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## Different estrus induction protocols and fixed time artificial insemination during the anoestrous period in non-lactating Kivircik ewes

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**ABSTRACT.** The efficiency of medroxyprogesterone acetate (MAP) sponges or norgestomet ear implants (half or entire) for synchronizing and inducing the estrous cycle in non-lactating Kivircik ewes was investigated during the natural non-breeding period. Ewes were treated for 11 days either with 60 mg MAP sponges (group 1,  $n=27$ ) or with 1.5 mg norgestomet (group 2,  $n=25$ ) or with 3 mg norgestomet (group 3,  $n=27$ ) ear implants. In addition, each ewe received an intramuscular injection of 500 IU of equine chorionic gonadotropin (eCG) and 125  $\mu$ g cloprostenol (PGF $2\alpha$ ), 48 h prior to progestagen removal. Double Cervical Artificial Insemination (AI) with diluted fresh semen was performed at a fixed time (36 and 48 h) following progestagen withdrawal. Mean values for estrous detection rates at the first  $12 \pm 6$  h and within 72 h, the time from progestagen removal to the onset of estrous, the duration of the induced estrous and pregnancy rate were found to be 46.8%, 86.1%,  $26.1 \pm 7.3$  h,  $27.0 \pm 10.7$  h and 27.8%, respectively. There were significant differences between groups 2 and 3 in the time of induced estrous onset ( $P<0.05$ ). These results indicate that, each of the three protocols was equally efficient in inducing and synchronizing estrus in non-lactating Kivircik ewes during the natural non-breeding period.

**Keywords:** anestrous, cloprostenol, eCG, ewe, norgestomet, MAP

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## INTRODUCTION

The majority of sheep breeds perform different reproduction activities depending on season changes, latitude/longitude, the length of the photoperiod, the nutrition, the male effect and other factors (Gordon, 1999; Dogan and Nur, 2006). Therefore, estrous synchronization and induction together with AI in ewes is important for the improvement of reproductive efficiency and management processes (Gordon, 1999; Gokcen et al., 2000; Wildeus, 2000; Menchaca and Rubianes, 2004). Controlled breeding of sheep involves artificial control of estrus and ovulation with exogenous hormone treatments (Keisler and Buckrell, 1997; Menchaca and Rubianes, 2004). Intravaginal sponges impregnated with progesterone or its synthetic analogues, namely medroxyprogesterone acetate (MAP) and fluorogestone acetate (FGA) are usually inserted over periods of 6 to 14 day in conjunction with eCG, especially during natural anestrus period. Sometimes prostaglandin F<sub>2</sub> $\alpha$  injected at sponge removal or 48 h prior to sponge removal (Gordon, 1999; Wildeus, 2000; Ungerfeld and Rubianes, 2002; Dogan and Nur, 2006; Ustuner et al., 2007). Moreover, norgestomet ear implants can easily be used in sheep for estrous synchronization (Cardwell et al., 1998; Awel et al., 2009; Ataman et al., 2009; Garoussi et al., 2012; Uslu et al., 2012; Blaschi et al., 2014). Gonadotrophins, such as equine chorionic gonadotrophin (eCG), have been shown to stimulate follicular growth, increase ovulation rate and fertility and induce a tighter synchrony of ovulation in both anestrus (Maurel et al., 2003; Dogan and Nur, 2006) and cycling sheep (Ustuner et al., 2007). Prostaglandin F<sub>2</sub> $\alpha$  and its synthetic analogs are luteolytic factors that can be used, particularly in breeding period (Keisler and Buckrell, 1997; Wildeus, 2000; Menchaca and Rubianes, 2004; Dogan and Nur, 2006). Estrous synchronization or induction in sheep has been accomplished using several protocols with varying degrees of success (Scaramuzzi and Martin, 1984; Menchaca and Rubianes, 2004). Furthermore, the effects of these protocols on fertility rates are variable (Wildeus, 2000). Hormone cost is one of the biggest factors limiting estrus induction and synchronization in sheep. Thus, many farmers are reluctant to use this technique. Lowering the cost can

make both AI and estrus synchronization more common. The purpose of this study was, therefore, to evaluate the effectiveness of half or entire doses of norgestomet implants in combination with eCG and cloprostenol: a) on estrous induction and synchronization rates and b) on the fertility rates obtained after AI, compared to that of the MAP sponges (in combination with eCG and cloprostenol) in non-lactating Kivircik ewes, during the natural non-breeding period.

## MATERIAL AND METHODS

The study was carried out at village Bursa (latitude 40° 13' E, longitude 29° 00' N, altitude 100 m), located in Inegol, in western Turkey, during March (the natural non-breeding period) under natural lighting. A total of 79 non-lactating Kivircik ewes, 2 to 4 years old, weighing 35 to 58 kg and with body condition scores ranging from 2.5 to 3.5 [evaluate on a scale of 0 to 5, according to Morand-Fehr et al. (1989)], were used in this study. In addition, 7 Kivircik rams of proven fertility and 5 teaser rams were used in the present study. The sheep were allowed to graze on natural pasture from 07:30 to 11:30 h and from 12:30 to 17:30 h and kept in pen overnight. Water and a mineral salt lick were provided *ad libitum*. In addition, the ewes received 0.5 kg concentrate per ewe per day during the entire period of this study. The management of the ewes did not change throughout the entire experimental period.

These females were equally assigned, according to age, body weight and body condition scores, to one of three 11 day progestagen treatments. Group 1 received a 60 mg MAP (Esponjavet, Hipra, Spain) vaginal sponge (group 1;  $n=27$ ). The rest ewes received either a half (group 2;  $n=25$ ) or an entire (group 3;  $n=27$ ) ear implant (impregnated with 3 mg of norgestomet, Crestar, Intervet, Netherlands). The half (0.2 cm in diameter and 1.5 cm in length) and the entire (0.2 cm in diameter and 3 cm in length) implants were inserted subcutaneously into the upper side of the ear using the implanting device provided by the manufacturer. All ewes received an intramuscular injections of 500 IU eCG (Chrono-Gest, Intervet, Netherlands) and 125  $\mu$ g cloprostenol (Dalmz-

in, Fatro, Italy) 48 h prior to progestagen removal. Estrous was monitored with the aid of 5 teaser rams, every 6 h from 12 to 72 h following progestagen sponge and implant withdrawal. The ewes were considered in estrus when they were mounted by the teaser rams. Estrous onset was defined as the time elapsed between sponge/implant removal and the first accepted mount. Estrous duration was defined as the time between the first and last accepted mount, within the same estrous period.

One ejaculate from each ram was collected by electroejaculation. During collection and examination, the semen was protected from temperature shock. Each ejaculate was immediately evaluated for volume and wave motility (Mylne et al., 1997). Only ejaculates with a volume higher than 0.5 ml and good wave motility ( $\geq 3$ ) were used. The volume was determined in the collection tube, which was graduated in divisions of 0.1 ml and the motility was assessed by depositing a drop of semen on a slide and was examined under the phase contrast microscopy (x40; Nikon, Japan) equipped with a warm stage (35°C). The semen sample was scored using a scale ranging from 0 (no wave movement) to 5 (extreme wave

movement). Only ejaculates with scores of 3 and higher were used. The density was determined with the aid of a haemocytometer. The semen was diluted (one step dilution) at 30°C to a sperm concentration of  $800 \times 10^6$  motile cells/ml. The diluent consisted of sterilized cow skim milk, 1000 IU/ml sodium G penicillin and 1000 µg/ml dihydrostreptomycin sulfate. Thereafter, diluted semen samples were pooled in the same test tube. The diluted semen was then cooled to 4°C over a 90 min period and kept at this temperature until insemination. All ewes were inseminated intracervically twice at a fixed time 36 and 48 h following sponge or implant withdrawal with a 0.25 ml straw containing  $200 \times 10^6$  spermatozoa. All ewes were restrained in a standing position and the external opening of the cervix was located with the aid of a speculum and a head lamp. The AI gun was carefully inserted as far as possible into the cervical canal without force, where the semen was slowly deposited. All ewes were inseminated by the same inseminator. Seventy-five days post AI ewes were screened for pregnancy diagnosis using a real-time ultrasound equipped with a 3.5- MHz linear array transabdominal transducer (Honda, HS-1500, Japan). The fetal heartbeat and the fetal image were checked for the

**Table 1.** The mean estrous detection rate, time from progesterone removal to estrous onset and, estrous duration ( $\pm$  S.E.), and pregnancy rate in Kivircik ewes following different estrous synchronization treatments and AI at a fixed time

Treatment group	n	Estrous detection rate (%)		Time from progesterone removal to onset of estrous (h)	Estrous duration (h)	Pregnancy rate (%)
		12 $\pm$ 6 h	within 72 h			
Group 1	27	(17/10) 37.0 <sup>a</sup>	(6/21) 77.8 <sup>a</sup>	26.3 $\pm$ 6.1 <sup>ab</sup>	26.6 $\pm$ 9.4 <sup>a</sup>	(19/8) 29.7 <sup>a</sup>
Group 2	25	(14/11) 44.0 <sup>a</sup>	(1/24) 96.0 <sup>a</sup>	28.3 $\pm$ 8.6 <sup>a</sup>	24.5 $\pm$ 10.2 <sup>a</sup>	(19/9) 36.0 <sup>a</sup>
Group 3	27	(11/16) 59.3 <sup>a</sup>	(4/23) 85.2 <sup>a</sup>	24.0 $\pm$ 6.3 <sup>b</sup>	30.0 $\pm$ 12.0 <sup>a</sup>	(22/5) 18.5 <sup>a</sup>
Total	79	(42/37) 46.8	(11/68) 86.1	26.1 $\pm$ 7.3	27.0 $\pm$ 10.7	(57/22) 27.8

a,b means in the same column with different superscripts indicate a significant difference (P<0.05)

Group 1: MAP impregnated sponge plus eCG and PGF2 $\alpha$ , Group 2: half norgestomet ear implant plus eCG and PGF2 $\alpha$ , Group 3: entire norgestomet ear implant plus eCG and PGF2 $\alpha$ .

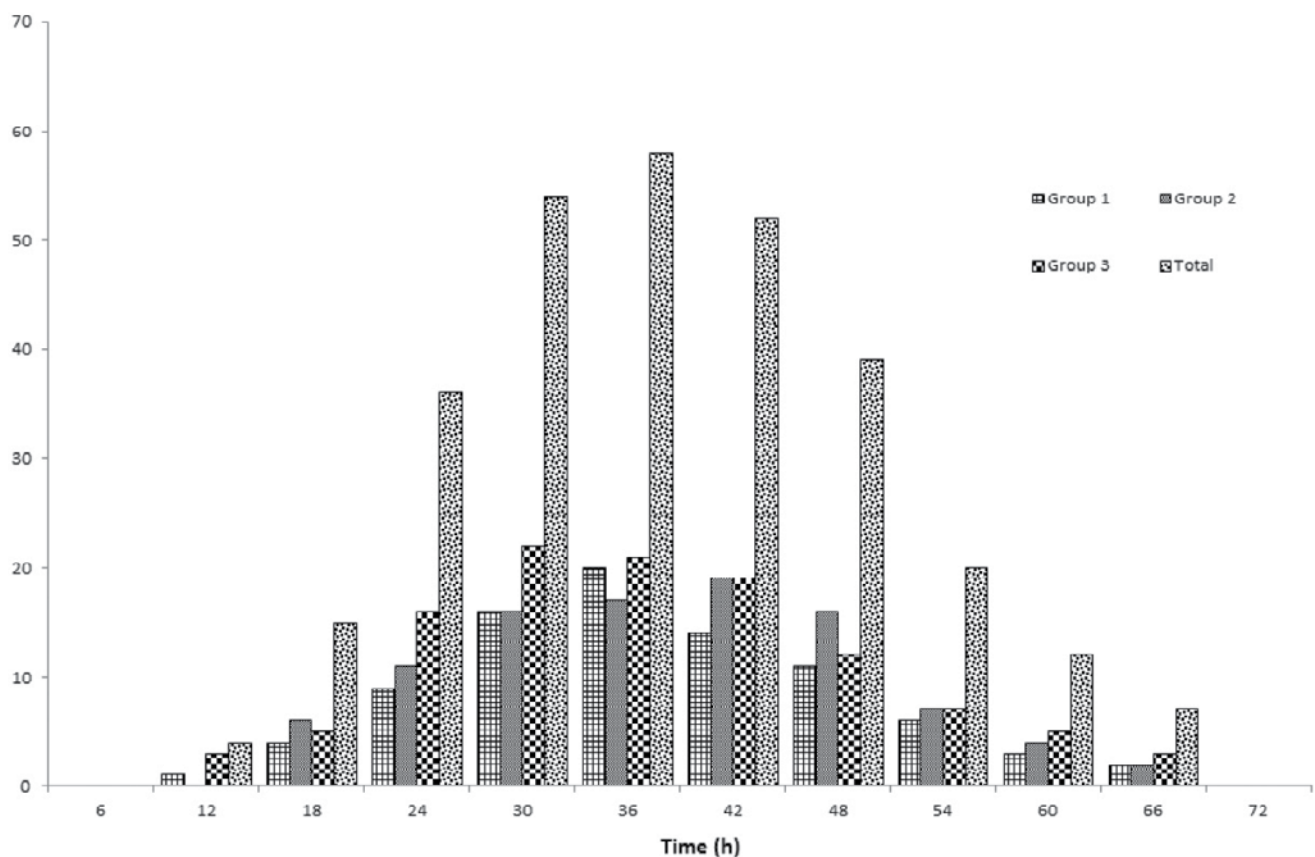
diagnosis of pregnancy.

The time from progesterone removal to onset of estrus and the duration of induced estrous periods were subjected to analyses of variance (one-way ANOVA). The differences between means were tested for significance with the Fisher's PLSD. Estrous detection and pregnancy rates were analyzed using the *chi-square test* and *Fisher's exact test*, respectively; the 95% significance level was noted. SPSS 10.0 software was used for statistical analyses (Instat, 1990-1993).

## RESULTS

The mean values of estrous rates for the first  $12.0 \pm 6.0$  h and within 72.0 h, the time from progesterone withdrawal to the onset of estrus, the duration of the induced estrous and the pregnancy rates are presented

in Table 1. Figure 1 illustrates the percentage of ewes in different groups that detected in estrus at different intervals after sponge or implant removal. Six ewes from Group 1, one ewe from Group 2 and four ewes from Group 3 did not show any overt signs of estrus during the observation period. Estrous onset for the rest ewes occurred between 12 and 72 h after the end of treatment. Only the time from progesterone withdrawal to the onset of estrus was significantly different between groups 2 and 3. All the other parameters were not significantly different among the 3 treatment groups. Thus, the data were pooled and the overall (for the three groups) estrous detection rate for the first  $12 \pm 6$  h period and within 72 h was 46.8% and 86.1%, respectively. The overall (in the 3 groups)



**Figure 1.** Percentages of ewes at different intervals between sponge or implant removal and the onset of estrus in Kivircik ewes after different progestogen treatments.

Group 1: MAP impregnated sponge plus eCG and PGF2 $\alpha$ , Group 2: half norgestomet ear implant plus eCG and PGF2 $\alpha$ , Group 3: entire norgestomet ear implant plus eCG and PGF2 $\alpha$ .



mean time from progesterone withdrawal to onset and the duration of estrous were  $26.1 \pm 7.3$  h and  $27.0 \pm 10.7$  h, respectively. The overall mean pregnancy rate 75 days after AI was 27.8%. Three of the 11 ewes did not show any estrous signs, but diagnosed pregnant at day 75 after AI.

## DISCUSSION

The three treatments used in this study were found to be efficient for estrous induction and synchronization of ewes during the non-breeding period. Although there was no significant difference among the three groups, the estrous detection rate obtained in Group 3 (59.3%) was the highest at the first  $12.0 \pm 6.0$  h. Furthermore, the estrous detection rate at the first  $12.0 \pm 6.0$  h period was higher in ewes treated with half (44.0%) or entire (59.3%) norgestomet implant in comparison with those treated with MAP (37.0%) sponges. Taking in mind these results, it could be concluded that ewes receiving norgestomet showed estrus earlier and closer synchrony compared to those received MAP sponges. This could be explained by the easier absorption of ear implant containing norgestomet compared with sponges. Similarly, Ataman et al. (2009) reported earlier estrous detection rate in Akkaraman cross bred ewes treated with ear implant containing 3 mg norgestomet, compared to ewes treated with intravaginal sponge containing 30 or 40 mg FGA at the transition from non-breeding to breeding period. Considering our overall estrous detection rate for the first  $12.0 \pm 6.0$  h period (46.8%), our result was higher than that reported by Ungerfeld and Rubianes (1999), Das et al. (2000), Simonetti et al. (2000), Dogan and Nur (2006), Hashemi et al. (2006), Ustuner et al. (2007) and Blaschi et al. (2014). There were no significant differences among the 3 groups in terms of estrous detection rate within 72 h after progesterone removal. The estrous detection rate recorded during the 72 h observation period following the cessation of treatment (overall mean rate 86.1%) is within the range of 42.0-100% quoted in treatment with progesterone (Das et al., 2000) or FGA or MAP intravaginal sponges alone (Ungerfeld and Rubianes, 1999; Simonetti et al., 2000; Ataman et al., 2009) or in com-

ination with eCG (Zarkawi et al., 1999; Gokcen et al., 2000; Ungerfeld and Rubianes, 2002; Zeleke et al., 2005; Hashemi et al., 2006; Ustuner et al., 2007) and PGF2 $\alpha$  (Dogan and Nur, 2006) or norgestomet ear implants alone (Ataman et al., 2009) or in combination with eCG (Cardwell et al., 1998; Awel et al., 2009; Garoussi et al., 2012; Uslu et al., 2012) and PGF2 $\alpha$  (Blaschi et al., 2014), in different breeds of ewes under different environmental conditions.

It has been reported that the onset of estrous occurred within 18-144 h following withdrawal of progesterone impregnated sponges (Das et al., 2000), MAP or FGA impregnated sponges (Ungerfeld and Rubianes, 1999; Simonetti et al., 2000; Gokcen et al., 2000; Dogan and Nur, 2006; Hashemi et al., 2006; Ustuner et al., 2007) or norgestomet ear implants (Cardwell et al., 1998; Ataman et al., 2009; Blaschi et al., 2014). In the present study, ewes detected in estrous between 12 and 72 h after sponge and implant removal; the highest incidence of estrous onset occurred between 30 and 42 hours (Fig. 1). The distribution of estrus in our groups was similar to that reported by Zarkawi et al. (1999), Simonetti et al. (2000), Dogan and Nur, (2006), Hashemi et al. (2006), Ataman et al. (2009) and Blaschi et al. (2014). Nevertheless, these results are not in agreement with Ungerfeld and Rubianes (1999) who found the highest incidence of estrous onset occurring between 72 and 96 h after MAP sponge withdrawal. This difference could be due to the different rate of absorption and metabolization of each progestagen or progesterone. In the present study, the mean overall interval to the onset of estrus following progestagen removal was  $26.1 \pm 7.3$  h and it was significantly longer in the half implant group, compared to the entire implant group ( $P < 0.05$ ) (Table 1). The mean overall interval obtained in this trial is in agreement with the findings of Hashemi et al., (2006), who reported a 29.6 h interval to the onset of estrus when using 60 mg MAP sponges and 500 IU eCG. As concern the onset of estrous, on the other hand, longer periods have been reported by Ungerfeld and Rubianes (1999), Das et al. (2000), Simonetti et al. (2000), Ungerfeld and Rubianes (2002), Zeleke et al. (2005), Dogan and Nur (2006) and Ustuner et al. (2007), where ewes synchronized with progesterone

or MAP or FGA impregnated sponges. In a previous study, Cardwell et al. (1998) used norgestomet alone or in combination with eCG and reported an estrous onset interval of 46.0 and 32.6 h, respectively. Furthermore, Usta et al. (2012) reported an interval of 45.6 h for the onset of estrus, after using 1.5 mg norgestomet ear implant treatment for 10 days with 500 IU eCG, in lactating Morkaraman ewes during non-breeding period. Similarly, Blaschi et al. (2014) reported a 44.0, 41.9 and 34.2 h interval to estrus onset in Santa Inês x Texel cross bred ewes, by using 1.5 mg norgestomet for 5, 9, and 14 days, respectively, in combination with 400 IU eCG and 22.5 µg D-cloprostenol at the time of implant removal. The reason for these discrepancies is indefinite; it may be attributed to differences in breed, nutrition, season, hormone-based protocols (Hashemi et al., 2006), use of gonadotrophins and presence of the male after sponge removal (Ungerfeld and Rubianes, 1999). All these factors are known to influence this parameter (Gordon, 1999; Das et al., 2000; Wildeus, 2000; Maurel et al., 2003; Zeleke et al., 2005, Blaschi et al., 2014). Having in mind all these result, our protocols lead to earlier estrus onset.

The mean overall duration of the induced estrous period ( $27.0 \pm 10.7$  h) recorded in this study is similar to that reported by Dogan and Nur (2006) and Blaschi et al. (2014), longer than that reported by Das et al. (2000), Zeleke et al. (2005) and Hashemi et al. (2006) and shorter than that reported by Ustuner et al (2007). The shortest mean estrus duration was recorded in the half-implant treatment group ( $24.5 \pm 10.2$  h), but it was not significantly different compared to the other 2 treatment groups. Maurel et al. (2003) reported high blood oestrogen levels after induced luteolysis and stimulation of follicular growth due to FSH or exogenous eCG. Thus, blood oestrogen concentration could be related to the contradictory results among our finding and those of previous studies. Further research is needed to confirm the reasons for these differences.

None of the treatment protocols showed any significant advantage over the other as concern the conception rate. These results are in agreement with Ataman et al. (2009), who did not record any differ-

ences between FGA sponges and norgestomet ear implants. The overall post-treatment conception rate obtained after AI with fresh diluted semen in this study was 27.8%. The pregnancy rate obtained in this study is not within the range of 33.3 to 100% reported for ewes synchronized with intravaginal progestagen sponges during the breeding (Gokcen et al., 2000; Simonetti et al., 2000; Ustuner et al., 2007), non-breeding (Dogan and Nur, 2006) and transition period (Zeleke et al., 2005) after AI with fresh diluted semen. Furthermore, the present results were lower than those of Blaschi et al. (2014), who recorded a pregnancy rate of 47.8, 60.9 and 83.3 % in short (5 days), medium (9 days) and long-term (14 days) norgestomet implant treated ewes after fixed-time AI with fresh diluted semen, during the natural breeding period. Nonetheless, the current pregnancy rates are not in agreement with none of those reported in the literature. However, pregnancy rates depend on many parameters, such as the breed, the time of AI, the synchronization protocols, the lifespan of spermatozoon and oocyte, and the overall managerial conditions (Gordon, 1999; Wildeus, 2000; Menchaca and Rubianes, 2004; Zeleke et al., 2005). Further research is needed to determine the exact reasons.

Three of 11 ewes did not show any overt signs of estrus, but were diagnosed pregnant at day 75 after AI. This finding is in agreement with a previous report in Anatolian black goats (Dogan et al., 2005) and Kivircik ewes (Dogan and Nur, 2006), synchronized with MAP or FGA sponges. Allison and Robinson (1970) suggested that these silent ovulations may be related to inadequate endogenous progesterone levels with consequent inability of the ewe to respond to endogenous oestrogen. Besides, absence of estrus and ovulation may be due to insufficient gonadotrophic hormone released by the pituitary, to a poor ovary response to the exogenous eCG or to the variation in responsiveness of animals to eCG (Maurel et al., 2003; Menchaca and Rubianes, 2004; Bartlewski et al., 2011).

## CONCLUSIONS

In conclusion, it can be said that estrous detection rate and pregnancy rate were not significant-

ly different among MAP impregnated sponges, half and entire norgestomet ear implants groups. However, the time from progesterone removal to estrus onset and the duration of induced estrus were significantly different between half and entire norgestomet groups. Therefore, protocols used in the present study are equally efficient in synchronizing

and inducing estrus in non lactating Kivircik ewes during the natural anoestrous period. Half implant could be used to reduce the cost of the treatment.

#### **CONFLICT OF INTEREST**

There is no conflict of interest to declare. ■



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