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Effects of Short-Term Melatonin or Progestogen with Gonadotropic Treatments on Reproductive Performance, Hormonal Levels and Ovarian Activity of Ewes

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ABSTRACT

The study compares the effects of short-term application of melatonin alone with a commonly used progestogen + gonadotropin on the reproductive performance, serum hormone concentrations, and ovarian activity of Improved Wallachian sheep. To induce ovarian cyclicity, 382 ewes were allocated into two treatment groups. Ewes of treatment group 1 (n=26) received dietary melatonin (5mg/head/day) for 18 days. Ewes of treatment group 2 (n=356) received intravaginal sponges (FGA, flugestone acetate 20mg) for 12 days followed by injection of eCG (equine chorionic gonadotropin, 500IU i.m.) at the time of sponge withdrawal. Forty-eight hours after sponge or melatonin withdrawal, sheep were exposed to rams, and recorded reproductive performance. Six ewes from each group were selected for hormone assessment and their ovaries removed for histological analysis. Ewes receiving melatonin had lower fertility, fecundity, smaller follicles (0 – <1mm), and lower follicular atresia compared to group 2. In addition, the concentration of progesterone and IGF-I, ovulation rate, and the number of healthy follicles were increased compared to FGA+eCG-treated ewes. Our results indicate that short-term application of melatonin is effective in inducing ovarian cyclicity in ewes with an increased ovulation rate despite low fertility and fecundity.

Key words: Fertility, Follicular atresia, Melatonin, Ovarian hormones, Ovulation rate.

INTRODUCTION

Due to photoperiodism in northern regions sheep come into oestrus in the early autumn, in response to the shortening daily length. Regulation of reproduction in sheep is achieved by either limiting access to the ram, photoperiod control, and flushing. These interventions can be aided by pharmacological interventions (e.g. progestogens, prostaglandins, gonadotropins, and melatonin). Natural methods may be viewed as more ecologically friendly and economic-advantageous. In contrast, pharmacological approaches are both more efficient and more precise for the timing of oestrus and ovulation (Haresign 1992; Henderson and Robinson 2000; Martin et al. 2004).

Melatonin is a hormone produced under photoperiodic control. The hormone has been used to induce early seasonal breeding of ewes in several European countries and Australia and New Zealand (Cognie 1990; Henderson and Robinson, 2000; Martin et al. 2004; deNicolo et al. 2008; Abecia et al. 2012). Melatonin is produced by the pineal gland during the increasingly dark autumn phase when the shortening days result in increased secretion of gonadoliberin from the hypothalamus and consequently the secretion of gonadotropic hormones from the pituitary gland, and thereby ovarian activity (Abecia et al. 2012). Melatonin has been reported to influence follicular development (Tamura et al., 2009), steroidogenesis (Lima et al. 2015) and luteinisation of granulosa cells (He et al. 2016).

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Exogenous melatonin is most commonly used in form of subcutaneous ear implants in combination with other interventions, such as the introduction of the ram into a melatonin-treated ewe flock (Cognie 1990; Henderson and Robinson 2000) or ewes treated with progestogen/ progesterone in combination with various doses of a gonadotropin (deNicolo et al. 2008). To advance the onset of the breeding season in sheep, ear implants are usually applied for at least five weeks (Henderson and Robinson 2000; deNicolo et al. 2008). In our country, this method is rare because of limited availability and cost. Sheep breeders most commonly use methods based on progesterone analogue-vaginal sponges used in combination with a gonadotropin (Vlčková et al. 2008).

The aim of this study was to compare the effect of a short-term dietary supplement of melatonin alone, or a commonly used progestogen + gonadotropin treatment and observe the reproductive performance, serum steroid concentrations, and ovarian activity of Improved Wallachian sheep.

MATERIALS AND METHODS

Animal Welfare Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

Animals and Treatments

The experiment was conducted in field conditions in sheep rearing area of the sub-mountainous region of the Low Tatras, Slovak Republic (48°40'0" N/19°30'0" E, altitude 600-1000m). Ewes of Improved Wallachian breed were 4-6 years old and weighted 45±5kg with body condition scores (BCS) between 2.5-3.5 (Calavas et al. 1998). Ewes were pastured during the day with an addition of a maize grit (750g/head/day), and at night, they were penned ad libitum access to hay and water. During the experiment, the ewes were kept as one flock. All procedures were approved by the Ethical Committee of the State Veterinary and Food Administration of the Slovak Republic (Approval No. 2371/08-221).

In preparation for the approaching breeding season and the production of milk lambs for the Easter market, 382 ewes were allocated between two groups. Ewes in the Melatonin group (n=26) received melatonin (5mg//head/day; Spánok melatonin INSOM, Tachyon Technol Pharm, Slovak Republic), from the 8th August for 18 days (to the 25th August). Melatonin tablets were crushed for easier application and mixed into the maize grit. Ewes in the FGA+eCG group (n=356) received intravaginal sponges (FGA, flugestone acetate 20 mg, Chrono-gest Intervet, Netherlands) on the 13th August for 12 days and intramuscular (i.m.) injection of eCG (500 IU; Sergon a.u.v., Bioveta a.s., Czech Republic) at the time of sponge withdrawal (25th August). Forty-eight hours after sponge or melatonin withdrawal (27th August), sheep were mated (ratio 1 ram: 14 ewes) In addition,6 ewes from each group were employed for assessment of hormonal and ovarian status. In ewes exposed to rams, the following reproductive parameters were observed: fertility (number of ewes that lambed), fecundity (number of lambing per group), birth rate (number of lambs born per ewe), twinning rate (number of twin lambs per total number of lambs born), and lamb mortality rate (number of lambs died before birth or before weaning as a percentage of the total number).

Blood Collection and Hormone Assay

Blood was collected routinely by jugular venepuncture immediately before euthanasia (27th August; intravenous application of T61 inj. a.u.v., 4-6mL/head (Intervet International B.V., Boxmeer, Netherlands). Samples were centrifuged at 3500 rpm for 15min after coagulation at room temperature (2h at 20°C). Blood serum was stored at -20ºC until assayed. Progesterone (P4) and oestradiol-17β (E2) concentrations were determined by RIA, while insulin-like growth factor I (IGF-I) was measured by ELISA commercial kits according to the producer's instructions (Vlčková et al. 2014).

Necropsy and Morphometric Analysis

After euthanasia, a necropsy was performed on the ovaries collected. They were measured using a manual Empire Vernier calliper (Germany) for volume calculation, and ovulation rates were estimated as the number of corpora lutea (per ewe) by visual inspection light microscopy analysis.

Light Microscopy and Image Processing

Ovaries were processed using standard histological methods as described previously (Vlčková et al. 2014). The 5μm thick wedge sections were stained with hematoxylin and eosin (H-E) for evaluation using a digital camera (ProgResCapture Pro 2.7.7, Jena, Germany) and light microscopy (Nikon Eclipse E200, connected to a PC System for Image Analysis NIS Elements, (both Japanese manufacture). Every 20th section was evaluated per ovary. All antral follicles were counted and classified into the following size categories: 0-<1 mm, 1-<3 mm, and 3-5mm. The follicular diameter was calculated from a mean of two perpendicular measurements. Each antral follicle was assessed as healthy or atretic, based on microstructural characteristics according to the criteria of Marion et al. (1968). In addition, the type of atresia was determined according to the following criteria: early, collapsing, contractile, cystic, and late atresia.

Statistical Analysis

Reproductive parameters are expressed as percentages. The differences in reproductive parameters (fertility, fecundity, and litter size) between the groups were analysed by the $\chi 2$ test. Differences in hormone concentrations, ovary volumes, ovulation rates, and numbers of follicles between the groups were assessed by a paired t-test (Graph Pad Prism 3.0 for Windows, Graph Pad Software, San Diego, California, USA). The differences in the mean sizes of ovarian follicles between the groups were compared using an unpaired t-test. All data are shown as means with SEM. Differences between the groups tested were marked with superscript asterisks and considered significant at $P<0.05$ and $P<0.01$.

Table 1: Reproductive performance of Improved Wallachian ewes treated with melatonin or a combination of FGA+eCG for induction of breeding season

Treatment	FGA+eCG	Melatonin
Number of ewes	350	20
Fertility %	100	50**
Fecundity %	120	70*
Birth rate %	120	140
Twins %	20	27
Lamb mortality %	2	2

Asterisks in rows indicate significantly different mean values at *P<0.05, **P<0.01; FGA, flugestone acetate; eCG, equine chorionic gonadotropin.

Table 2: Concentrations (Mean±SEM) of progesterone (P4), oestradiol-17β (E2), and the insulin-like growth factor I (IGF-I) in the blood serum, ovary volume, and ovulation rate of ewes treated with melatonin or a combination of FGA and 500IU eCG

Treatment	FGA+eCG	Melatonin
P4 ($ng.l^{-1}$)	0.39±0.04	0.85±0.19*
E2 ($pg.l^{-1}$)	75.32±10.27	98.6±20.73
IGF-I ($ng.l^{-1}$)	44.00±5.89	79.56±10.87**
Ovary volume (cm^3)	2.41±0.35	2.54±0.57
Ovulation rate	0.17±0.17	0.67±0.30*

Asterisks in rows indicate significantly different mean values at *P<0.05, **P<0.01; FGA, flugestone acetate; eCG, equine chorionic gonadotropin.

Table 3: Mean±SEM Numbers of ovarian follicles of ewes grouped according to size and treated with melatonin or a combination of FGA and 500IU eCG

Treatment	FGA+eCG	Melatonin
Total No of follicles within size groups	27.50±4.10	20.67±7.07
No F 0 – <1 mm	16.50±3.31	11.67±4.40
No F 1 – <3 mm	10.33±2.43	7.67±2.69
No F 3 – 5 mm	0.00±0.00	0.33±0.21
Size F 0 – <1 mm	0.67±0.02 (n=100)+	0.60±0.02* (n=70)
Size F 1 – <3 mm	1.46±0.06 (n=58)	1.50±0.06 (n=48)
Size F 3 – 5 mm	0.00±0.00	3.04±0.04 (n=2)

Asterisks in rows indicate significance between mean values at *P<0.05; FGA, flugestone acetate; eCG, equine chorionic gonadotropin; F, follicle; +n, number of follicles analysed.

Table 4: Mean±SEM numbers of healthy and atretic follicles on the ovaries of ewes treated with melatonin and combination of FGA and 500 IU eCG

Treatment	FGA+eCG		Melatonin	
Number	Healthy F	Atretic F	Healthy F	Atretic F
Total	3.67±0.92	24.17±3.42	6.17±2.06	13.00±4.98
F 0 – <1mm	2.83±0.70	13.67±3.00	3.83±1.62	7.83±2.99
F 1 – <3mm	0.83±0.40	9.50±2.39	2.50±0.85*	5.33±2.36

Asterisks in rows indicate significantly different mean values at *P<0.05; SEM, standard error of the mean; FGA, flugestone acetate; eCG, equine chorionic gonadotropin; F, follicle.

RESULTS

Reproductive Performance

The reproductive performance of ewes treated with either melatonin or a combination of FGA+eCG is shown in Table 1. Melatonin-treated ewes lambed in two phases, at the end of January and continuously in the period from the 8th to 20th February. In the FGA+eCG-treated group, lambing took place continuously from the 29th January to the 3rd March, peaking at the end of January and on the 17th and 24th February. Of the 20 ewes treated with melatonin, only half lambed compared to FGA+eCG - treated ewes (fertility; P<0.05). In the melatonin group, also fecundity was significantly lower (P<0.05), although the birth rate and twinning rate tended to be greater than in the FGA+eCG group (P>0.05).

Hormone Concentrations and Morphometry of Ovaries

There were no differences between the groups for serum concentrations of E2, ovary volume, and ovulation rate (OR) (Table 2). The serum concentrations of P4 and IGF-I in melatonin ewes were almost twice the level (P<0.01) of the FGA+eCG group (Table 2).

Microscopic Analysis of Follicles

There were no differences between the groups in the number of follicles or in follicle mean size (Table 3), except for the follicles 0 – <1 mm category. The latter follicle type was smaller in the melatonin group (P<0.05) than in the FGA+eCG group (Table 3). There were only two follicles in category 3-5 mm found on the ovaries of melatonin ewes, while in the FGA+eCG treated ewes follicles of this category were not observed.

The number of healthy and atretic follicles on the ovaries of ewes treated with melatonin or a combination of FGA+eCG are shown in Table 4. The number of healthy follicles in the melatonin group was higher (P<0.05) than in the FGA+eCG group. There were no significant differences between the groups in total numbers of such follicles and follicles 0 – <1 mm category. Significant increases (P<0.05) in the number of healthy follicles were found in the melatonin group compared to the FGA+eCG group with no differences in the atretic follicle distribution (Fig. 1).

DISCUSSION

Melatonin treatments are reported to improve the reproductive performance of sheep and reduce the lambing period (Abecia et al. 2012; Mura et al. 2017). They have been widely used to induce oestrus by implanting in the ear for at least 35 days before a planned breeding season (Lalliotis et al. 1998; Henderson and Robinson 2000; deNicolo et al. 2008). The present experiment was performed to determine whether the short-term application of melatonin alone would improve the reproductive performance of Improved Wallachian ewes compared to commonly used progestogen + gonadotropin treatment. In the present study, melatonin was applied as a dietary supplement for 18 days with unexpected results. Melatonin-treated ewes lambed over 25 days in two phases, while ewes receiving the FGA+eCG-treatment lambed over about 33 days in three phases. This finding is in agreement with the report from Mura et al. (2017). Treatment with melatonin could be advantageous through shortening the lambing period. However, ewes receiving the progestogen + gonadotropin treatment in our study had double the level of fertility and almost double the level of fecundity compared to the ewes treated with melatonin. Similarly, the fertility of FGA+eCG (100%) and melatonin (50%) ewes were higher than the fertility after treatment with CIDR+eCG (60%) or melatonin + progesterone (11%) in Romney composite ewes reported by deNicolo et al. (2008). Moreover, Cosso et al. (2021) observed fertility of 92% in Sarda ewes treated with melatonin alone.

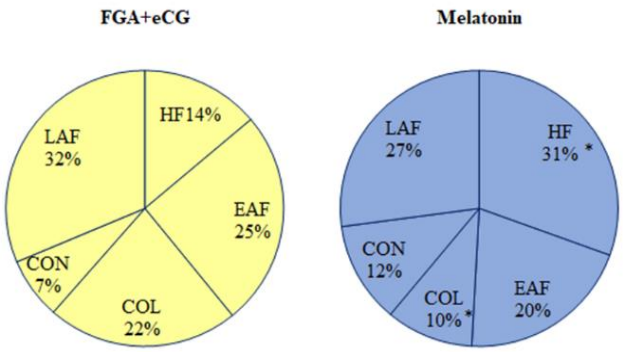

Fig. 1: Distribution of several types of follicular atresia on the ovaries of ewes treated with melatonin and the combination of FGA+eCG. Asterisk indicates significantly different mean values at $^{*}P<0.05$; HF: healthy follicle; EAF: early atretic follicle; COL: collapsing atretic follicle; CON: contracting atretic follicle; LAF: late atretic follicle.

The low fertility in melatonin treated sheep can be enhanced when used in conjunction with other techniques, such as ram effect exposure (Cognie 1990) or progestogen-impregnated intravaginal sponges (Henderson and Robinson 2000). In our study, a higher number of lambs (140%), and a tendency for a higher twinning rate, were observed after melatonin application. Our results are consistent with the study of Lalliotis et al. (1998), who incorporated melatonin implants into a MAP-based synchronization system during the anoestrous period. However, they are not in accordance with another study (Cosso et al. 2021) that reported a lambing rate of 112%.

Melatonin is a stimulator of ovarian steroidogenesis (Sirotkin 2014; Lima et al. 2015), luteinisation (He et al. 2016), proliferation (Sirotkin 2014), and production of IGF-I in human ovaries (Rai and Gosh 2021). The short-term dietary administration of melatonin to anoestrous sheep increased the concentrations of IGF-I andP4, but not E2, which were similar to our previous observations in Tsigai ewes treated with melatonin for 30 days (Kaľatová et al. 2009). However, P4 concentrations found by Vázquez et al. (2010) were higher in melatonin-treated sheep than was in the present study.

The average size of follicles in the 1–<3 mm category was larger in the group treated with melatonin. Follicles of 3-5 mm were only present in this group and are referred to as oestrogen-active follicles, their number determined by the ovulation rate (OR) (Webb et al. 1999). shorter application of melatonin (18 days) would induce ovine oestrus for earlier onset of the breeding season and increases both the OR and the number of twins. Higher OR was also reported after a 35-day application of melatonin implants (Lalliotis et al. 1998; Henderson and Robinson 2000; deNicolo et al. 2008; Abecia et al. 2012; Mura et al. 2017). However, the present results are not consistent with some other studies (Maurel et al. 2003; deNicolo et al. 2008). These studies reported that the combination of melatonin + progestogen + eCG can increase ovulation rates. Therefore, combining both methods used in our study could be a suitable means of increasing the number of lambs born per ewe treated out of season.

In sheep treated with a combination of progestogen and 500 IU eCG, we found a higher number of follicles on the ovaries, but with a lower incidence of healthy follicles and a higher proportion of atretic follicles (86%) than in the melatonin treated ewes (69%). Similar to another report (Rai and Gosh 2021), melatonin stimulated IGF-I production. Moreover, melatonin reduces oxidative stress in follicles and CL thereby preserving their health and inhibiting atresia (Reiter et al. 2013; Tamura et al. 2013). We found an increased incidence of atresia in the FGA+eCG treated sheep, up to 100% for follicles in the 3-5 mm category. This is in accordance with the observations of Hsueh et al. (1994) who reported up to 99.9% occurrence of follicle atresia. Atretic changes in follicles were observed at all levels of folliculogenesis, manifested as degeneration of the oocyte, granulosa cells, and theca interna cells as described in the literature (Rosales-Tores et al. 2000; Armstrong et al. 2001). In the group of sheep treated with the combination of preparations, we observed a higher proportion of follicles undergoing early, collapsing, and late atresia compared to the melatonin group. We reported similar results in our previous study performed on puerperal ewes (Vlčková et al. 2014).

Conclusion

Our results point to the possibility of using the short-term application of melatonin to induce breeding season in Improved Wallachian sheep in our conditions. Despite low fertility and fecundity, there is a significant birth rate, which indicates an increase in the ovulation rate and in the number of twin lambs after this treatment. Shortening the melatonin application to 18 days seems sufficient to induce ovarian cyclicity and reproductive performance of the ewes. The costs of treatment are also reduced. However, a more user-friendly method of administering the melatonin would be more advantageous for use, therefore subcutaneous implants with continuous prolonged release of the active substance should be investigated.

Conflict of Interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Author's Contribution

R. Vlčková: Validation, Formal analysis, Investigation, Writing - Original draft, J. Pošivák: Investigation, I. Valocký: Investigation, D. Sopková: Investigation, Z. Andrejčáková: Software, Visualisation, Z. Kostecká: Investigation, K. Kozioł: Writing - Review & Editing, A. Seidavi: Writing - Review & Editing. All authors read and confirmed the final version of manuscript.

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