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The Effect of Short-Term or Long-Term Progesterone-Based Estrus Synchronization on Fertility Parameters in Tuj Ewes outside the Breeding Season

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ABSTRACT The objective of this study was to investigate the impact of estrus synchronization on certain fertility parameters in Tuj ewes during the non-breeding season, using short-term (ST) or long-term (LT) progesterone (P4)-impregnated sponge and equine chorionic gonadotropin (eCG). Forty-eight clinically healthy Tuj ewes were selected and divided into three groups: Group I (G1, n=15), Group II (G2, n=18), and Group III (G3, n=15). G1 and G2 received hormonal treatment with a P4-impregnated sponge inserted into the vagina on day 0, and 500 IU eCG was administered intramuscularly five or ten days later, respectively. The sponges were removed from the vagina seven days after insertion for G1 and 12 days after insertion for G2. G3 did not receive any hormone treatment. All groups were exposed to the ram 24 hours after sponge removal. Estrus was monitored every six hours for five days, and pregnancy was confirmed by transrectal ultrasonography. The estrus rate, estrus onset time, and pregnancy rate were significantly different between G1 and G3 and between G2 and G3 ($p<0.05$). However, there was no difference between the treatment groups (G1 and G2) in terms of fertility parameters ($p>0.05$). In conclusion, ST or LTP4-impregnated vaginal sponge treatment was equally effective on fertility parameters in Tuj ewes during the non-breeding season.

Keywords: *Estrus synchronization, Medroxyprogesterone acetate, Pregnancy rate, Sheep.*

ÖZ

Üreme Mevsimi Dışında Tuj Koyunlarında Kısa Süreli veya Uzun Süreli Progesteron Temelli Östrus Senkronizasyonunun Fertilite Parametrelerine Etkisi

Bu çalışmanın amacı, kısa (ST) veya uzun süreli (LT) progesteron (P4)-emdirilmiş sünger ve kısrak koriyonik gonadotropin (eCG) kullanarak östrus senkronizasyonunun Tuj koyunlarında bazı fertilite parametreleri üzerindeki etkisini araştırmaktır. Klinik olarak sağlıklı 48 Tuj koyunu seçildi ve üç gruba ayrılmıştır: Grup I (G1, n=15), Grup II (G2, n=18) ve Grup III (G3, n=15). G1 ve G2, P4-emdirilmiş sünger vaginaya yerleştirilerek hormon tedavisi aldı ve sırasıyla beş veya on gün sonra 500 IU eCG intramusküler olarak uygulandı. Süngerler, G1 için yerleştirme tarihinden yedi gün sonra ve G2 için yerleştirme tarihinden 12 gün sonra vaginadan çıkarıldı. G3 herhangi bir hormon tedavisi almadı. Tüm gruplara, sünger çıkarılmasından 24 saat sonra koç katımı yapıldı. Östrus, beş gün boyunca her altı saatte bir izlendi ve gebelik transrektal ultrasonografi ile doğrulandı. Östrus oranı, östrus başlangıçzamanı ve gebelik oranı G1 ve G3 ve G2 ve G3 arasında anlamlı şekilde farklıydı ($p<0.05$). Fakat tedavi grupları arasında (G1 ve G2) arasında fertilite parametreleri açısından fark tespit edilmedi ($p>0.05$). Sonuç olarak, kısa veya uzun dönem P4-emdirilmiş vaginal sünger tedavisi, Tuj koyunlarında üreme sezonu dışında fertilite parametreleri açısından eşit derecede etkilidir.

Anahtar Kelimeler: *Gebelik oranı, Koyun, Medroksiprogesteron asetat, Östrus senkronizasyonu*

INTRODUCTION

Seasonally polyestrous sheep exhibit multiple estrous cycles that occur only during a specific season of the year, provided that pregnancy does not interrupt the cycles. In sheep, cyclic activity begins as the amount of darkness in a day increases from late summer to early autumn, and they become sexually receptive (Bartlewski et al. 2011; Abecia et al. 2012). There are various methods for exogenous progesterone (P4) administration, with the most common being the use of an intravaginal device or oral feed additives that slowly release P4. Currently, in small ruminants, medroxyprogesterone acetate (MAP), fluorogestone acetate (FGA), and intravaginal P4-releasing devices [such as controlled internal drug release (CIDR)] are widely used for both in-season and out-of-season

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estrus synchronization purposes (Kuru et al. 2018; Biehl et al. 2019; Kuru et al. 2022; Arya et al. 2023).

Progesterone and its analogues are widely used for induction and synchronization of estrus in sheep. In this method, P4-impregnated sponges or CIDRs are inserted intravaginally for 12-14 days. The sheep will exhibit estrus approximately 30-48 hours after the removal of the P4-impregnated device. To achieve higher fertility parameters, equine chorionic gonadotropin (eCG) should be administered on the day of P4 withdrawal. In recent years, short-term (ST) P4 protocols have also been developed in small ruminants. In this approach, P4-impregnated sponges or CIDR are inserted into the vagina for a period of 5-7 days, which is shorter than the half-life of the corpus luteum in the ovaries. The response to ST treatments can be similar to that of long-term (LT) treatments (Abecia et al. 2012; Kuru et al. 2018; Garoussi et al. 2020; Hameed et al. 2021; Kuru et al. 2022). However, we did not come across any studies investigating the effect of short-term P4-based estrus synchronization on fertility parameters in Tuj ewes outside the breeding season in our literature review.

The aim of this study was to investigate the effect of ST (7 days) or LT (12 days) intravaginal P4 sponge and eCG treatment on some fertility parameters in Tuj ewes during the non-breeding season.

MATERIAL AND METHODS

This study was conducted following the approval of the Local Ethics Committee for Animal Experiments of Kafkas University (KAÜ-HADYEK 2019/130, Decision date: 24.10.2019), Kars, Türkiye.

Animals

Forty-eight clinically healthy Tuj ewes aged between 2 and 4 years, weighing between 40 and 50 kg, with a body condition score (BCS) ranging from 2.5 to 3.5 (1=emaciated, 5=obese), and having completed the postpartum period without any complications were included in the study. Furthermore, eight Tuj rams (n=8) were used for mating during estrus detection.

Nutrition

Sheep were pastured in the fields during the daylight hours and then returned to the barn in the evening throughout the study period. During synchronization, the sheep were fed with dry hay and barley when they returned from the pasture. In addition to the regular feeding during pregnancy, we added 0.4 kg of concentrated feed per sheep per day, which contained 16% crude protein and 2700 kcal of metabolizable energy (Hasel Koyun Yemi®, Hasel Yem, Türkiye) (Table 1). Water and mineral licking buckets were provided *ad libitum* during the study period.

Estrus Synchronization Protocol

The estrus synchronization procedures in the study were carried out during the non-breeding season (March-May). Prior to synchronization, the sheep were divided into three groups, balanced in terms of age, BCS, and weight.

Group I (G1, n=15, Short-term P4 + eCG, 7-day group): A P4-impregnated sponge (60 mg medroxyprogesterone acetate, MAP, Esponjavet®, Hipra Animal Health, Türkiye) was inserted into the vagina on day 0. On the fifth day, 500 IU eCG (Oviser®, Hipra Animal Health, Türkiye) was injected intramuscularly. The sponges were removed from the vagina on day 7, and 24 hours later, mating with the ram was performed (Figure 1).

Group II (G2, n=18, Long-term P4 + eCG, 12-day group): A P4-impregnated sponge (60 mg, MAP) was inserted into the vagina with a special applicator on day 0. On the tenth day, 500 IU eCG was injected intramuscularly. The sponges were removed from the vagina on day 12, and 24 hours later, mating with the ram was performed (Figure 1).

Group III (G3, Control, n=15): No intervention was performed in this group, and mating with the ram was performed at the same time as the other groups (Figure 1).

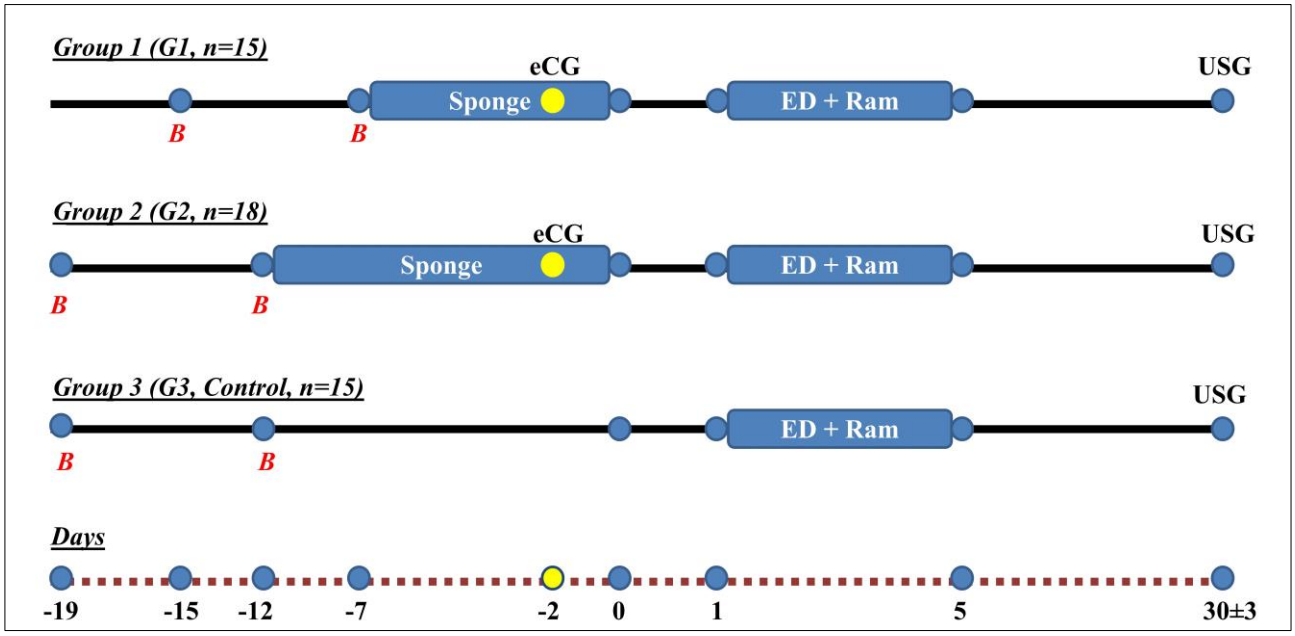

Figure 1: Estrus synchronization procedures and blood sampling days in the groups. '**eCG**: Equine chorionic gonadotropin, **USG:** Ultrasonography, **ED**: Estrus detection, **B:** Blood.

Estrus Detection and Mating

Estrus detection was initiated 24 hours after removal of the sponges in all groups. Heat was observed every 6 hours for 5 days. Ewes that allowed ram mating were accepted in estrus and recorded. Ewes that mated two or three times were separated from the group and placed in a separate area with rams.

Pregnancy Diagnosis

Pregnancy diagnosis was performed using transrectal ultrasonography (5-7.5 MHz, SonoSite Vet 180Plus®, SonoSite, USA) 30±3 days after sheep were mated. The diagnosis of pregnancy was confirmed by visualizing the embryo.

Blood Sampling and Processing

Blood samples were collected from the jugular veins of ewes 7 days prior to sponge treatment and on the day of intravaginal sponge insertion. Samples were collected using a holder (BD Vacutainer®, Becton, Dickinson and Company, USA) and a holder needle (BD Vacutainer®) into 8.5 mL vacuum tubes with gel (BD Vacutainer®). After collection, the tubes were centrifuged at 3000 rpm for 15 min using a centrifuge (NF 400R®, Nüve, Türkiye). Serum samples were then transferred to microcentrifuge tubes and stored at -18 °C until P4 concentration was determined.

Serum Progesterone Analysis

Serum P4 concentration was determined using a commercial Enzyme-Linked Immunosorbent Assay (ELISA) kit (Bioassay Technology Laboratory, China) specifically designed for sheep. The ELISA kit was read with an ELISA plate reader (Epoch®, Biotek, USA). The standard range of the commercial kit was between 0.05 ng/mL and 15 ng/mL, with a sensitivity value of 0.027 ng/mL.

Fertility Parameters and Lamb Yield Traits

Fertility parameters, including time of onset of estrus, duration of estrus, estrus rate, pregnancy rate, conception rate, lambing rate, twinning rate, fecundity, lamb yield, and survival rate were determined. The relevant parameters and traits were calculated according to the formulas (Kuru et al. 2017; Kuru et al. 2022; Yılmaz et al. 2022). To determine the survival rate of lambs, a follow-up was conducted for 30 days after delivery.

Statistical Analysis

In the study, the mean±standard error (SE) of estrus onset time, estrus duration, and P4 concentration were provided. Estrus onset time and duration, which were determined to have a normal distribution according to the Kolmogorov-Smirnov test, were compared using one-way ANOVA and post-hoc Tukey Honestly Significant Differencetest. Two-way analysis of variance (ANOVA) was conducted to analyze the data for the measured P4 concentration in the groups, including group effect, time effect, and group × time interactions.

Tukey's multiple comparisons test was used for pairwise comparisons of group days and between-group days. For the comparison of estrus rate, pregnancy rate, conception rate, lambing rate, twinning rate, fecundity, and lamb yield, the Pearson chi-square test or Fisher's exact test was used. All analyses were performed using SPSS® (Version 26.0, SPSS Inc./IBM Group, Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). Differences between groups were considered significant at $p<0.05$ after statistical analysis.

RESULTS

Serum Progesterone Concentration

Serum P4 concentration was less than 1 ng/mL in all groups 7 days before treatment and on the day of sponge insertion (Figure 2). In one ewe from G2, serum P4 concentration was higher than 1 ng/mL. This ewe was excluded from the study as it showed cyclic activity.

Table 1: Characteristics of concentrate feed.

Nutrients	**%**
Crude protein	16
Crude cellulose	10
Crude fat	3.2
Crude ash	9
Insoluble ash in HCL	1
Molasses	5
Salt	1
Vitamins	**IU/kg**
Vitamin A	15000
Vitamin D	5000
Vitamin E	30000
Minerals	**%**
Calcium	1.6
Phosphorus	0.4
Sodium	0.4
Trace minerals	**mg**
Manganese	50
Copper	10
Iron	50
Iodine	800
Zinc	50

Fertility Parameters

Both short- and LT treatments using P4-impregnated sponges (G1 and G2) resulted in significantly higher rates of estrus induction compared to the control group (G3). The estrus rates were 73.33%, 70.59%, and 15.38% in G1, G2, and G3, respectively (p=0.001, Figure 2).

Estrus onset time was also significantly different between the groups, with 36.5±2.76 hours in G1, 32.9±1.36 hours in G2, and 55.0±5.0 hours in G3 (p=0.002). Estrus onset time was statistically different between G1 and G3 (p=0.006), and between G2 and G3 (p=0.001). However, estrus duration did not differ statistically between the groups (p=0.991, Figure 2).

Outside the breeding season, P4-impregnated sponge treatment (G1 and G2) increased the pregnancy rate compared to the control group (G3), with pregnancy rates of 46.67% in G1, 58.82% in G2, and 6.67% in G3 (p=0.007). ST or LTP4-based estrus synchronization did not have a statistically significant effect on pregnancy rate (p=0.492). There was a statistically significant difference in pregnancy rate between G1 and G3 (p=0.035) and between G2 and G3 (p=0.002, Figure 2).

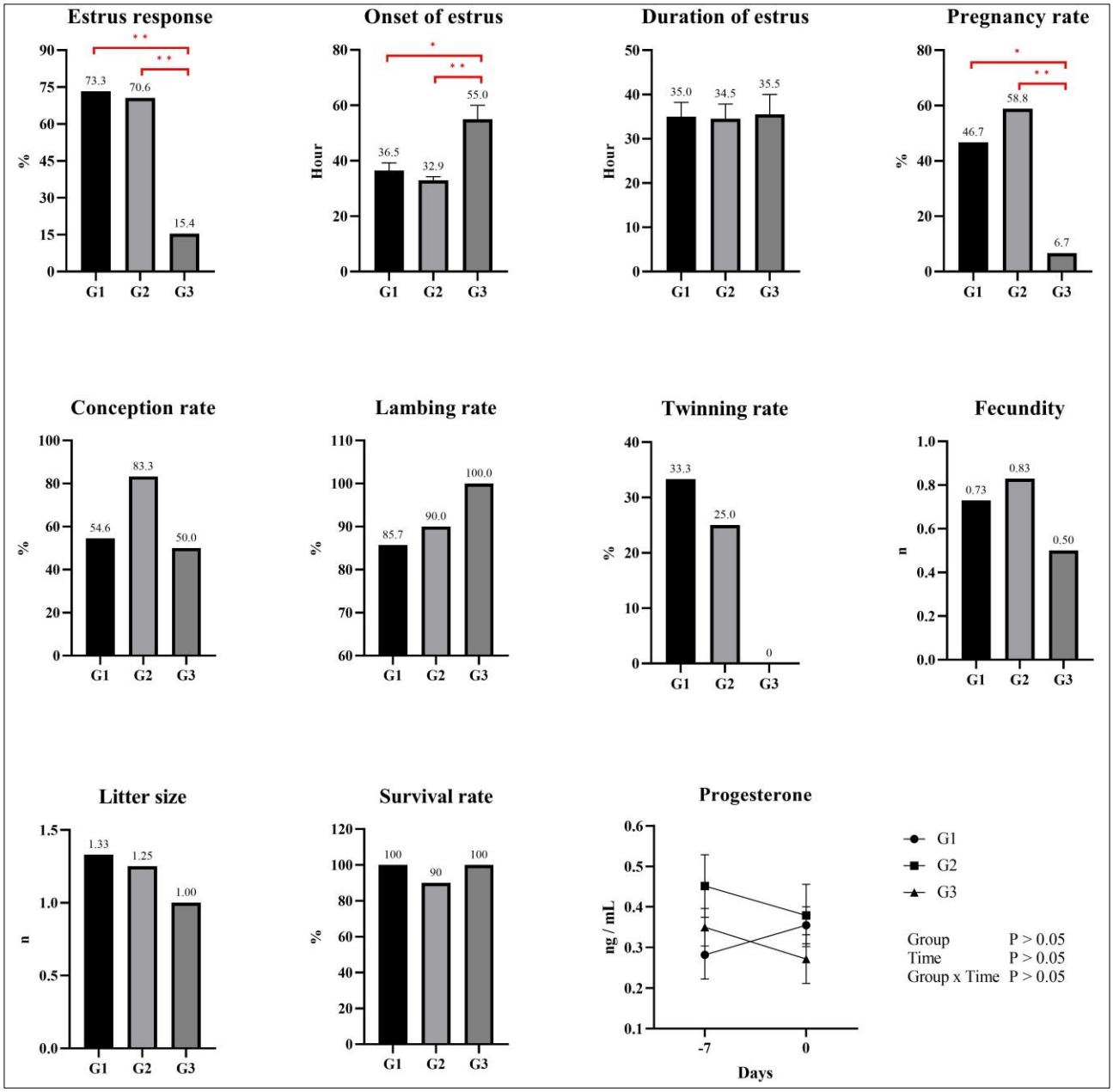

Figure 2: Changes in fertility parameters among groups.

Serum progesterone concentration in the groups before 7 days of treatment and on the day of sponge insertion. According to Pearson's chi-squared test, there was a statistically significant difference in estrus response between G1 vs G3 and G2 vs G3 (p=0.001). Short- or long-term progesterone-impregnated sponge treatments did not have a significant effect on estrus rates. Post-hoc Tukey HSD test revealed a statistically significant difference in estrus onset time between G1 vs G3 and G2 vs G3. Pearson's chi-squared test showed a statistically significant difference in pregnancy rate between G2 vs G3 (p=0.002). Fisher's exact test demonstrated a statistically significant difference in estrus rates between G1 vs G3 (p=0.035). G1 refers to the group receiving short-term (7-day) progesterone and equine chorionic gonadotropin (eCG) treatment, G2 refers to the group receiving long-term (12-day) progesterone and eCG treatment, and G3 refers to the control group. The asterisks indicate statistical significance between groups, *: p<0.05 and **: p<0.001.

Long-term (12 days) P4 treatment had a positive effect on conception rate, but there was no statistically significant difference in conception rate between the groups (p=0.285). Lambing rates did not differ statistically between the groups (Figure 2) (p=0.901).

STP4 treatment had a positive effect on twinning rate, but there was no significant difference in twinning rate between the groups (p=0.788). LTP4 treatment appeared to increase fecundity numerically, but there was no statistically significant difference between the groups (p=0.917, Figure 2).

Progesterone and eCG treatment had a positive effect on twinning rate, which in turn had a positive impact on lamb yield. Litter size in G1, G2, and G3 was 1.33, 1.25, and 1, respectively (p=0.981). Lamb survival did not differ statistically between the groups (p=0.622, Figure 2).

Raw materials of concentrate feed include barley, corn, wheat bran, sunflower seed meal, cottonseed meal, molasses, minerals, salt, calcium carbonate, and vitamins.

DISCUSSION AND CONCLUSION

The Tuj ewes are one of the local breeds of Türkiye, which is raised in the Northeastern Anatolia region. Particularly, the Tuj ewes raised in Kars and its surrounding areas is categorized as a breed with a fatty thigh among sheep breeds. This breed, which has delicious meat, has adapted to the harsh and tough climatic conditions and geography of its region (Aksoy et al. 2001). Despite the adverse

environmental conditions, various synchronization protocols have been applied to stimulate estrus both during and outside the breeding season in Tuj ewes (Ozturkler et al. 2003; Gungor et al. 2007; Kaçar et al. 2008; Kaya et al. 2013; Kamiloğlu et al. 2017). However, literature research has not found any studies on estrus synchronization treatment using ST P4-containing intravaginal sponges in Tuj ewes outside the breeding season.

This study aims to determine the effect of short (7-day) or long (12-day) intravaginal P4-impregnated sponges with eCG on some fertility parameters in Tuj ewes outside the breeding season.

Different synchronization protocols using P4 or its analogues for 12 or 14 days in Tuj ewes can result in estrus rates varying from 50-100% outside the breeding season (Gungor et al. 2007; Kaçar et al. 2008; Kaya et al. 2013). The study revealed estrus rates of 73.33%, 70.59%, and 15.38% in G1, G2, and G3, respectively (p=0.001). Moreover, no statistically significant difference in estrous response was observed between the ST and LT groups. The obtained findings align with the previously reported research (Gungor et al. 2007; Kaçar et al. 2008; Kaya et al. 2013) data pertaining to Tuj ewes, as described above, with the exception of the control group.

In our study, the induction of estrus with P4-impregnated sponges and eCG outside the breeding season was quite remarkable. The results of this study indicate that the use of a ST (7-day) P4-impregnated sponge treatment in Tuj ewes outside the breeding season yielded a similar estrus response to the LT (12-day) treatment, suggesting the efficacy of this protocol in Tuj ewes. As a high estrus response is crucial in affecting other fertility parameters, this finding is of particular importance. Given the notably low estrus response in the control group, the use of P4+eCG treatment may be necessary to achieve a satisfactory estrus response in Tuj ewes during a non-breeding season.

Progestogens are commonly used in small ruminants for estrus synchronization outside the breeding season. Injection of eCG one or two days prior to sponge removal increases fertility (Kuru et al. 2018). Following sponge removal, the onset of estrus in Tuj ewes during studies conducted outside the breeding season occurs within a range of 34-59 hours (Gungor et al. 2007; Kaçar et al. 2008; Kaya et al. 2013). Kuru et al. (2017) and Kuru et al. (2020) reported that in the local breed of sheep known as Pirlak, estrus onset occurred within 34-45 hours or 31-38 hours following sponge removal. However, ST P4 treatment may lead to a delayed onset of estrus compared to LT treatment (Özyurtlu et al. 2011; Texeira et al. 2016; Kuru et al. 2022), although some studies have reported no statistical difference between the two protocols (Ataman et al. 2006; Amer and Hazzaa 2009). In our study, the group that received ST P4 exhibited a numerically later onset of estrus compared to the LT group. Furthermore, combined P4 and eCG treatment resulted in a more synchronized onset of estrus compared to the control group.In our study, the onset of estrus differed significantly between the groups, with 36.5±2.76 hours in G1, 32.9±1.36 hours in G2, and 55.0±5.0 hours in G3 (p=0.002). Furthermore, there was no statistically significant difference in the onset of estrus between our ST and LT protocols. According to Özyurtlu et al. (2011), the estrus onset occurred around 53 and 41.5 hours following the ST and LT P4 treatments, respectively. Similarly, Texeira et al. (2016) reported that the estrus initiation happened at 46 and 32 hours for the respective protocols. In contrast, Kuru et al. (2022) observed estrus starting at 40.5 and 32.9 hours after the ST and LT treatments, while Ataman et al. (2006) and Amer and Hazzaa (2009) determined the onset of estrus at 46.3 and 45.6 hours, and 37.4 and 32.9 hours, respectively. These findings indicate conflicting results among different studies, likely attributed to variations in protocols used and the animal populations under investigation. Moreover, the discrepancies in previous studies might also be influenced by the differences in progestin (P4) treatment durations and dosages employed.

The duration of estrus in Tuj ewes after synchronization outside the breeding season can vary between 23 to 42 hours (Gungor et al. 2007) and 32 to 36 hours (Kaya et al. 2019). In sheep, there may be no difference in estrus duration after ST or LT P4 treatment outside the breeding season (Özyurtlu et al. 2011; Texeira et al. 2016), or LT P4 treatment may lead to a shorter estrus duration (Sareminejad et al. 2014). In the present study, we observed results (35-35.5 hours) that align with the previously reported duration of estrus in Tuj ewes from earlier investigations (Gungor et al. 2007; Kaya et al. 2019). Furthermore, our examination revealed that the administration of both ST and LT P4 protocols did not exert any significant influence on the duration of estrus.

Currently, the aim of many estrus synchronization protocols is to increase fertility rates and subsequently improve pregnancy rates. This not only results in economic gains but also allows for the most efficient use of breeding stock. Previous studies have shown that LT (9-14 days) P4 treatment outside the breeding season resulted in pregnancy rates of 46.6% (Kaya et al. 2013),46.7% (Kaya et al. 2019), and 50% (Kaçar et al. 2008) in Tuj ewes. ST P4 treatment in ewes outside the breeding season can either be advantageous (Sareminejad et al. 2014), disadvantageous (Silva et al. 2021), or similar (Özyurtlu et al. 2011; Texeira et al. 2016; Kuru et al. 2022) in terms of pregnancy rates compared to LT treatment. In our investigation, the pregnancy rates were recorded as 46.67% in G1, 58.82% in G2, and 6.67% in G3. Moreover, we observed that the pregnancy rate among Tuj sheep synchronized with P4 outside the breeding season in our study demonstrated a noteworthy similarity with the rates reported in the existing literature (Kaçar et al. 2008; Kaya et al. 2013; Kaya et al. 2019).Additionally, there were no statistically significant differences in both pregnancy and conception rates between the groups receiving short or LT P4 treatment. However, the pregnancy and conception rates in G2 were numerically higher, which was an interesting finding. Although statistically similar pregnancy rates were observed with both ST and LT P4-impregnated sponge treatment, a short protocol may be preferred in Tuj ewes to alleviate the stress caused by intravaginally inserted devices (Kuru et al. 2015; Kuru et al. 2018).

There is currently no study on the effect of ST P4 treatment on lambing rate in Tuj ewes outside the breeding season, but the use of LT P4-impregnated sponges has resulted in lambing rates ranging from 46.6% to 100% (Kaçar et al. 2008; Kaya et al. 2013; Kaya et al. 2019). In addition, previous studies on ewes have shown that ST or LT P4 treatment did not have a significant effect on lambing rate (Ataman et al. 2006; Amer and Hazzaa 2009; Özyurtlu et al. 2011). In our study, the lambing rate was 85.7% and 90% after ST or LT P4 treatment, respectively, and consistent with the literature (Ataman et al. 2006; Amer and Hazzaa 2009; Özyurtlu et al. 2011), the

duration of P4 treatment did not have a significant effect on lambing rate in Tuj ewes.

Tuj ewes have a low rate of twinning and rarely give birth to triplets both during and outside the breeding season. Many studies have shown that the genetic capacity of Tuj ewes is not suitable for triplets or higher order multiple births (Aksoy et al. 2001; Öztürkler et al. 2003; Kaçar et al. 2008; Kaya et al. 2013; Kamiloğlu et al. 2017). In our study, the twinning rate was 33.3% and 25% after ST or LT P4 treatment, respectively. Although the rates were statistically similar between groups, it can be said that ST P4 treatment had a positive effect on the twinning rate. Similarly, this situation also had a positive effect on litter size, and litter size in G1 was numerically higher than in G2, although there was no statistically significant difference. When future comprehensive studies determine how ST or LT P4 treatments affect follicular dynamics and hormone levels related to follicular development in Tuj ewes outside the breeding season, the effects on both twinning rate and litter size will be better understood.

In our previous studies, we found that various P4-based estrus synchronization protocols at different durations outside the breeding season had no effect on lamb survival rates. These rates ranged from 86.67% to 100%, as reported in our studies (Kuru et al. 2017; Kuru et al. 2020). However, our current study shows that lamb survival rates during the first month after birth were quite high, ranging from 90% to 100%. This indicates that ST or LT P4-containing synchronization protocols can be used in Tuj ewes to meet the market demand for lamb.

Although statistical analysis did not reveal any significant difference, it may be preferable to use ST protocols that result in high litter size and survival.

In conclusion, the use of short (7 days) or long (12 days) P4-containing intravaginal sponges with eCG treatment effectively stimulated estrus and achieved a more synchronized onset of estrus with higher pregnancy rates in Tuj ewes outside the breeding season compared to the control group. However, there was no superiority of either short or long intravaginal sponge treatments in terms of the investigated fertility parameters. Therefore, ST protocols are preferred in synchronization protocols using P4-containing sponges in Tuj ewes outside the breeding season. This would allow the sheep to be less affected by vaginitis and stress caused by prolonged sponge use. Additionally, increasing lamb production with such synchronization protocols during high-demand periods in the market may be more advantageous for farmers.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Idea / Concept: ET, MK
Supervision / Consultancy: MK
Data Collection and / or Processing: ET, BBK, MK
Analysis and / or Interpretation: ET, BBK, MK
Writing the Article: ET, MK
Critical Review: ET, BBK, MK

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