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Effects of oestrus synchronisation using melatonin and norgestomet implants followed by eCG injection upon reproductive traits of fat-tailed Morkaraman ewes during suckling, anoestrus season☆

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ABSTRACT

The aim of this study was to compare the efficacy of different oestrus synchronisation protocols using melatonin (MEL), norgestomet (NOR) and melatonin + norgestomet (MELNOR) treatments (further, all animals from the groups MEL, NOR and MELNOR were supplemented with equine chorionic gonadotropin, eCG) in fat-tailed Morkaraman ewes during the non-breeding season. Forty healthy suckling ewes were assigned, from mid-May onwards, randomly to one of four experimental groups. Ten ewes were assigned to the control group CON ($n = 10$) and received no treatments. Ten ewes were assigned to the MEL group ($n = 10$) receiving a subcutaneous melatonin ear implant for 35 days, followed by a 500 IU intramuscular (i.m.) injection of eCG. Ten ewes were assigned to the NOR group ($n = 10$) and received norgestomet implants placed subcutaneously in the ear for 10 days and followed by a 500 IU i.m. injections of eCG after removal of the implant. The last 10 ewes were assigned to the MELNOR group ($n = 10$) receiving a subcutaneous melatonin ear implant for 35 days plus a subcutaneous norgestomet ear implant, inserted on the 25th day of the melatonin implant, for 10 days. It was then followed by a 500 IU i.m. injection of eCG after removal of the norgestomet implant and by introduction of rams to the flock concurrently. Ewes exhibiting oestrous signs by 7 days were hand-mated with rams known to be fertile. Blood samples were collected at the time of first implantation (baseline), on day 35 (norgestomet implant removal and eCG injection) and when ewes expressed oestrus in order to determine plasma progesterone (P_4) concentrations. The values of oestrus response, onset of oestrus post-treatment, pregnancy/lambing rates and litter sizes were recorded. The results showed that the oestrus rates were identical for all synchronisation protocols (100%) and the oestrus rates ($40 \pm 16.3\%$) were significantly lower ($P < 0.001$) in the control group. The onset of oestrus shortened slightly in the hormone-treated groups. The P_4 concentrations on the day of eCG injections (at the end of synchronisation) were significantly higher ($P < 0.01$) in the hormone-treated groups (varying from 2.45 to 2.91 ng/ml)

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compared to the control group (0.45 ng/ml). Pregnancy/lambing rates were slightly higher in the synchronised groups (varied from 60 to 80%), compared to the control group. The results of oestrus synchronisation with melatonin and norgestomet ear implants (supplemented with the eCG) showed that: (i) an oestrus onset could be shortened significantly ($P < 0.001$) with melatonin and/or norgestomet, and (ii) significantly higher ($P > 0.01$) P_4 concentrations and higher lambing rates could be obtained in all hormone-treated ewes when compared to the un-treated Morkaraman ewes during the suckling (anoestrus) season.
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1. Introduction

Sheep breeding is one of the main sources of income for farmers, especially in rural/mountain areas with large grazing-pasture-lands in Turkey. Morkaraman sheep is the second most common breed across the country, and is the most common in our region, Eastern Anatolia. Ewes of this breed or fat-tailed breeds such as Tuj are distinctly seasonally polyoestrous. Ewes mate in fall (mostly from mid-October onwards), and lamb (mostly single offspring) in spring (mostly from mid-March onwards) (Ucar et al., 2005; Celik and Özdemir, 2006). They are used for both milk and meat production, with body weights for ewes and rams reaching a minimum of 40–60 kg (Celik and Özdemir, 2006).

Seasonal anoestrus reduces the reproductive efficiency and hinders productivity. However, melatonin implants and ram effects are used to stimulate the oestrus at the beginning of the breeding season (Wildeus, 1999). Furthermore, the mating season can be prolonged, with reasonable success, using gestagen (such as vaginal sponges) and eCG (Ucar et al., 2005).

Norgestomet ear implants can easily be used in sheep and goats for oestrus synchronisation (Woody et al., 1983). Melatonin, administered either orally (Wheaton et al., 1990) or subcutaneously (s.c.) (Forcada et al., 2002), has been used to induce oestrus activity in ewes and these ewes became successfully pregnant afterwards.

Therefore, the objective of this study was to investigate the efficacy of different synchronisation protocols using norgestomet and melatonin implants, either alone or in combination, all followed by an eCG injection to achieve successful pregnancy rates in Morkaraman ewes during the anoestrus season.

2. Materials and methods

2.1. Location

The study was conducted at the Experimental Research and Practice Farm at the Yuzuncu Yil University where the ewes ($n = 40$) were kept for the study. The farm was located close to the province of Van, Turkey (latitude 42°40′ and 44°30′ East, longitude 37°43′ and 39°26′ North). The region has an altitude of 1727 m and is characterised by an annual temperature between 13 and 17 °C on average.

The study was carried out during May, June and July when the daylight length ranges from 13.5 to 14.5 h.

2.2. Animals

40 mature clinically healthy Morkaraman ewes in anoestrus (suckling) season were used in this study. Body weights of ewes varied from 45 to 56 kg (49.9 ± 1.2 kg, at average). The body condition scores ranged from 3.0 to 4.0 with an average of 3.5 ± 0.4 (using 1–5 scale, 1-emaciated to 5-obese) (Ucar et al., 2005). The ewes were housed in indoor shelters during winter and lambed, as expected, from mid-March onwards. After lambing, the lambs remained with their mothers, and they both were allowed to graze on pasture in May. The animals were housed in indoor shelters overnight. Medium quality grass hay and clean drinking water were available at all times. Ewes were not milked, but their lambs were allowed to suckle. Prior to the study period, rams ($n = 8$) with known fertility (according to farm records) were separated from the flock until re-introduction. At the end of oestrus synchronisation, i.e. starting from the day of eCG injections in treated groups, they were then re-introduced to the flock concurrently to detect oestrous signs (details to be given below) of ewes and were kept with them afterwards. All the flock were maintained under natural lighting conditions in Eastern Anatolia, Turkey.

2.3. Experimental groups

The ewes ($n = 40$) were randomly divided into four groups, which are: control (CON), melatonin (MEL), norgestomet (NOR), and melatonin plus norgestomet (MELNOR) groups (Fig. 1). Treatments were initiated on May 22nd.

Schematic diagram of experimental protocols are given in Fig. 1. The protocols used are as follows:Group CON ($n = 10$): The animals were kept as a control group and received no hormonal treatment.Group MEL ($n = 10$): The ewes received double melatonin implants (18 mg × 2) (Regulin®, Sanofi Animal Health, Hoechst, Germany) on May 22nd (Day −35). Each of the implants contained 18 mg melatonin and were placed s.c. in the ears. The implants were not removed and on June 26th (Day −0), the ewes were given a 500 IU i.m. injection of eCG.Group NOR ($n = 10$): The animals were treated with 1.5 mg norgestomet ear implants (Crestar®-Intervet, Boxmer-Netherlands), on June 16th (Day −10) for 10 days. Upon the removal of the implants, the ewes received a 500 IU i.m. injection of eCG (Day −0).Group MELNOR ($n = 10$): The ewes were treated s.c. with melatonin and norgestomet implants. In this group, the animals received two s.c. melatonin implants on May 22nd (Day −35) and, 25 days after the melatonin administration, they also received 1.5 mg norgestomet on June 16th (Day −10). Ten days later (June 26th, Day 0), the implants were removed and the ewes received a 500 IU i.m. injection of eCG.

On the day of eCG injections, the fertile rams ($n = 8$) were introduced to the flocks and the ewes in the control and experimental groups were all visually monitored twice a day (morning and evening) for the presence of oestrus signs. The oestrus was monitored daily, for 7 days after the introduction of the rams to the flock. The occurrence of mounting as well as uneasiness, increased occurrence of repeated tail wagging, frequent urination, abnormal amount of bleating, reddish and swollen vulva, and mucus under the tail were all considered as true signs of oestrus. The main criteria for signs of oestrus were attractivity and receptivity of ewes (standing still to be mounting by the ram) (Ucar et al., 2005). The ewes in oestrus were allowed to mate. A multi-ram breeding (rotational mating by 4 rams daily) system was used for inseminations.

Additionally, blood samples form the jugular vein were collected from all ewes on the days of: (i) treatment with hormone implants, (ii) implant removal, and (iii) on the onset of oestrus for measuring progesterone (serum) levels, using a radio immune assay (RIA) method (Noakes et al., 2001). To confirm ovarian activity and pregnancy, a commercially available progesterone test kit (Progesterone RIA, DSL-USA) was used according to the instructions of manufacturer.

The criteria used to evaluate the efficacy of hormonal treatments were oestrus response, onset (post-treatment interval) of oestrus, pregnancy/lambing and litter sizes. The presence of pregnancy was diagnosed 35 days after mating using real-time ultrasonography (Honda HS-1500, Japan).

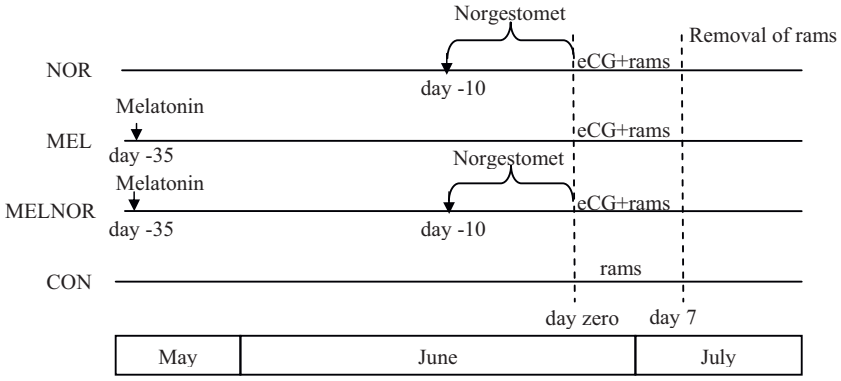


Fig. 1. Schematic diagram of the overall experimental design.

2.4. Statistical analysis

Data presented as mean ± SEM, except for progesterone levels, are given as mean ± SD. The values from the rates of oestrus, onset of oestrus, pregnancy/lambing rates, litter sizes and plasma P_4 concentrations of different synchronisation groups (CON, MEL, NOR, MELNOR) were analysed by regression analysis using MINITAB statistical software package (Minitab, 1996). Differences between the groups were considered significant when $P < 0.05$.

3. Results and discussion

Results from the oestrus response, onset of oestrus, pregnancy/lambing and litter sizes of ewes in the control and treatment groups are presented in Table 1. The rates of oestrus in the treated groups (100%) were significantly higher ($P < 0.001$) than those in the control group (40 ± 16.3%). There were no significant differences for the other parameters examined.

Some researchers, studying the effects of melatonin upon the stimulation of ovarian activity in anoestrous ewes, argue that successful stimulation of ovarian activity is unlikely during the seasonal anoestrus (English et al., 1986; Arendt et al., 1988). However, other authors reported that satisfactory oestrus rates (from 81 to 100%) were achievable (Wigzell et al., 1988; Haresign, 1992). We observed a 100% oestrus rate in ewes ($n = 30$) in all the hormone-treated groups. In goats, a similar rate was also achieved using melatonin and norgestomet combinations (Cetin et al., 2009). This high oestrus response could be due to the synergetic effects of melatonin and progesterone. Surprisingly, we achieved a 40% oestrus in the control group, differing from previous reports (Bastan, 1995; Cetin et al., 2009; Uyar and Alan, 2008). This discrepancy could be due to the fact that the control ewes might have been influenced by the inclusion of rams for the treatment groups. Indeed, some of the control ewes could have been sexually stimulated by the rams (through combined effects of visual, auditory and olfactory factors) and they led them to show oestrus. Further, Yildiz et al. (2004) reported similar findings earlier.

The interval to onset of oestrus following the eCG injection on day 35 was shorter (41–50 h) in the hormone-treated groups in comparison to the control group (78 h). In previous studies, different durations of intervals, 33–46 h (Cardwell et al., 1998) or 36–72 h with implants (Wildeus, 1999), or 12–72 h with sponges (O'Doherty and Crosby, 1990; Oyediji et al., 1990; Crosby et al., 1991; Ucar et al., 2005) were reported. Differences between intervals were related mainly to the synchronisation protocols, i.e. the implant content, route of implant/sponge, supplementation with eCG (Ritar, 1993; Freitas et al., 1996; Ucar et al., 2005), or the use of $PGF_{2\alpha}$. Cetin et al. (2009) showed an interval of 51 h using melatonin plus eCG treatments in goats and an interval of 46 h when the protocol given was combined with CIDR-G. Other co-factors, such as breeds, species (sheep/goat) and ages (parity) of females as well as the geographical locations would all further affect the ultimate results of any synchronisation programme chosen.

In previous studies, pregnancy rates ranging from 85 to 100% were achieved after melatonin administration

Table 1
The response and interval of oestrus as well as the lambing rate and litter size following oestrus synchronisation by melatonin and norgestomet implants (supplemented with the eCG) in anoestrous ewes.

Parameters	Groups				Statistics		
	CON ($n = 10$)	MEL ($n = 10$)	NOR ($n = 10$)	MELNOR ($n = 10$)	*F*-ratio	*P* value	Sign.
Live weight, kg	48.60 ± 1.27	51.30 ± 1.19	50.60 ± 1.44	49.20 ± 1.45	0.03	0.857	NS
Oestrus, %	40[a] ± 16.3	100[b] ± 0.0	100[b] ± 0.0	100[b] ± 0.0	17.69	0.000	$P < 0.001$
[a]Onset of oestrus, h	78 ± 11.5	40.80 ± 6.25	45.60 ± 7.55	50.40 ± 7.55	0.87	0.358	NS
[a]Lambing, %	50 ± 28.9	70 ± 15.3	60 ± 16.3	80 ± 13.3	0.75	0.393	NS
[a,b]Litter size	1 ± 0.0	1 ± 0.0	1 ± 0.0	1.1 ± 0.1	1.32	0.264	NS

CON: control, MEL: melatonin, NOR: norgestomet, MELNOR: melatonin plus norgestomet ear implants. Means (±SEM) having different superscripts (a, b) within the same row are significantly different from each other ($P < 0.05$). NS: not significant ($P > 0.05$).

[a] For controls, only the values from those ewes ($n = 4$) showing oestrus signs were considered for the analyses.

[b] For litter size, only the values from those ewes ($n = 23$) lambing were considered for the analyses.

Table 2
Mean values (±SEM) of blood progesterone levels following oestrus synchronisation using melatonin and norgestomet implants (supplemented with the eCG) in anoestrous ewes.

Groups (n = 10 each)	Progesterone, ng/ml			Statistics		
	P_4-baseline	[a]P_4-after 35 days	P_4-oestrus	F-ratio	P value	Sign.
CON	0.3973 ± 0.0692	0.4543[a] ± 0.0670	0.5773 ± 0.0855	3.02	0.093	NS
MEL	0.3574 ± 0.0523	2.450[b] ± 0.795	0.660 ± 0.146	0.15	0.699	NS
NOR	0.3973 ± 0.0692	2.725[b] ± 0.588	1.142 ± 0.250	1.24	0.275	NS
MELNOR	0.3594 ± 0.0592	2.914[b] ± 0.365	0.853 ± 0.134	0.69	0.413	NS

CON: control, MEL: melatonin, NOR: norgestomet, MELNOR: melatonin plus norgestomet ear implants. Means (±SEM) having different superscripts (a, b) within the same column are significantly different from each other ($P < 0.05$). NS: not significant ($P > 0.05$).

[a] Norgestomet withdrawal and/or eCG injection.

(Bastan, 1995; Horoz et al., 2003; Emrelli et al., 2003). In this study, a 70% pregnancy rate was achieved in the MEL group. In the NOR group, there was 60% pregnancy rate. In previous reports, a range from 39 to 100% pregnancy rate was reported (Jabbar et al., 1994; Horoz et al., 2003; Awel et al., 2009). When melatonin was combined with progesterone (supplemented with the eCG), there was a marked increase in the pregnancy rate (Dunston et al., 1989). Indeed, Horoz et al. (2003) obtained a 95% pregnancy rate by combining melatonin and progesterone. In this study, the MELNOR group achieved a pregnancy rate of 80%. Differences may be due to various factors such as: (i) the time of the year (earlier, in this study) and (ii) route of administration (s.c. or intravaginal), (iii) breed type with fat-tail (Morkaraman, as used herein) or lean-tail (for Kivircik breed, as used by Horoz et al., 2003) and (iv) geographic location (with relative seasonality of Kivircik reared in Western Turkey, in the latter study). In the MEL group, twinning rate did not change, but the rate of pregnancy/live birth was relatively higher in comparison to the NOR group. This was attributed to the stimulatory effect of melatonin upon the corpus luteum, thereby increasing the progesterone production during the luteal phase, which ultimately sustains the embryonic development (McEvoy et al., 1998; Abecia et al., 2002).

Oestrus synchronisation studies using melatonin and norgestomet administrations have been conducted widely in ewes both in- and out of the breeding season (Bastan, 1995; Freitas et al., 1997). However, there exist only a few studies using their combination in ewes. Horoz et al. (2003) achieved a 95% pregnancy rate by the combination of melatonin and a vaginal sponge (60 mg medroxyprogesterone). The present results were similar with their report, the relatively lower lambing rates observed in this study may be due to the differences in breeds used and the commencement of our study earlier in the suckling season (before deep anoestrus). In this respect, some researchers claim that ewes had to be exposed to long durations of daylight in order to achieve optimal results during the anoestrus season (English et al., 1986; Haresign, 1992). Additionally, Nowak et al. (1990) observed that ongoing lactation had unfavourable effects upon early melatonin treatments. These findings indicate the effect of how co-factors can potentially affect the outcome of a given synchronisation programme.

The mean plasma P_4 concentrations were given in Table 2. There were no significant differences of the concentrations measured on different days between the groups (given in rows). However, considering the column statistics for P_4-Day 35, control values (0.45 ± 0.07) were significantly ($P < 0.05$) lower than all the hormone-treated groups, 2.45 ± 0.80, 2.73 ± 0.59 and 2.91 ± 0.37 in the MEL, NOR and MELNOR groups, respectively ($F = 10.13$, $r^2 = 0.21$, $P = 0.003$). Furthermore, the levels on the day of oestrus in the NOR group (>1 ng/ml) tended to be higher ($P = 0.091$) than those (<1 ng/ml, all) in both other hormone-treated groups and in the control group. Finally, there was a significantly positive correlation between the P_4 levels on day 35 and the day of oestrus ($F = 7.32$, $r^2 = 0.162$, $P = 0.010$).

As expected, all the initial (baseline) P_4 levels of anoestrous (control) ewes and those in oestrus were below 1 ng/ml, except for the NOR group (1.14 ng/ml). The concentration of 1 ng/ml was normally considered as a critical threshold value for the ovarian activity in cows (Polat et al., 2009). Likewise, the low initial plasma P_4 concentrations observed in suckling (anoestrus) season can be considered normal, due presumably to the lower circulating FSH/LH concentrations (negligible luteal activity of the ovaries) during the anoestrus in ewes (Ucar et al., 2005). However, for all the hormone-treated groups, the concentrations (>2.45 ng/ml, all) at the end of synchronisation (on day 35) were markedly higher ($P > 0.01$) than those (0.45 ng/ml) in the control group. This latter situation simply indicates the effectiveness of synchronisation protocols used herein. Furthermore, these high P_4 levels obtained by exogenous hormones (melatonin and/or norgestomet) were very similar to those in previous reports (Bastan, 1995; Forcada et al., 1995). It is probable that blood P_4 concentrations at higher levels could successfully be achieved by using any of the protocols in this study. Also, regarding the marked relationship ($P \leq 0.01$) between the P_4 levels on day 35 and those in oestrus, it is expected for all groups that high blood P_4 levels could be sustained for a short-time (up to 2–3 days), until further (final) blood sampling on the day of oestrus. These high levels of P_4 are mandatory before re-gaining the sexual cyclicity in ewes during the anoestrus season that can ultimately result in conception and lambing at a proposed time.

In summary, the findings of oestrus synchronisation by melatonin and norgestomet ear implants (supplemented with the eCG) suggest that: (i) the oestrus response with a shorter onset could successfully be induced in all hormone-treated ewes, (ii) markedly higher progesterone concentrations (on day 35, post-treatment) and relatively higher lambing rates were obtained in treated groups (particularly with the melatonin plus norgestomet protocol)

when compared to un-treated Morkaraman ewes during the suckling, non-breeding season. This study shows that melatonin ear implants can be applied to induce oestrus approximately four months earlier (mid-May onwards) in fat-tailed Morkaraman ewes, which normally bred in fall (mid-October onwards).

Nevertheless, future evaluations of the melatonin plus norgestomet administration in a larger number of ewes from different breeds and in- and out of the breeding seasons should be performed before more reliable conclusions can be drawn.

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