

Effect of exogenous melatonin on metabolic profile and reproductive performance in undernourished suckling Merino ewes in the early postpartum period

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Abstract: This study aimed to determine whether serum metabolite and hormones of ewes with low body condition scores subjected to the melatonin plus progesterone-based protocol (MPE) would be similar to a single progesterone-based protocol (PE) and whether melatonin ear implant supplementation could increase pregnancy rate. Ninety suckled Merino ewes with singleton lamb were equally allocated in two experimental groups (MPE, PE) and a control group (CON) on days 53 ± 3 (D0) postpartum. Ewes in MPE group were subjected to exogenous melatonin plus progesterone-based protocol [Melatonin (day 0) + P4 insertion (day 35) + 500 IU eCG (day 42)]. Ewes in the PE group were subjected to a progesterone-based protocol [P4 insertion (day 35) + 500 IU eCG (day 42)]. Ewes in the CON group received no application. Blood samples (10 ewes/group) were collected to determine serum leptin, cortisol, insulin-like growth factor 1 (IGF-1), beta-hydroxybutyric acid (BHBA), and prolactin levels on days 0, 35, and 42. A pregnancy diagnosis was performed on days 72 and 102. Neither the synchronisation protocol nor the duration of lactation affected body condition score, (BCS; 2.30 ± 0.05), serum leptin (3.49 ± 0.30 ng/mL), cortisol (6.28 ± 1.10 ng/mL), IGF-1 (53.38 ± 2.09 ng/mL) and BHBA levels (0.24 ± 0.02 mmol/L) at the beginning of study (p > 0.05). However, prolactin levels reduced (p = 0.043) by the time (111.80 ± 12.49, 97.40 ± 3.77, 66.30 ± 4.85 ng/mL) in the MPE group. The oestrus response (p < 0.0001) and pregnancy rate (p = 0.018) were higher in the MPE (70%, 30%) and PE (60%, 30%) groups than in the CON group (10%, 3.3%), respectively. In conclusion, adding a melatonin implant to a progesterone-based protocol decreased prolactin levels but did not change other hormones and BHBA levels. Although both synchronisation protocols increased oestrus response and pregnancy rate, melatonin implant did not contribute to the increment of pregnancy rate compared to a single progesterone-based protocol in suckled Merino ewes with low BCS during the early postpartum period.

Key words: Melatonin implant, metabolic profile, undernutrition, body condition score, synchronisation, ewes

1. Introduction

Ewes conceive late or do not conceive until the following breeding season after lambing in conventional production systems [1]. The prolongation of the interval between lambing and breeding (mating) causes reduced lambing frequency and profitability [2]. However, the accelerated lambing system is suggested to shorten the time from lambing to breeding [3,4]. Early weaning of lambs without suckling is impractical in the conventional system. Thus, the requirement of conventional suckling prevents the system from reaching its theoretical potential of accelerating the lambing system [5]. Rapid breeding strategies including natural or hormonal protocols require candidate mothers to become pregnant during the early postpartum period in suckled ewes [4,6]. However, the

inhibitory effect of suckling on fertility is associated with increased prolactin levels [7]. Higher prolactin delayed ovarian activity by reducing melatonin secretion from the pineal gland in ewes [8].

On the other hand, ewes require more energy during early lactation and experience varying degrees of negative energy balance (NEB). In lactating ewes, unfavourable nutritional conditions cause more use of body energy reserves, resulting in a decrease in body condition score [9]. It was clearly demonstrated that NEB has a direct adverse effect on the 'hypothalamic-pituitary axis' and ovary [10]. Reproductive control of ewes is usually focused on oestrus synchronisation with exogenous hormones to improve fertility in ewes [2]. Melatonin-based synchronisation may be useful to improve fertility

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by decreasing prolactin levels in postpartum ewes [11,12]. However, various experimental studies demonstrated the negative effect of restricted nutrition on fertility [9,13,14]. Little is known about the influence of exogenous melatonin on the metabolic profile and fertility in postpartum undernourished ewes. In a previous study, it was reported that treatment with melatonin increased embryo viability in postpartum undernourished ewes [15].

Given the possible favourable effects of exogenous melatonin on ovary and prolactin levels in ewes, this study aimed to determine whether a combination of melatonin ear implant and progesterone-based oestrus synchronisation protocol might increase the pregnancy rate in ewes with low BCS during suckling period.

2. Materials and methods

Balikesir University Animal Care Committee approved the experimental procedures used (reference number: 2021/3-3).

2.1. Animals and management

Ninety lactating Merino ewes, ranging in age from 3 to 5 years, that had singleton lamb, were enrolled in the study. The study was carried out between November 19 and December 21, 2022, on a commercial farm in Balikesir (39° 33' N, 26° 58' E) which is located on the coastline of the Aegean Sea and the Sea of Marmara in Türkiye. A commercial dairy farm having an average of 3000 ewes was managed under a semi-intensive production system. In this study, ewes had a 3.10 ± 0.05 of mean BCS at lambing and they were individually kept together with their singleton lambs in pens of 3 m² per ewe with 3 for three weeks after lambing. As a rule of thumb, lambs were weaned at 85–90 days of age. During the preweaning period (from 21 to 85 days postpartum), 30 ewes and their lambs were partially housed. Ewes were grazed for 2 to 3 h a day and were kept with lambs indoors at night. During the experiment, ewes received 0.2 kg of barley grain (91.0% DM), 0.5 kg of corn silage (25.0 DM), and 1 kg of barley straw (88.0% DM) per ewe daily, as providing 2.1 Mcal/kg of ME and 6.2% CP. Lambs had access to fresh clean water ad libitum and concentrate commercial feed, as providing 16.55% CP and 2.53 Mcal/kg ME). Feeding and management practices were applied equally to all ewes and their singleton lambs over the study period.

2.2. Experimental design

BCS of ewes was determined by the palpation of the spinous and transversal processes of lumbar vertebrae at the time of lambing and postpartum synchronisation. It is based on a scale of 1–5 units, such that score 1 was considered very skinny (cachexia) and score 5 was very fat (obese) [16]. Merino ewes (n = 90) with a singleton lamb were equally assigned to two experimental groups [melatonin + progesterone + eCG (MPE) and progesterone + eCG

(PE)] and one control (CON) group on days 53 ± 3 (D0) postpartum. In the MPE group, an ear melatonin implant (18 mg, Regulin, CEVA, Türkiye) was subcutaneously applied on days 53 ± 3 (D0) postpartum for 35 days. After administration of the implant, medroxyprogesterone acetate (60 mg, Esponjavet, HIPRA, Turkey) impregnated intravaginal sponge was administered on days 88 ± 3 postpartum (D35) for 7 days. In the PE group, an intravaginal progesterone sponge was administered on days 88 ± 3 postpartum (D35) for 7 days. After sponge removal, 500 IU of equine chorionic gonadotropin (eCG) was administered in the MPE and PE groups on days 95 ± 3 postpartum (D42). In the CON group, ewes received no hormonal application.

The ram-to-ewe ratio was determined as 1/5 in this study. Eighteen fertile rams (2 to 3 of age and 3 to 3.5 of BCS) with mating crayon marks were introduced to the flocks for natural mating on D42. An ewe was considered to be in oestrus when crayon marks were observed on the rump. Ewes in oestrus were recorded twice a day (08.00 am and 5.00 pm) for 5 days after the introduction of rams to the flock. After sponge removal, pregnancy diagnoses were performed on days 30 (D72) and 60 (D102) via transrectal ultrasound, USG (Hasvet 838, Hasvet, Türkiye).

Ewes diagnosed pregnant at the first examination on day 30 but not pregnant at the second USG examination on day 60 were defined to have pregnancy loss.

2.3. Blood samples and analysis of metabolic parameters

Blood samples were collected at 08.00 to 10.00 pm from the subgroups (10 ewes per each group) via jugular vein using serum tubes to determine serum leptin, cortisol, insulin-like growth factor 1 (IGF-1), beta-hydroxybutyric acid (BHBA), and prolactin levels on days 53 ± 3 (D0), 88 ± 3 (D35), and 95 ± 3 (D42) postpartum. Serum samples were obtained by whole blood centrifugation for 15 min at $2000 \times g$ at 4 °C, transferred to 1.5 mL Eppendorf tubes, and stored at –20 °C for analyses.

Measurement of serum leptin levels was analysed with the Sheep Leptin ELISA kit (BT Lab, Bioassay Technology Laboratory, Zhejiang, China). Intraassay and interassay coefficients of variation (CV) were 2.96% and 2.96%, respectively. Sensitivity of the assay was 0.52 ng/mL. Prolactin, IGF-1, and cortisol levels were measured using Abbott i2000 automatic chemiluminescence immunoassay analyser (Abbott Diagnostics, Abbott Park, IL, USA). The prolactin, IGF-1, and cortisol intraassay coefficients of variation (CV) were 4.3%, 3.9%, and 5.1% while interassay CVs were 7.1%, 6.3%, and 8.5%, respectively. Sensitivities of the assays were 0.35, 0.23, and 0.12 ng/mL, respectively. Serum BHBA levels were measured using hand-held meter Precision Xceed (Abbott Diagnostics, Abbott Park, IL, USA).

2.4. Statistical analysis

Data were analysed by using the SPSS® 25.0 package program (SPSS Inc., Chicago, IL, USA) for statistical analyses. The chi-square test was used to compare the oestrus response and pregnancy rate among the groups. The repeated measures analysis of variance (ANOVA) was used to analyse the measurements of prolactin, leptin, IGF-I, BHBA, cortisol levels, and BCS. Results were reported as least square means and standard error of means (SEM). The significance level was considered as $p < 0.05$ for all analyses.

3. Results

The BCS (mean \pm SEM) of ewes was 2.30 ± 0.05 . There was no significant difference ($p > 0.05$) between the BCS of ewes among groups (MPE; 2.33 ± 0.05 , PE; 2.27 ± 0.05 , and CON; 2.31 ± 0.05) at the beginning of the study. Neither synchronisation protocol nor time affected the mean BCS ($p > 0.05$, Table 1).

Overall oestrus response as well as pregnancy rates on days 30 and 60 were 46.7% (42/90), 21.1% (19/90), and 20% (18/90), respectively. Apparently, oestrus response was higher ($p < 0.0001$) in both MPE (70%, 21/30) and PE (60%, 18/30) groups than that in the CON group (10%, 3/30). Further, the pregnancy rate on day D72 was also greater ($p = 0.018$) in both MPE (30%, 9/30) and PE (30%,

9/30) groups than that in the CON group (3.3%, 1/30). Unfortunately, a pregnancy loss was also observed in one ewe in the PE group (Table 2).

At the beginning of the study, the mean serum leptin, cortisol, IGF-1, BHBA, and prolactin levels were 3.49 ± 0.30 ng/mL, 6.28 ± 1.10 ng/mL, 53.38 ± 2.09 ng/mL, 0.24 ± 0.02 mmol/L, and 114.60 ± 6.97 ng/mL, respectively. Except for the prolactin levels, other hormones and metabolite levels in all groups did not significantly change ($p > 0.05$) over time. Synchronisation protocol statistically affected prolactin levels ($p < 0.05$). Although its level was not affected significantly by the time in PE (117.70 ± 12.49 , 109.10 ± 3.77 , 101.30 ± 4.85 ng/mL) and CON (114.30 ± 12.49 , 100.70 ± 3.77 , 111.90 ± 4.85 ng/mL) groups, significantly ($p = 0.043$) reduced levels (111.80 ± 12.49 , 97.40 ± 3.77 , 66.30 ± 4.85 ng/mL) were observed consistently in MPE group (Table 3).

4. Discussion

In early lactation, ewes experience varying degrees of NEB leading to loss of BCS that is considered normal [9,10]. It was found that Merino ewes experience a loss of 10 kg of live weight and a 0.20 unit of BCS after accessing adequate nutrition during three months of lactation. Furthermore, restricted nutrition caused even greater losses of 15 kg of live weight and 0.75 units of BCS in ewes [9]. As consistent

Table 1. Distributions of BCS (mean \pm SEM) of suckling Merino ewes in CON, PE, and MPE groups based on different days of administration in synchronisation protocol.

Days	CON	PE	MPE	Overall	p-value
Day 0	2.33 ± 0.13	2.27 ± 0.09	2.31 ± 0.05	2.30 ± 0.08	0.920
Day 35	2.39 ± 0.09	2.46 ± 0.10	2.35 ± 0.08	2.40 ± 0.07	0.502
Day 42	2.31 ± 0.05	2.40 ± 0.05	2.47 ± 0.05	2.39 ± 0.06	0.473

CON: Control group

PE: Progesterone + eCG group

MPE: Melatonin + Progesterone + eCG group

Table 2. Comparison of oestrus response and pregnancy rates based on different days of administration among groups in suckling Merino ewes.

Reproductive variables	CON	PE	MPE	Overall	p-value
Oestrus response	%10 ^a (3/30)	%60 ^b (18/30)	%70 ^b (21/30)	%46.7 (42/90)	0.0001
Pregnancy rate on Day 72	%3.3 ^a (1/30)	%30 ^b (9/30)	%30 ^b (9/30)	%21.1 (19/90)	0.018
Pregnancy rate on Day 102	%3.3 ^a (1/30)	%26.7 ^b (8/30)	%30 ^b (9/30)	20% (18/90)	0.022

CON: Control group

PE: Progesterone + eCG group

MPE: Melatonin + Progesterone + eCG group

Table 3. Comparison of metabolite and metabolic hormone levels (mean \pm SEM) in suckling Merino ewes that received different synchronisation protocols with the control group based on different days of administration

Parameters	Days	CON	PE	MPE	p-value
Leptin (ng/mL)	Day 0	3.36 \pm 0.54	3.73 \pm 0.54	3.37 \pm 0.54	0.490
	Day 35	2.94 \pm 0.61	3.98 \pm 0.61	3.63 \pm 0.61	
	Day 42	3.14 \pm 0.68	4.21 \pm 0.68	3.53 \pm 0.68	
Cortisol (ng/mL)	Day 0	7.92 \pm 1.95	6.05 \pm 1.95	4.88 \pm 1.95	0.313
	Day 35	9.17 \pm 1.77	4.37 \pm 1.77	7.48 \pm 1.77	
	Day 42	8.85 \pm 2.43	8.09 \pm 2.43	3.70 \pm 2.43	
IGF-1 (ng/mL)	Day 0	52.04 \pm 3.67	56.65 \pm 3.67	51.44 \pm 3.67	0.660
	Day 35	53.56 \pm 2.61	62.00 \pm 2.61	53.62 \pm 2.61	
	Day 42	60.86 \pm 3.27	54.37 \pm 3.27	48.44 \pm 3.28	
BHBA (mmol/L)	Day 0	0.24 \pm 0.04	0.18 \pm 0.04	0.31 \pm 0.04	0.377
	Day 35	0.23 \pm 0.03	0.24 \pm 0.03	0.22 \pm 0.03	
	Day 42	0.38 \pm 0.06	0.27 \pm 0.06	0.27 \pm 0.06	
Prolactin (ng/mL)	Day 0	114.30 \pm 12.49	117.70 \pm 12.49	111.80 \pm 12.49	0.043
	Day 35	100.70 \pm 3.77	109.10 \pm 3.77	97.40 \pm 3.77	
	Day 42	111.90 \pm 4.85 ^b	101.30 \pm 4.85 ^b	66.30 \pm 4.85 ^a	

CON: Control group

PE: Progesterone + eCG group

MPE: Melatonin + Progesterone + eCG group

with the previous study, the mean 0.80 unit of BCS, as decreased from 3.10 \pm 0.05 at lambing to 2.30 \pm 0.05 on days 53 \pm 3 postpartum, clearly indicated energetic restriction leading to a significant reduction in BCS in suckling Merino ewes in this study. The suboptimal BCS of ewes was not affected by the time during the experiment (from day 0 to day 35). However, previous studies reported that a BCS of 3.0–3.5 is optimal for better fertility in Merino ewes during the peri-conceptual period [9,16,17].

It is plausible that undernutrition is one of the major factors affecting fertility in ewes [14]. However, some metabolites of nonesterified fatty acid (NEFA) and BHBA reveal nutritional status and affect reproductive efficiency [10]. The increased BHBA concentration is one of the most important findings of NEB. As in BCS, the BHBA levels were not influenced by the time in this study. Although the BCS of ewes was suboptimal in this study, our findings for BHBA levels remained stable in all groups and were not close to the cut-off point of subclinical ketonemia (\geq 0.8 nmol/L) for ewes. Previous studies reported that ewes with BCS of 3.0 units have higher metabolic adaptive responses to undernutrition [18,19]. However, as consistent with our results, the BHBA levels did not change according to different levels of BCS. In a previous study, BHBA levels ranged from 0.41 to 0.51 nmol/L between 1.25, 2.0, 3.0,

and 4.0 units of BCS, respectively [18]. Thus, monitoring the BHBA level lower than 0.8 nmol/L may not indicate the severity of undernutrition in ewes with low BCS.

Undernutrition has a greater association with reduced fertility and suppression of metabolic hormones such as leptin and IGF-1 during the peri-conceptual period [20]. Basically, reduced leptin and increased cortisol levels help metabolic adaptation in undernutritional status in ewes [21]. The mean serum leptin levels were 3.49 ng/mL as ranged from 2.94 to 4.21 ng/mL in Merino ewes studied herein. Our finding was consistent with the previous results in Hampshire-Romanov crossbreed ewes during early lactation [22]. In that study, similar to our findings, ewes maintained BCS of 2.86 unit, and serum leptin levels (approximately 4 ng/mL) did not change during the lactation period [22]. Moreover, physiological condition and photoperiodic alterations changed leptin level which was at their peak at night and 50-h fasting reduced plasma leptin levels [23]. However, a recent study reported that leptin concentrations increased on long days in ewes [24]. In some studies, it was found that the concentrations decreased on short days and that the levels decreased to short-day levels in the case of food restriction on long days in Soay rams [25]. Other studies have reported no circadian change in plasma leptin levels in ewes [26,27]. As

consistent with this finding, we found no marked effect of exogenous melatonin on plasma leptin levels in the MPE group compared to others. The low frequency of blood collection may not have revealed the possible effect of melatonin administration on leptin concentration [23].

Negative energy balance triggers using animal's energy stores (glycogen, triglycerides, and protein) to meet the net nutrient deficit [10]. Fat and protein catabolism may increase depending on levels of nutritional restriction to support gluconeogenesis and glucose availability in undernourished ewes [20]. Both energy and protein availability are required for the maintenance of IGF-1. The effect of undernutrition on fertility is associated with reduced IGF-1 as secreted from the liver and other reproductive tissues [10]. Reduced IGF-1 secretion caused the decreased response of developing follicles to FSH [28]. Moreover, IGF-1 is crucial for embryo development, and undernutrition status caused a low number of embryo recovery and embryonic mortality in ewes subjected to subnutrition [19]. It was well known that undernutrition status is critical during peri-conceptual period. Our major presumption was that the exogenous melatonin increased IGFs expression and improved embryo quality in undernourished ewes since previous studies reported improvement in oocyte quality and embryo development in undernourished and optimally fed ewes [29,30]. In contrast, researchers of other studies have found that IGF-1 levels did not change in ewes that received exogenous melatonin during the early postpartum period [31] and in ewes exposed to altered postpartum photoperiod [32]. In the current study, IGF-1 level was not influenced by either exogenous melatonin or time effect. Further, all the factors of age, breed, nutrition, reproductive status (suckling), season, and blood sampling time at dark may also be responsible for the similar IGF-1 levels among groups studied herein.

Cortisol is one of the main indicators of stressful conditions. Suckling increases its levels and the increments have corresponding effects on mother-offspring relations in suckling ewes [7]. In the literature, nutritional stress slightly affected cortisol levels [33]. The levels increased on the second day of fasting in ewes but decreased on the fifth day of fasting compared to optimally fed ewes [28]. In a previous study, researchers found that exogenous melatonin administration decreased cortisol levels in ewes under an artificial light regime [34]. However, neither the time nor the exogenous melatonin implant changed the levels studied herein. Consistent with our results, it was found in a recent study that there were no seasonal differences in plasma cortisol levels in ewes [24]. The

ongoing suckling effect during administration [7], the long measurement interval [28], and the small number of samples leading to great variation may have masked the effect of melatonin on cortisol in our study.

It is well known that the intensity of mother-offspring relations by suckling induces strong prolactin stimulation [31]. However, Molik et al. [8] reported that the application of melatonin implant reduced the prolactin levels in suckling ewes during long days. Also, Elhadi et al. [31] demonstrated that exogenous melatonin implants also decreased prolactin levels in Manchega and Lacaune ewes during the early lactation period. Indeed, herein, application of the implants reduced prolactin levels in the MPE group compared to other groups after weaning of lambs (day D35). Similar to our study design, Molik et al. [35] administered melatonin in lactating ewes on days 56 postpartum. They found that prolactin levels did not change during 28 days after exogenous melatonin and low prolactin levels were found over the ongoing lactation stage. The reduction in melatonin secretion is accompanied by an increase in prolactin levels in ewes during long days. However, during short days, higher melatonin secretion has an inhibitory effect on prolactin secretion [31,35]. Melatonin modulates prolactin secretion with two mechanisms. The first mechanism of circadian rhythm affects prolactin storage in pituitary lactotopic cells. The second mechanism is that melatonin receptors are located in all cells of the pars tuberalis and prolactin secretion is sustained in pars tuberalis independently of the hypothalamus [35]. Reduced oestradiol concentration and lower LH release are one of the main reasons for delayed oestrus in suckling ewes during the postpartum period [7].

In addition to prolactin levels, breed, reproductive status (dry or lactating), age, and season are also major factors affecting fertility. Low oestrus response and pregnancy rate in the control group showed a strong seasonality effect in Merino ewes in this study. Monitoring BCS with optimisation of as many of these factors as possible improves fertility in ewes [36]. In this study, we presumed that supplementation of melatonin implant to progesterone-based synchronisation protocol would improve fertility in suckling ewes with low BCS during the postpartum period. Indeed, considering differences in synchronisation protocols used herein, oestrus synchronisation (progesterone plus 500 IU of eCG) in ewes increased oestrus response from 30% (control) to 70% (with melatonin) or to 60% (without melatonin) while pregnancy rate increased from 3.3% (control) to 30% (with/without melatonin, both on day D72). In progesterone-based synchronisation protocol, using progesterone as an intravaginal device mimics the luteal phase of the cycle and suppresses LH secretion from the

pituitary. Progesterone concentration abruptly ceases after removal of the intravaginal device and using eCG, has FSH and LH-like activities, at the time of removal induced oestrus behaviour and ovulation. Hence, considering the control group, it was clearly indicated that progesterone-based protocol induced higher oestrus response in undernourished suckling ewes in our study.

In a recent meta-analysis [12], researchers found that progesterone plus eCG protocol (60%) and melatonin protocol (64%) had higher pregnancy rates than the control group (37.5%) during the anoestrus season in ewes. Although our findings for oestrus response to both synchronisation protocols were in the range of previous reports, the pregnancy rate was lower than in the meta-analysis study given. The present results clearly showed the adverse effects of undernutrition on reproductive outcomes (only 30% of pregnancies following the progesterone-based synchronisation protocols with or without melatonin implants) in suckled Merino ewes. Indeed, the low BCS had a high unfavourable potential to affect fertility in mature ewes [37,38]. Although exogenous melatonin decreased prolactin levels, as expected, it did not have a markedly positive effect on IGF-1 levels and pregnancy rate in this study. One can presume that the discrepancies between the high oestrus response and low pregnancy rate obtained herein might have resulted from the reduced oocyte and embryo qualities. Firstly, it was well known that NEB has direct inhibitory effects on oocyte and embryo qualities [10,13]. The second reason could have been a strong relationship between undernutrition and reduced circulating plasma progesterone levels. Failure of supply of progesterone to the uterus leads to the failure of maternal recognition and embryo survival in mature ewes [10,39]. In this regard, aiming to use better-

conditioned (well-fed) ewes with BCS of 2.5 or higher (up to 3.50) units is advisable for achieving more acceptable (above 50% or more) fertility rates. Hence, a longer duration (12 or 14 days) of intravaginal sponge along with higher energy and/or protein intake (flushing) is advisable for a greater reproductive outcome for future trials.

In conclusion, our study showed that monitoring low body condition scores showed suboptimal nutritional status of females and undernutrition is one of the major detrimental factors to improving fertility in postpartum (suckling) Merino ewes. Stable low body condition score was consistent with unchanged serum leptin, BHBA, and cortisol levels throughout the experimental study. Although administration of exogenous melatonin implant reduced the prolactin levels, it did not markedly increase pregnancy rates and serum IGF-1 levels compared to ewes received the single progesterone-based protocol. Thus, it can be stated that progesterone-based protocol with or without melatonin implant increased fertility to shorten lambing interval in suckling undernourished Merino ewes during the early postpartum period.

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Conflict of interest

The authors have no conflicts of interest in this study.

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