the estradiol secretion.

Keywords: *Breeding season; Non-breeding season; Hormones; Nitric Oxide; Tuj ewes*

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INTRODUCTION

Tuj ewes, which are acclimated to high (1500-2000 m) and hilly terrain, are a regional ewe species unique to the Northeast of Turkey (Saatci et al., 2003; Kaya et al., 2013). Photoperiod is the most critical factor regulating reproduction and sexual activity in seasonally estrus ewes and goats (Cevik and Yurdaydın, 2001). They typically enter estrus period in the autumn and winter months, when days are shorter, and enter anestrus period during spring and summer months, when days are longer (Gordon, 1997). For this reason, estrus synchronization is performed with natural or hormonal methods to increase yield in ewes that are infertile most of the year. Progesterone and its derivatives have been used to synchronize and induce estrus in sheep outside of the breeding season since the 1960s (Powell et al., 1996). In particular, progestogen intravaginal sponges are very often used to synchronize estrus and ovulation, both in and out the natural breeding season (Romano et al., 2000; Hashemi et al., 2006).

The non-breeding season is associated with a lower fertilization rate and increased embryo mortality compared to the breeding season (Ritar et al., 1994; Gordon, 1997). This, can be due to the hormonal release difference between the reproductive control mechanisms of the two seasons. Therefore, it is important to ascertain and evaluate hormonal and chemical differences during induced estrus throughout both the breeding and non-breeding season. Most studies on estrus synchronization in small ruminants are focused on the profiles of ovarian steroids and gonadotropin release (Van Cleeff et al., 1998; Menegatos et al., 2002; Swellum et al., 2015). However, there are many hormonal or chemical components that affect the estrus and ovulation processes directly or indirectly. According to the findings of studies conducted in females of human and animal species, nitric oxide (Roselli et al., 1998; Dixit and Parvizi, 2001), melatonin (Kumar and Purohit, 2009), and serotonin (Battista and Condon, 1986; Payne et al., 1994) hormones impact ovarian steroidogenesis, ovulation, and corpus luteum function. The fact that these hormones are secreted concurrently with steroid hormones in small ruminants suggests that they regulate reproductive seasonality. Furthermore, whereas the annual pattern of melatonin (Chemineau et al., 1992) and serotonin (Le Corre and Chemineau, 1993) release is well known, little is known about the rhythm during peri-estrus and estrus. However, it is unknown whether a correlation exists between the secretion of melatonin, serotonin, and nitric oxide and steroidogenic activity in ewes. In this study, we aimed to determine and compare the alterations in the plasma concentrations of estradiol, progesterone, melatonin, serotonin and nitric oxide in Tuj ewes after induced estrus in breeding and non-breeding season. Thus, the relationship between the releases of these hormones during the peri-ovuluary during breeding and non-breeding period will be examined.

MATERIALS AND METHODS

This study was carried out after approval from Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK/2019-060).

Animals and experimental design

The experiment was conducted on Tuj ewes at the Faculty of Veterinary Medicine of Kafkas University, Prof. Dr. Ali Rıza AKSOY Education Research and Application Farm in the province of Kars, at an altitude of 1751 m and at latitude and longitude of 40°34’23” N - 43°02’27” E, respectively. The ewes grazed outside on fresh pasture from March to November. Only during the synchronization and blood collection procedures they were maintained in the closed barn system. Along with hay, the ewes were given barley at that period. Throughout the trial, water and mineral licks were provided *ad libitum*.

Nonlactating Tuj ewes between the ages of 1.5 and 2.5 years, with body condition score ranging from 2 to 3 (1: extremely thin and 5: obese) were used. The experiment was conducted in the natural photoperiod environment during the natural breeding season (October; n=10) and non-breeding season (May; n=10). During the breeding season, daylight was generally between 06:30 a.m. and 17:30 p.m. In the non-breeding season however, it was approximately between 05:00 a.m. and 19:30 p.m. Estrus synchronization was performed by insertion of intravaginal sponges containing 60 mg of medroxy-progesterone acetate (MAP, Esponjavet®, Hipra Animal Health, Spain) for 12 days. On the 12th day, sponges were removed, and 500 IU of equine Chorionic Gonadotropin (eCG-Oviser®, Hipra Animal Health, Spain) and 2.5 mL PGF2α (Dinoprost Tromethamine, Dinolytic®, Zoetis, ABD) were intramuscularly injected.

Blood Sample Collection

Blood samples were collected from the vena jugularis into vacuum heparinized test tubes. The first blood samples (0 h) were collected immediately after

intravaginal sponge removal (at 12:00 noon), the second 24 hours later, and the rest 10 samples at 4-hour intervals. Dark period blood sampling was performed under dim red light. The dark hours measured were between 17:30 a.m. and 06:30 p.m. [approximately after the sponge was removed, the 1st dark phase (16h) was between 28 and 44 hours, and the 2nd dark phase (12h) was between 52 and 64 hours] in the breeding season and between 19:30 a.m. and 05:00 p.m. [after removing the sponge, the 1st dark phase (12h) was between 32 and 44 hours, and the 2nd dark phase (12h) was between 52 and 64 hours] in the non-breeding season. The blood sampling was completed 64 hours after sponge removal and eCG + PGF2α injection. The blood plasma was kept at -20°C until it was examined for biochemical parameters and hormones. All assays were carried out in duplicate.

Twenty-four hours after the sponge withdrawal, two aproned rams were entered to the synchronized ewes, and estrus detection was performed at 4-hour intervals. The ewes in which the rams demonstrated sniffing and jumping responses were marked as estrous. Plasma samples from 7 animals in each season exhibiting the earliest and strongest estrus indications were collected and analyzed.

Blood analysis

Plasma progesterone (YL Biotech, measuring range 0.05-15 ng/mL with a precision of 0.027 ng/mL Shanghai, China), estradiol (Bioassay Technology Laboratory, measuring range 1-300 ng/L with an accuracy of 0.52 ng/L Shanghai, China), melatonin (Bioassay Technology Laboratory, measuring range 3-700 ng/L with a sensitivity of 1.52 ng/L Shanghai, China) and serotonin (Bioassay Technology Laboratory, measuring range 5-600 ng/mL with a sensitivity of 2.62 ng/mL Shanghai, China) concentrations were measured at 450 nm using commercial ELISA kits.

Plasma nitric oxide concentrations was determined colorimetrically by the method reported by Miranda et al. (2001). Nitrate was reduced to nitrite by vanadium (III) chloride. The colored complex diazonium compound formed as a result of the reaction of reduced nitrite and sulfanilamide with N-(1-naphthyl) ethylenediamine dihydrochloride in acidic medium, was measured at 540 nm. The nitrite and nitrate concentrations were determined separately from the standard curve obtained using sodium nitrite ($NaNO_2$, Merck) and sodium nitrate ($NaNO_3$, Merck), respectively, and the sum of the nitrate and nitrite concentrations represented the amount of nitric oxide (μmol/L).

Statistical analysis

The data were analysed using the SPSS for Windows 20.0 statistical software package. For each of the parameter evaluated, mean values and standard deviations were computed. The Friedman test was used within the season and the Mann Whitney U test was used between the seasons. The Spearman test was used to calculate the correlation coefficients between the two seasons' findings. Significant results were defined as those with a $P<0.05$ value (Tekin, 2003).

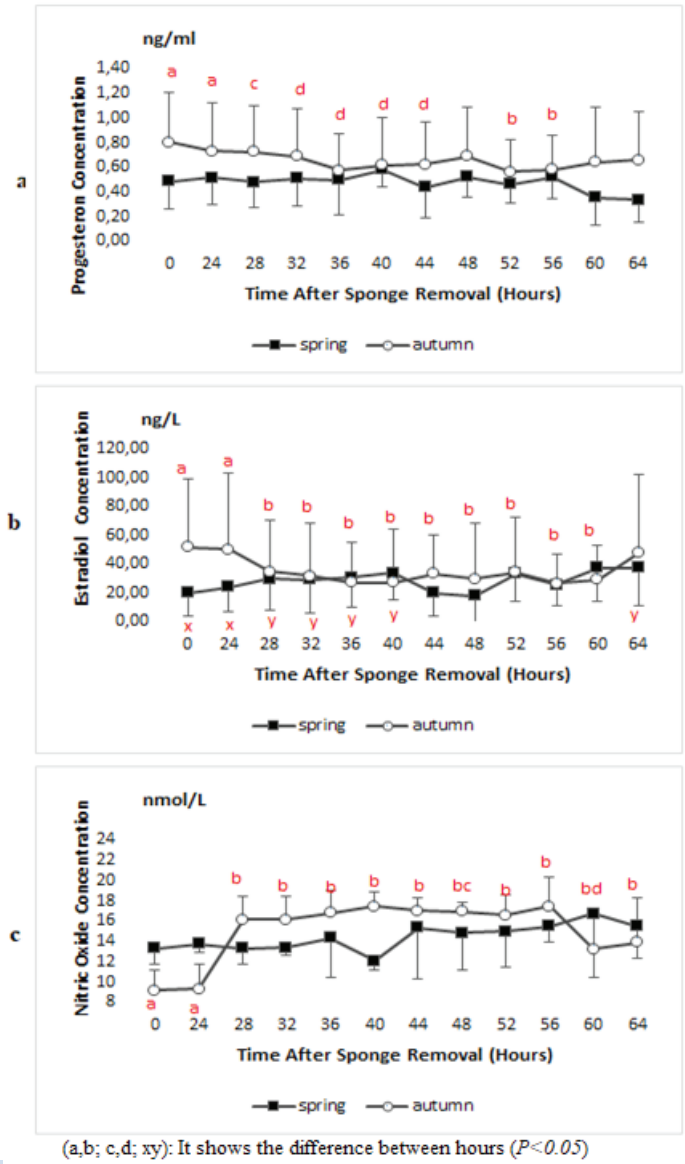
RESULTS

All ewes treated with sponge and eCG + PGF2α exhibited the major indications of estrus, including mounting by the ram. However, there were seasonal and individual variations in the degree and severity of estrus signs. The first signs of estrus appeared at approximately 36 hours in the breeding season and, 44 hours in the non-breeding season.

The alterations in plasma progesterone, estradiol, and nitric oxide concentrations at different times after the estrus synchronization in the breeding and the non-breeding season groups in Tuj ewes are presented in Figure 1 and the mean values according to the season (Table 1). Despite similar fluctuations in progesterone concentrations in both seasons, breeding season mean progesterone levels were higher ($P<0.05$) than in the non-breeding season. In addition, there were a differences ($P<0.05$) in progesterone concentration between blood sampling hours in the breeding season group.

As in progesterone, the breeding season mean plasma estradiol concentration was higher ($P<0.05$) compared to the non-breeding season (Table 1). A rapid increase in estradiol concentration was observed between 24 and 40 hours after the removal of the sponge in the non-breeding season (Figure 1b). In contrast, a decrease in estradiol concentration was observed 24 hours after the sponge was removal in the breeding season, and persisted at lower concentrations (Figure 1b).

Although plasma nitric oxide concentration was higher at the time of sponge withdrawal in the non-breeding season than in the breeding season (Figure 1c), the mean plasma nitric oxide concentration was significantly higher ($P<0.05$) in the breeding season (Table 1). In addition, there were differenc-



(a,b; c,d; xy): It shows the difference between hours ($P<0.05$)

Figure 1: Plasma progesterone (a), estradiol (b) and nitric oxide (c) concentrations at different hours after estrus induction in ewes at breeding (autumn) and non-breeding season (spring).

Table 1: Mean (±S.D.) plasma concentrations of progesterone, estradiol, melatonin, serotonin, and nitric oxide after estrus synchronization in and out of the natural breeding season (autumn and spring).

	Number of samples = 168				
	Parameters				
	Progesterone (ng/mL)	**Estradiol (ng/L)**	**Melatonin (ng/L)**	**Serotonin (ng/ml)**	**Nitric Oxide (µmol/L)**
Autumn	0.65±0.36 *	35.42±36.66 *	118.76±35.5 *	50.79±29.18	15.01±2.32 *
Spring	0.47±0.20	28.36±19.86	93.03±44.34	66.16±21.93 *	14.41±2.74

* : It shows the statistical difference among of season ($P<0.05$).

es ($P<0.05$) in nitric oxide concentration according to the blood collection hours in the breeding season (Figure 1c).

Plasma melatonin levels, which were significantly low during the light phase and high ($P<0.05$) during the dark phase in both seasons, demonstrated a circadian rhythm (Figure 2a). On the other hand, the breeding season plasma melatonin concentrations (Figure

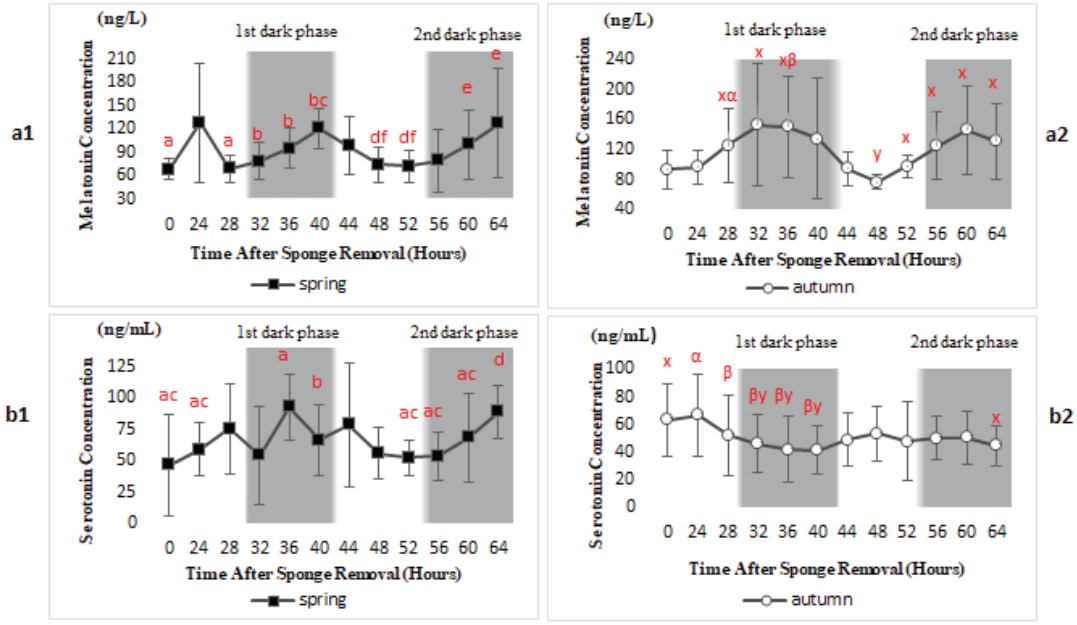


Figure 2: Alterations in plasma melatonin (a) and serotonin (b) concentration after the estrus induction in ewes, in and out the breeding season (autumn and spring).

Table 2: Correlation coefficients among plasma concentrations of progesterone, estradiol, melatonin, serotonin, and nitric oxide after estrus synchronization in and out of the natural breeding season (autumn and spring).

Parameters	Progesterone	Estradiol	Nitric Oxide	Melatonin	Serotonin
Progesteron		**,685***	**,088**	**-,149**	**,227**
Estradiol	-,420		**,535***	**-,342**	**,490**
Nitric Oxide	-,483	,231		**-,063**	**-,538**
Melatonin	,091	,448	,063		**-,352**
Serotonin	-,364	,420	,189	,671*	

*: Correlation is significant at the $P<0.05$ level. Bold font represents autumn, normal font represents spring values.

2a2) were higher ($P<0.05$) than the non-breeding season (Figure 2a1), at sponge withdrawal and the entire observation period.

Contrary to the plasma estradiol, progesterone, melatonin and nitric oxide concentrations, the mean serotonin concentration for the whole peri-ovulatory period analyzed was significantly higher during the non-breeding season than during the breeding season ($P<0.05$) (Table 1). Besides, serotonin at different blood collection hours present significant differences ($P<0.05$) in both seasons (Figure 2b). In addition, a positive correlation was recorded between plasma estradiol and progesterone (r=0.685; $P<0.05$) or nitric oxide (r=0.535; $P< 0.05$) in the breeding season; whereas a positive correlation was recorded only between plasma melatonin and serotonin concentrations (r=0.671; $P<0.05$) in the non-breeding season (Table 2).

DISCUSSION

Progesterone and its analogues are regarded as estrus cycle stimulants and regulators (Blache et al., 1996; van Werven et al., 2013). In the trial performed, estrus detected in all ewes, confirms the findings of other studies (Ritar et al., 1984; Ileri et al., 1996; Romano et al. 2000; Hashemi et al., 2006) that progestagens are effective in inducing and synchronizing estrus.

The mean estradiol concentrations were higher ($P<0.05$) in the breeding season than in the non-breeding season. However, elevated estradiol levels in breeding season resulted in earlier and more pronounced indications of estrus signs compared to the non-breeding group. In our study, the highest estradiol level was recorded at the time of sponge removal in the breeding season. However, this peak value was lower than that found by Menegatos et al. (2002)

and Swellum et al. (2015), who used different estrus synchronization protocols in other breeds of ewes during the mating period, and higher than the peak value reported by Vifloles et al. (2001). The estradiol peak in the non-breeding season group was recorded on the third day (64th hour) after sponge removal, and it was higher than the peak in Merino ewes (Emrelli et al., 2003), or lower than that in Kıvırcık breed ewes (Ekiz, 2006). These differences could be due to the ewe breed the synchronization protocol used and the time of application.

According to our data, mean plasma progesterone concentrations were increased in breeding period compared to non-breeding season (P<0.05). The seasonal variation in progesterone levels can be caused by the amount of progesterone released from the corpus luteum in the breeding season, or by photoperiodic alterations. Coelho et al. (2006), found that plasma progesterone levels were higher during the autumn and winter months than during spring and summer months and attributed it to the corpus luteum formation after natural estrus during the breeding season. The mean plasma progesterone concentrations below 1 ng/mL at all blood collection hours in both seasons are consistent with the findings of many researchers using similar to our study synchronization protocols to our study (Leyva et al., 1998; Menegatos et al., 2002; Emrelli et al., 2003; Swellum et al., 2015). The differences between the breeding and non-breeding period for the above mentioned plasma progesterone and estradiol concentrations are also an indication of a different steroidogenic capacity of the ovary during the two periods.

In mammals, nitric oxide contributes to follicular growth, regulation of female reproductive processes such as ovarian hormone release, implantation, pregnancy maintenance, and lambing in addition to its vasodilatory and increase uterine blood flow (Veille et al., 1996; Dixit and Parvizi, 2001; Khan et al., 2015). A positive correlation between estradiol and nitric oxide was related to the follicular growth in the pre-ovulatory period (Khan et al., 2015; Nath and Maitra, 2018), which is consistent with our findings in the breeding season. This correlation between estradiol and nitric oxide may be the cause of a higher (P<0.05) mean nitric oxide concentration in the breeding period compared to the non-breeding season. All these results indicate that nitric oxide, along with estradiol, plays a significant role in reproduction and especially during estrus in ewes.

The photoperiod's fluctuating duration is the major environmental signal determining reproductive seasonality in small ruminants. While prolonged photoperiods suppress reproduction, short photoperiods enhance reproduction (Mura et al., 2017). The pineal gland converts photoperiod impulses to melatonin, a hormone signal (Willams et al., 1993; Abecia et al., 2012; Mura et al., 2019). Melatonin secretion and blood levels are low during the day and high at night (Luridiana et al., 2015). Plasma melatonin levels were substantially higher in the breeding season, than in the non-breeding season (P<0.05). The annual pattern of plasma melatonin and progesterone levels in several ewe breeds was higher in autumn and winter months than spring and summer months (Misztal et al., 1996; Coelho et al., 2006; Brunet et al., 2008), which supports our study results. Furthermore, our study included two light and two dark phases. Plasma melatonin levels were low in both seasonal groups during light phase hours but considerably increased during dark hours (P <0.05) and, it is consistent with other research findings (Misztal et al., 1996; Coelho et al., 2006; Todini et al., 2011). At the same time, the lowest plasma melatonin level in both seasons was measured during the daytime hours, and the highest level during the night hours.

Ewes enter an anestrus period with the suppression of LH hormone secretion depending on seasonal alterations (Callaghan, 1999). There are two mechanisms by which LH is suppressed during anestrus: steroid-dependent and steroid-independent suppression. Serotonin receptors, which are implicated in the steroid-independent pathway, are responsible for the suppression of LH secretion during seasonal anestrus due to photoperiodic variations (Le Corre and Chemineau, 1993; Forcada and Abecia, 1996; Olivier et al., 2019). Whisnant and Goodman (1990) reported that by applying a serotonin antagonist to ovariectomized sheep in the anestrus period the LH pulse frequency increased, and that serotonin administration could suppress LH secretion in sheep. In the present study, the mean and peak plasma serotonin levels of the non-breeding season group after induced estrus were higher (P<0.05) than those of the breeding season group. The increase level of serotonin in the non-breeding season group may be due to the long-day photoperiods in the non-breeding season and its role in suppressing the LH hormone. However, in our study nighttime and daytime serotonin concentration alterations were significant in both season and exhibited a circadian rhythm. Similar to our results, plasma

serotonin levels increase and decreases in the light and dark phase (Piccione et al., 2008) in goats kept under a 12-hour light/12-hour dark. As far as we know, no reports were found on seasonal alteration in serotonin hormone levels of ewes. However, in other species (Goda et al., 2015; Quay, 1963, Sarrias et al., 1989), the serotonin hormone was found to be higher in the non-breeding, similar to our study. All these findings lead us to the logical assumption that serotonin suppressors could be studied in estrus synchronization in ewes during natural anestrus, and that more studies are needed on this subject.

CONCLUSION

In conclusion, the increased levels of estradiol, progesterone, melatonin, and nitric oxide in Tuj ewes in the breeding season, and the increased serotonin in the non-breeding season could be related to seasonal differences on reproduction. The seasonal variation of serotonin hormone in sheep after induced estrus was revealed for the first time in this study. The fact that serotonin hormone is higher at non-breeding season despite estrus stimulation could be an indication that this hormone is a natural suppressor of estrus. However, further studies are needed to support our findings about the suppression effect of serotonin on estrus in sheep. Furthermore, serotonin suppressive substances could be investigated in estrus synchronization protocols.

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CONFLICT OF INTEREST

None declared

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