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Effect of pre-synchronisation with progestogen and eCG on reproductive activity in synchronised ewes during anoestrous season

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Abstract: The objective of the study was to investigate the effect of pre-synchronisation on the occurrence of the oestrus and pregnancy rate after fixed time artificial insemination (FTAI) in synchronised ewes during the anoestrous season. Kivircik ewes (n = 84) were randomly assigned to one of the two treatment groups with (PRE; n = 42) or without (SYN; n = 42) pre-synchronisation. In the SYN group, the ewes were subjected to a 7-d short-term protocol (P4 insertion-6d-PGF2α-1d-P4 removal + eCG). In the PRE group, the same short-term protocol as in the SYN group was applied with 7 days apart for a pre-synchronised synchronisation protocol. A cervical FTAI was performed with fresh semen at 54 h after sponge removal. At the beginning of the synchronisation protocol, the oestrous response (66.7% vs. 0.0%) and cyclicity rates (64.3% vs. 14.3%) based on progesterone (P4) were higher in the PRE group compared to those in the SYN group, respectively (P < 0.01). However, the oestrous response after synchronisation was lower within 96 h (57.1% vs. 95.2%; P < 0.01) in the PRE group compared to that in the SYN group. Although the pregnancy rate after the FTAI was significantly (P < 0.05) lower in the PRE group (14.3%) than the SYN group (35.7%), the overall pregnancy rate after natural mating was not different (95.2%) between the groups. In conclusion, the pre-synchronisation decreased the oestrous response leading to a lower pregnancy rate after the FTAI in the synchronised ewes during the anoestrous season. Thus, attention should be paid to two consecutive administrations of eCG in a pre-synchronisation and synchronisation protocol in ewes.

Keywords: artificial insemination; pregnancy rate; progesterone; sheep

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Ewes exhibit a seasonal breeding pattern and this effect is a significant barrier in obtaining offspring throughout the year. Besides, farmers take advantage of producing lambs by inducing the oestrous cycle during anoestrous season (Abecia et al. 2012). In this aim, the reproductive management of ewes is generally controlled with synchronisation protocols rather than natural methods to maintain the profitability and raise lambs during the anoestrous period (Ungerfeld and Rubianes 2002; Rosasco et al. 2019). Progesterone or progestogen releasing devices are commonly used for 12–14 days by mimicking the luteal phase of the oestrous cycle in the anoestrous period (Ungerfeld and Rubianes 2002).

Although a long-term synchronisation protocol induced a high rate of oestrous response (80–90%), the high oestrous rate did not result in high pregnancy rate (30–50%) after fixed time artificial insemination (FTAI) in previous studies in ewes (Ungerfeld and Rubianes 2002; Miranda et al. 2018). Long-term synchronisation protocols result in low fertility because of several reasons such as the sub-luteal levels of progesterone, persistent preovulatory follicles, and delayed new follicular wave compared to short-term protocols (Vinoles et al. 1999; Vinoles et al. 2001). However, there has been no consensus on obtaining a higher fertility with a short-term protocol compared to a long-term protocol in literature (Blaschi et al. 2014; Biehl et al. 2019).

In addition to the length of the synchronisation protocol, the absence of oestrus and ovulation prior to the FTAI may be limiting factors for the discrepancy between the low pregnancy rate and the high oestrous response in ewes during the anoestrous season (Kaya et al. 2013). In cattle, the stage of the oestrous cycle at the beginning of the synchronisation protocol affects the fertility, and pre-synchronisation protocols have been developed to align cows to dioestrus at the onset of FTAI synchronisation protocols (Dirandeh 2014). Thus, pre-synchronisation is a critical factor to increase the synchronisation success and pregnancy rates for FTAI protocols in cows (Gumen et al. 2012). It was reported that achieving higher fertility rates with pre-synchronisation protocols by inducing the oestrus, the formation of the *corpus luteum* after oestrus, and the initiation of the synchronisation under a high progesterone concentration is a significant target in cows (Richardson et al. 2016). For the same target, there has been no study investigating the effect of pre-synchronisation on the efficacy of oestrous synchronisation protocols and fertility in ewes in the literature. The objective of the present study was to increase the effectiveness of the oestrous synchronisation protocol and fertility with pre-synchronisation in Kivircik ewes during the anoestrous season.

MATERIAL AND METHODS

The experimental procedures were approved by the Balikesir University Animal Care Committee (Reference No. 2019-7/1).

Animals and management

The Kivircik ewes display seasonal breeding activity in Balikesir, located on the coastline of the Aegean Sea and the Sea of Marmara in Turkey. This study was carried out in a sheep flock at the Balikesir University, Experimental Farm (39°28'N, 28°02'E). The ewes were isolated from contact with rams for three months within the farm. All the ewes were grazed on natural pasture prior to the experiment. Trace mineral salt blocks and water were given *ad libitum*. During the experiment, the ewes were maintained on a natural pasture with an additional 1.5 kg of dried grass hay/ewe/day and 600 g of barley/ewe/day as nutritional flushing from D-14 to D7 for 21 days. Day 0 (D0) indicated the day of the synchronisation initiation.

Study design

The experiment was conducted from 26th March to 25th April, 2019. The body condition score (BCS) was evaluated by the palpation of the spinous and transverse processes of the lumbar vertebrae at the beginning of the study (Kenyon et al. 2014). The hind legs of the ewes were restrained at an appropriate height to prevent lateral or forward movement. Non-lactating ewes (n = 90), 2–5 years old, were randomly assigned to one of the two treatment groups [synchronisation with (PRE) or without (SYN) pre-synchronisation] at the onset of the study (D-14). However, six ewes were removed due to losing their ear tags during the experiment, and the study was completed with 84 ewes.

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For the pre-synchronisation protocol in the PRE group (n = 42), an intravaginal sponge, containing 60 mg of medroxyprogesterone acetate (MPA; Esponjavet®, Hipra, Turkey), was inserted for seven days (between D-14 and D-7). The ewes received 500 IU of equine chorionic gonadotropin (eCG; Gonaser®, Hipra, Turkey) at D-7 and 75 µg of D-cloprostenol (Gestavet Prost®, Hipra, Turkey) at D-8. No natural mating or artificial insemination (AI) was performed after removing the first sponge (between D-7 and D0) in the PRE group. There was no hormonal intervention in the SYN group during the pre-synchronisation period in the PRE group (between D-14 and D-7). Teaser rams (n = 5) with anti-mating aprons were used for the oestrous detection for one hour at 12-h intervals between 24 h to 96 h (D-6 to D-3) after removal of the vaginal sponge in both groups. The time of the oestrous onset was assumed to be the midpoint (six hours before) following the detection of the first standing position of the ewes.

For the synchronisation protocol in the PRE group (n = 42) and SYN group (n = 42), all the ewes in both groups received an intravaginal sponge from D0 to D7 (Figure 1). Injections of 75 µg of D-cloprostenol were applied at D6 to prevent the occurrence of luteolysis at different times and 500 IU of eCG was applied at D7 to prevent delay of the oestrus after the sponge removal (Kaya et al. 2013). Secondly, teaser rams (n = 5) with anti-mating aprons were used for the oestrous detection for one hour at 12-h intervals between 24 h and 96 h (between D8 and D11) after removal of the vaginal sponge in both groups. The time of the oestrous onset was assumed to be the midpoint following the detection of the first standing position of the ewes.

Fixed time artificial insemination was cervically performed with fresh semen (250×10^6 spermatozoa) at 54 ± 0.5 h after the sponge removal regardless of the oestrus manifestation (Figure 1). Insemination was performed using a duck-billed speculum with a light source and an ovine insemination catheter (Minitube, Tiefenbach, Germany) was passed through into the cervical canal in order to prevent retrograde flow in the outer chamber of the cervix. Twelve days later from the FTAI, rams with mating marks were introduced to the flock to detect natural mating for 30 days.

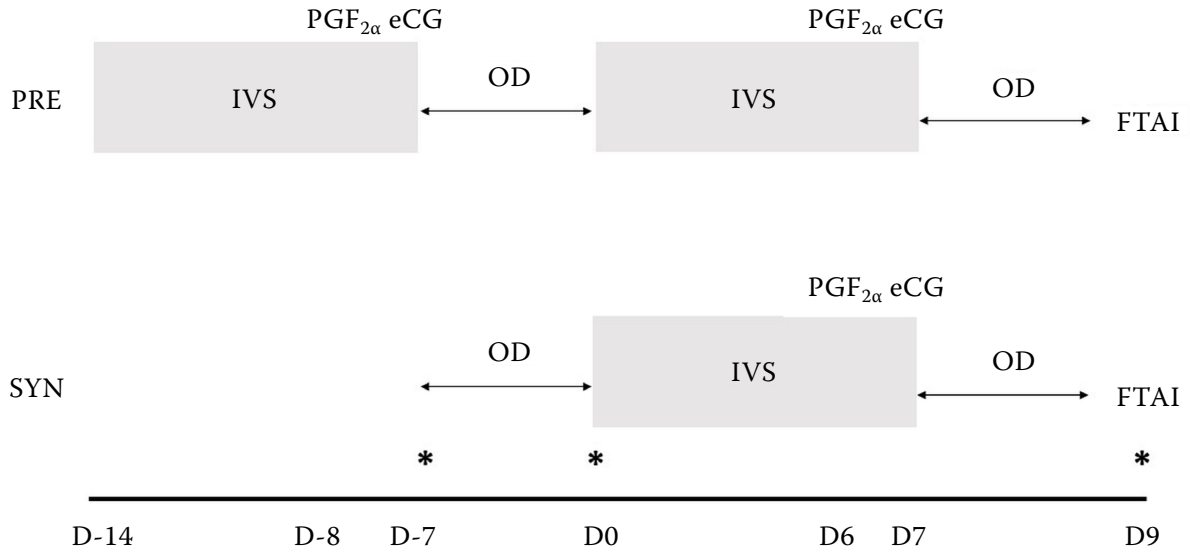

Figure 1. Schematic representation offers a comparison of two protocols in the present study

Pre-synchronisation (PRE; n = 42) performed with a 7-d short-term protocol between D-14 and D-7 in the PRE group. The pre-synchronised ewes received the same short-term 7-d protocol at D0. Synchronised ewes (SYN; n = 42) received only a short-term protocol during the same time as the PRE group exposed the synchronisation. Oestrous detection was performed with teaser rams every 12 ± 1 h between 24 and 96 h for 1 h after removal of the vaginal sponge in both groups. The cervical fixed time artificial insemination (FTAI) was performed with fresh semen at 54 h at D9. Blood samples (*) were collected to determine the serum progesterone concentration on D-7, D0, and D9

eCG = equine chorionic gonadotropin; OD = oestrous detection; FTAI = fixed time artificial insemination; IVS = intravaginal sponge; $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$, D-cloprostenol; PRE = group with pre-synchronisation; SYN = group without pre-synchronisation

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A pregnancy diagnosis was performed via transrectal ultrasound (Ibex Pro equipped with a 5.0–7.5 MHz; E.I. Medical Imaging, Loveland, CO, USA) at 25 days post-FTAI. The presence of a clear anechoic embryonic vesicle with a viable embryo (heartbeat) was evaluated as a positive pregnancy diagnosis. Pregnancies were confirmed at 50 days after the FTAI to determine the embryonic loss. Non-pregnant ewes from the FTAI were re-scanned at days 50 and 70 to determine the pregnancy status following the natural mating. The lambing rate was defined as the proportion of lambing to pregnant ewes.

Collection and processing of semen

In this study, the semen of five rams were collected at three days (D4) before the insemination time by hand-made electro-ejaculation using a probe 12 cm in length, 2.5 cm in diameter, and 12 volts. The ejaculate volume, spermatozoa motility, and spermatozoa density from all the rams were evaluated before the insemination procedure (Tsakmakidis 2010). Three days prior to insemination, the rams were neither ejaculated nor used for natural breeding. The percentage of the motility and concentration of the spermatozoa were examined by a phase-contrast microscope with heated stage at 37 °C and magnification 400 × after dilution. Three ejaculates of five rams having acceptable parameters, providing an adequate volume (> 1.0 ml), mass activity (3+), concentration (> 3.0×10^9 spermatozoa/ml), and motile cells (> 70%), were pooled at 37 °C to prevent the individual ram effect prior to insemination (Casali et al. 2017). The pooled semen from three rams had the following characteristics: 3.8 ml in volume with 4+ mass activity and including 82% motile cells. The pooled semen was diluted with ultra-heat treated (UHT) skimmed milk as an extender. The diluted semen, containing 250×10^6 spermatozoa in 0.25 ml/straw, was stored at 30 °C up to within one hour of insemination (Gibbons et al. 2019).

Blood sampling and serum progesterone concentration

Blood samples were collected from all the ewes (n = 84) via jugular venepuncture at one-week intervals prior to the synchronisation (D-7 and D0) to determine the cyclicity status based on the progesterone (P4) concentration in both groups. Blood samples were also collected at the time of the FTAI (D9) to verify the luteolysis (P4 concentrations > 1 ng/ml).

The samples were centrifuged at 3 000 g for 15 min after collection and stored at –20 °C until analysis.

The extraction method was applied before the analysis of the P4 concentration. For this purpose, all the samples (500 µl) were placed in Eppendorf tubes, then n-butanol (500 µl) was added to the tubes. They were shaken on a vortex for 4 h, and samples were frozen overnight at –47 °C. The supernatant was evaporated to dryness at 40 °C, then 500 µl of phosphate-buffered saline (PBS) was added in the tubes and shaken for 4 h at room temperature.

The extracted samples were used in the determination of the P4 concentration with an enzyme-linked immunosorbent assay (ELISA) using commercial kits (Elabscience Biotechnology, Co, Ltd, Huston, TX, USA). The inter-assay and intra-assay coefficients of variation for the progesterone assay were 1.6% and 5.9%, respectively. Ewes having < 1 ng/ml of a circulating P4 concentration indicated the absence of a functional *corpora lutea*. Ewes having lower (< 1 ng/ml) P4 concentrations in two consecutive samples (D-7 and D0) prior to the synchronisation protocol were considered as non-cyclic. The alteration of the circulating P4 concentration in two consecutive samples prior to synchronisation from < 1 ng/ml to > 1 ng/ml was accepted as induced cyclicity (Yuthasastrakosol et al. 1975).

Statistical analysis

The oestrus onset following the sponge removal was analysed by a general linear model (GLM) with PROC. The GLM procedure of SAS v9.4 software (SAS Institute Inc., Cary, NC, USA) and the results were reported as the least square means and standard error of means. The other category outcomes (oestrous response, pregnancy rate, lambing rate, cyclicity) were analysed by chi-square analyses with the PROC FREQ procedure of SAS. The significance level was considered as $P < 0.05$ for all the analyses.

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RESULTS

There was no difference in the age (SYN = 3.12 ± 0.19, PRE = 2.98 ± 0.18) and body condition score (SYN = 2.80 ± 0.05, PRE = 2.78 ± 0.06) in the groups ($P > 0.05$).

Following pre-synchronisation, the oestrous response was 66.7% (28/42) in the PRE group. None of the ewes showed oestrus for the first 24 h after the sponge removal, and the onset of oestrus was 45.1 ± 0.5 h after pre-synchronisation (between D-7 and D0) in the PRE group. Oestrus was not observed during D-7 and D0 in SYN group.

Following synchronisation in both groups, the overall oestrous response was 78.0% (64/84) in the present study.

The oestrous response in the first 24 h and the first 96 h were significantly ($P < 0.01$) higher in the SYN group (26.2%, 11/42; 95.2%, 40/42) compared to those in the PRE group (2.3%, 1/42; 57.1%, 24/42) after the sponge removal at D7, respectively. The interval between the sponge removal and the onset of oestrus was significantly ($P < 0.01$) longer in the PRE group (47.8 ± 0.3 h) compared to the SYN group (37.8 ± 0.4 h; Table 1). Furthermore, the P4 concentration was lower (< 1 ng/ml) at the time of the FTAI in all the ewes in both groups.

The pregnancy rate was lower in the PRE group (14.3%, 6/42) compared to the SYN group (35.7%, 15/42) ($P < 0.05$). There was no pregnancy following the FTAI in the ewes without signs of oestrus in the PRE (0.0%, 0/18) and SYN (0.0%, 0/2) groups. The pregnancy rates in the ewes with detected oestrus did not differ ($P > 0.05$) between the PRE (25.0%, 6/24) and SYN (37.5%, 15/40) groups. In the PRE group, 40.5% (17/42) of the ewes showed oestrous and 16.7% (7/42) of the ewes did not show oestrous after both applications (pre-synchronisation and synchronisation) (Table 2). While 26.2% (11/42) of the ewes were detected as being in oestrus following the pre-synchronisation only, 16.7% (7/42) of the ewes were detected as being in oestrus following synchronisation only in the PRE group (Table 2). Only ewes exhibited oestrus following both pre-synchronisation and synchronisation became pregnant (35.3%, 6/17) in this study. The distribution of the oestrous response following the pre-synchronisation, synchronisation, both pre-synchronisation and synchronisation or either of them are depicted in Table 2. Moreover,

Table 1. Comparison of the oestrous responses and onset of oestrus of Kivircik ewes after oestrous synchronisation with or without pre-synchronisation

Oestrus response	Experimental groups		Total (%) (*n*/*n*)	*P*-value
	PRE (%) (*n*/*n*)	SYN (%) (*n*/*n*)		
First 24 h (%)	2.3 (1/42)	26.2 (11/42)	14.3 (12/84)	< 0.01
Within 96 h (%)	57.1 (24/42)	95.2 (40/42)	78.0 (64/84)	< 0.01
Onset of oestrus (mean ± SEM)	47.8 ± 0.3 h	37.8 ± 0.4 h	46.4 ± 0.8 h	< 0.01

PRE = group with pre-synchronisation; SYN = group without pre-synchronisation

Table 2. Distribution of the pregnancy rates following the FTAI with respect to the oestrous response (yes/no) following pre-synchronisation and synchronisation in the PRE group

Parameters	Oestrous response				
	yes/yes	yes/no	no/no	no/yes	*P*-value
Proportion of ewes (%) (*n*/*n*)	40.5[a] (17/42)	26.2[ab] (11/42)	16.7[b] (7/42)	16.7[b] (7/42)	< 0.05
Pregnancy rate (%) (*n*/*n*)	35.3[c] (6/17)	0.0[d] (0/11)	0.0[d] (0/7)	0.0[d] (0/7)	< 0.05

[a–d]Values with different superscripts within a row differ at $P < 0.05$

FTAI = fixed time artificial insemination; PRE = group with pre-synchronisation

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the pregnancy rates following the FTAI in these subgroups are given in Table 2. The pre-synchronisation significantly increased ($P < 0.01$) the cyclicity rate in the PRE group (64.3%, 27/42) compared to that in the SYN group (0.0%, 0/0) at the beginning of the synchronisation protocol. However, the pregnancy rate after the FTAI was not different in the cyclic ewes (14.8%, 4/27) compared to that in the non-cyclic ewes (13.3%, 2/15) in the PRE group. Similarly, the cyclicity did not change the pregnancy rate after the FTAI in the cyclic ewes (16.7%, 1/6) and non-cyclic ewes (38.9%, 14/36) in the SYN group.

There was no embryo or foetal loss between the first (D25) and the second pregnancy examinations (D50) following the FTAI. The pregnancy rates after the first (63.9% vs. 77.8%; $P > 0.05$) and the second (84.6% vs. 66.7%; $P > 0.05$) natural mating were not statistically different in the PRE and SYN groups, respectively. The overall pregnancy rates (following one FTAI and two natural matings) at the end of the experiment were equal (95.2%, 40/42) between the PRE and SYN groups ($P > 0.05$). The lambing rates were also 95.2% in both groups in this study (Table 3).

Table 3. Reproductive outcomes of Kivircik ewes after the oestrous synchronisation without or with an additional pre-synchronisation protocol including an intravaginal sponge and eCG

Fertility results	PRE	SYN	*P*-value
FTAI			
Pregnancy rate (%) (*n*/*n*)	14.3 (6/42)	35.7 (15/42)	< 0.05
First natural mating			
Pregnancy rate (%) (*n*/*n*)	63.9 (23/36)	77.8 (21/27)	NS
Second natural mating			
Pregnancy rate (%) (*n*/*n*)	84.6 (11/13)	66.7 (4/6)	NS
Overall pregnancy rate (%) (*n*/*n*)	95.2 (40/42)	95.2 (40/42)	NS
Lambing rate (%) (*n*/*n*)	95.2 (40/42)	95.2 (40/42)	NS

eCG = equine chorionic gonadotropin; FTAI = fixed time artificial insemination; NS = not significant; PRE = group with pre-synchronisation; SYN = group without pre-synchronisation

DISCUSSION

Pre-synchronisation with a short-term protocol induced the oestrous (66.7%) and increased the cyclicity (64.3%) at the beginning of the synchronisation. Previous studies reported a higher oestrous response (≥ 80%) after a short-term protocol (Ungerfeld and Rubianes 2002; Ozyurtlu et al. 2011; Martinez-Ros et al. 2018). Our pre-synchronisation resulted in a lower oestrous response compared to the results of previous studies. The possible explanation for the decreased oestrous response after the first sponge removal in the PRE group may be the low BCS due to the short flushing period. The short-term nutritional supplementation did not have a stimulatory effect on the IGF-I which had a significant role on the folliculogenesis (Scaramuzzi et al. 2006). Besides, detection of a high oestrous response (95.2%) following the three-week flushing after the synchronisation in the SYN group supported the beneficial effect of an adequate flushing period on the oestrous response in the present study.

There was considerable variation in the pregnancy rate after the cervical insemination in ewes in previous studies (Zeleke et al. 2005; Dogan and Nur 2006; Ustuner et al. 2007; Masoudi et al. 2017; Dogan et al. 2018). Although all cervical inseminations were conducted in the same breed in the previous and current studies, the pregnancy rates varied on the synchronisation protocol, time of the FTAI, nutritional status, and management status (Menchaca and Rubianes 2004; Scaramuzzi et al. 2006; Dogan et al. 2018). While the pregnancy rate was reported as 72.3% after cervical insemination with 200×10^6 spermatozoa between 53–55 h after the sponge removal during the transition period in Dorper ewes (Zeleke et al. 2005), the pregnancy rate was determined as 33.3% after two cervical inseminations with 250×10^6 spermatozoa at 40 h and 60 h following the sponge removal during the breeding season in Awasi ewes (Ustuner et al. 2007).

In the present study, the pregnancy rate in the SYN group after the cervical insemination was lower than that in the study reported by Dogan and Nur (2006), but higher than that in the study reported by Dogan et al. (2018) in Kivircik ewes. However, the pregnancy rate after the short-term protocol in the SYN group was in the range of the previous studies.

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Pre-synchronisation has been widely used to increase pregnancy rates in cows. In an ovular cows, the pregnancy rate after FTAI is considerably lower compared to cyclic cows. Thus, inducing the formation of the *corpus luteum* at the beginning of the FTAI protocol is a plausible approach to improve the fertility (Herlihy et al. 2012). The reason for the discrepancy between the high oestrous response and low pregnancy rate has been unclear except for the length of the progesterone exposure during different synchronisation protocols (Vinoles et al. 2001; Ozyurtlu et al. 2011). Our strategy was planned similar to a cow model to increase the pregnancy rate by inducing the oestrus and cyclicity with pre-synchronisation at the initiation of a short-term protocol. Although pre-synchronisation induced the oestrous response and cyclicity at the onset of the synchronisation, the synchronisation with the same short-term protocol reduced the oestrous response in the first 24 h (26.2% vs. 2.3%) and within 96 h (95.2% vs. 57.1%) in the PRE group compared to the SYN group. Although the onset of oestrus was delayed by about 10 h (47.8 ± 0.3 h vs. 37.8 ± 0.4 h) in the PRE group compared the that in the SYN group, the onset of oestrus in both groups was in agreement with previous studies that reported a wide range (18–144 h) (Das et al. 2000; Ungerfeld and Rubianes 2002; Ustuner et al. 2007; Ozyurtlu et al. 2011; Biehl et al. 2019).

There have been limited studies investigating the effects of cyclicity with different hormones at the beginning of timed artificial insemination (TAI) protocols in ewes (Titi et al. 2010; Kaya et al. 2013). Kaya et al. (2013) reported an increased pregnancy rate with the use of hCG seven days before and seven days after synchronisation during the anoestrous period. Titi et al. (2010) reported an increased lambing rate (47% vs. 13%) with additional GnRH as the pre-synchronisation at the time of the vaginal sponge insertion during breeding season. In FTAI protocols, eCG has been widely used to induce oestrus and ovulation (Titi et al. 2010). In our study, consecutive synchronisation with MAP + eCG for pre-synchronisation and synchronisation significantly reduced the first pregnancy rate (14.3% vs. 35.7%) after the FTAI. Similar to our results, a recent study reported a lower pregnancy rate (19.4%) after repeated MAP + eCG administration applied at 20-day intervals for resynchronisation in Corriedale ewes (Cosentino et al. 2019). The reason for the low fertility rate following the repeated eCG administrations could be associated with the presence of circulating anti-eCG antibodies in ewes (Roy et al. 1999a) and in goats (Roy et al. 1999b). Humoral immune responses against recurrent eCG could be different within breeds and within individuals of the same breed (Roy et al. 1999a; Roy et al. 1999b). The fertility decreased from 65.0% to 33.3% after development of an immune response against the eCG application in repeated synchronisations in Alpine goats (Baril et al. 1993). The reason for reduced fertility in the PRE group could be delayed preovulatory luteinising hormone (LH) surge at the FTAI leading to a lower oestrus expression following the use of two consecutive eCG applications in the present study. In the PRE group, a lower pregnancy rate among ewes detected in the oestrus following the pre-synchronisation and synchronisation could reveal a lower ovulatory response due to the developed anti-eCG antibodies. The accumulation of residual anti-eCG antibodies has no effect on successive natural matings in ewes (Bodin et al. 1997). Similarly, no difference in the overall pregnancy rates between the PRE and SYN groups could indicate no long-term detrimental effect of the anti-eCG antibodies on the fertility following two successive natural matings in the present study.

In conclusion it could be stated, that pre-synchronization decreased the oestrous response and, depending on it, also the pregnancy rate after the FTAI in the synchronised ewes during the anoestrous season. However, no difference in the overall pregnancy rates following two successive natural matings could indicate no long-term detrimental effect of the anti-eCG antibodies in the ewes in this study. Thus, attention should be paid to the use of repeated eCG applications with a short interval for a pre-synchronisation protocol in ewes.

Conflict of interest

The authors declare no conflict of interest.

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