Melatonin deprivation modifies follicular and corpus luteal growth dynamics in a sheep model

Maria Elena Manca, Maria Lucia Manunta, Antonio Spezzigu¹, Laura Torres-Rovira, Antonio Gonzalez-Bulnes², Valeria Pasciu, Peter Piu, Giovanni G Leoni, Sara Succu, Didier Chesneau^{3,4}, Salvatore Naitana and Fiammetta Berlinguer

Department of Veterinary Medicine, University of Sassari, Via Vienna 2, 07100 Sassari, Italy, ¹Embryo Sardegna, Technology, Reproduction, and Fertility, 07034 Perfugas, Località Suiles (SS), Italy, ²Department of Animal Reproduction, INIA, Avda. Puerta de Hierro s/n, 28040 Madrid, Spain, ³Institut National de la Recherche Agronomique (INRA), UMR85, F-37380 Nouzilly, France and ⁴CNRS, UMR 7247, Université François Rabelais de Tours, F-37041 Tours, France

Correspondence should be addressed to F Berlinguer; Email: berling@uniss.it

Abstract

This study assessed the effect of melatonin deprivation on ovarian status and function in sheep. Experimental procedures were carried out within two consecutive breeding seasons. Animals were divided into two groups: pinealectomised ($n=6$) and sham-operated ($n=6$). The completeness of the pineal gland removal was confirmed by the plasma concentration of melatonin. Ovarian status was monitored by ovarian ultrasonography for 1 year to study reproductive seasonality. Follicular and corpus luteal growth dynamics were assessed during an induced oestrous cycle. As the effects of melatonin on the ovary may also be mediated by its antioxidant properties, plasma Trolox equivalent antioxidant capacity (TEAC) was determined monthly for 1 year. Pinealectomy significantly extended the breeding season (310 ± 24.7 vs 217.5 ± 24.7 days in controls; $P < 0.05$). Both pinealectomised and sham-operated ewes showed a well-defined wave-like pattern of follicle dynamics; however, melatonin deficiency caused fewer waves during the oestrous cycle (4.3 ± 0.2 vs 5.2 ± 0.2 ; $P < 0.05$), because waves were 1 day longer when compared with the controls (7.2 ± 0.3 vs 6.1 ± 0.3 ; $P < 0.05$). The mean area of the corpora lutea (105.4 ± 5.9 vs 65.4 ± 5.9 mm²; $P < 0.05$) and plasma progesterone levels (7.1 ± 0.7 vs 4.9 ± 0.6 ng/ml; $P < 0.05$) were significantly higher in sham-operated ewes compared with pinealectomised ewes. In addition, TEAC values were significantly lower in pinealectomised ewes compared with control ones. These data suggest that melatonin, besides exerting its well-known role in the synchronisation of seasonal reproductive fluctuations, influences the growth pattern of the follicles and the steroidogenic capacity of the corpus luteum.

Free Italian abstract

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Introduction

Seasonal breeders have developed mechanisms to restrict fertility to a particular time of the year. This ensures the birth of the offspring during the most favourable season, generally when the resources needed to support the requirements of lactation and post-weaning growth of the offspring are most abundant. Domestication may have attenuated or suppressed some of the various physiological expressions of seasonality, but domesticated small ruminants have retained most of them.

Sheep, like the majority of seasonal breeders, use photoperiod as the main environmental cue to establish

the time of year. Ewes have a breeding season characterised by a succession of 16–18-day long oestrous cycles, which usually start in late summer or at the beginning of autumn and end in late winter or at the very beginning of spring, in the northern hemisphere but varies according to breed and latitude (Ortavant *et al.* 1985). Thereafter, the anoestrous season takes place without behavioural or ovarian cyclicity. Ambient photoperiod is transduced by a photoneuroendocrine system composed of the retina, the suprachiasmatic nucleus (seat of the master circadian clock) and the pineal gland (Malpoux *et al.* 2001). The latter releases the hormone melatonin exclusively at night, so that the duration of secretion varies according to day length

and provides an endocrine representation of photoperiod (Malpaux *et al.* 2001, Simonneaux & Ribelayga 2003). Melatonin transmits day length information to the neuroendocrine–gonadal axis exerting indirect actions on the Kiss1/GPR54 system responsible for controlling reproduction via the neural axis by tuning circulating gonadotrophin and sex steroid levels (Revel *et al.* 2007).

As described above, it is generally accepted that melatonin exerts its primary reproductive action at the level of the brain and pituitary. However, the presence of high melatonin levels in follicular fluid (Brzezinski *et al.* 1987) and the presence of receptors in granulosa cells (Yie *et al.* 1995, Niles *et al.* 1999, Woo *et al.* 2001, Soares *et al.* 2003a, Wang *et al.* 2012, Barros *et al.* 2013) have suggested a role of melatonin on ovarian function. This hypothesis has been confirmed by recent studies reporting strong evidence for melatonin action on the ovary, such as modulation of ovarian steroidogenesis (mainly progesterone (P₄) production; Taketani *et al.* 2011) and contribution in the maintenance of a proper follicular structure and function (Soares *et al.* 2003a,b, Barros *et al.* 2013, Maganhin *et al.* 2013). In a previous study, we demonstrated that melatonin supplementation during the anoestrous period modifies the pattern of follicular waves by increasing the turnover of dominant follicles (Berlinguer *et al.* 2009).

The main role of melatonin within the follicle may be also to act as a free radical scavenger. The antioxidant properties of melatonin have been extensively studied (Reiter *et al.* 2013, Tamura *et al.* 2013). The scavenger activity of melatonin or of its metabolites may thus account for its beneficial effect on follicular and oocyte development (Tamura *et al.* 2008).

This study aimed to investigate the effect of melatonin deprivation on follicular and corpus luteal growth dynamics in sheep. In addition, the effect of melatonin deprivation on blood total antioxidant capacity was determined.

Materials and methods

Chemicals

All reagents and media were purchased from Sigma Chemical Co. unless otherwise specified.

Animals and experimental design

Approval was obtained from the Institutional Animal Care and Use Committee, and all procedures conformed to the National Institutes of Health Guidelines for the Care of Laboratory Animals. All the animals used were adult multiparous Sarda ewes from a uniform flock ($n=12$; 2.5–5 years old, body weight 39.5 ± 7.7 kg) housed outdoors with indoor access and fed with a live-weight maintenance ration following nutritional requirements recommended by NRC (2007) for an adult ewe of 40 kg body weight. This study was conducted at the experimental facilities of the Department of Veterinary

Medicine of the University of Sassari, Italy (latitude 40°43'N). These facilities meet the requirements of the European Union for Scientific Procedure Establishments.

All animals received physical and neurological examinations, complete blood count, serum biochemical analysis and urine analysis. Animals considered unhealthy were excluded from the study. Ewes were randomly divided into two groups: i) pinealectomised ewes ($n=6$) and ii) sham–pinealectomised ewes ($n=6$).

Plasma melatonin concentration was determined in each animal before the surgery, and 60 days and 12 months after the surgery. One year after the surgery, ewes were enrolled in the study for the evaluation of the effects of melatonin deprivation on ovarian status. The observational period was comprised within two consecutive natural breeding seasons (from late August 2010 to late December 2011) described for this breed at this latitude. To determine the effect of melatonin deprivation on reproductive seasonality, ovarian status was determined weekly during a complete natural year (starting on August 22) by transrectal ultrasonography. The presence of ovulation, and thus of oestrous cyclicity, was confirmed by the visualisation of corpora lutea. During this period, jugular blood samples were withdrawn every 30 days to evaluate total antioxidant capacity throughout a complete natural year in pinealectomised and sham-operated ewes.

At the end of the observation period (December), follicular and corpus luteal growth dynamics were monitored during an induced oestrous cycle in pinealectomised and sham-operated ewes. Ovulation was synchronised in all the ewes with two doses of 125 µg prostaglandin analogue (cloprostenol, Estrumate, Essex Animal Health, Friesoythe, Germany) given 10 days apart. Corpora lutea and follicular development were assessed by daily transrectal ultrasonography performed from the day of oestrus detection with vasectomised rams until the next ovulation. Coincidentally, luteal cell function was evaluated daily by determining plasma P₄ concentrations from jugular blood samples.

Surgical procedure

Pre-medication, induction, maintenance of anaesthesia, analgesic treatment and intraoperative monitoring

The ewes were kept off feed 24 h prior to surgery but had free access to water for up to 3–4 h prior to surgery. Ewes received 2.0 mg/kg i.v. ketoprofen (Vet-Ketofen 1%, Merial, Assago, Italy), 0.4 mg/kg i.v. diazepam (Diazepam 0.5%, Intervet GmbH, Unterschleißheim, Germany) and 6 µg/kg i.v. fentanyl (Fentanest, Actavis, Nerviano, Italy). The scalp and the subcutaneous tissue were infiltrated with 10 ml mepivacaine 2% (Mepibil, Hospira, Naples, Italy). For induction, sheep received 8 mg/kg thiopental sodium i.v. (Pentothal; Intervet GmbH) followed by orotracheal intubation.

Ewes were maintained with sevoflurane (Sevoflo; Abbott Laboratories) in oxygen (O₂)/air through a small animal circle system (Fabius GS, Dräger, Lübeck, Germany). Animals were maintained in mechanical ventilation and the ventilator was adjusted to maintain the end tidal CO₂ between 28 and 32 mmHg (moderate hyperventilation).

Crystalloid solution (NaCl 0.9%; Acme, Cavriago, Italy) was infused at a rate of 5–10 ml/kg per h to support circulating blood volume. After the craniectomy, 1 ml of 2% mepivacaine was dropped over the dura 1 min before its incision. At the end of the surgery, a bolus of i.v. buprenorphine (0.02 mg/kg; Temgesic, Schering–Plough S.p.A., Segrate, Italy) was administered to ensure an adequate post-operative analgesia and this procedure was repeated after 4 h. Just before the extubation i.v. lidocaine (1 mg/kg in bolus; Lidocaina 2%, Fort Dodge, Aprilia, Italy) was administered to avoid an increase in intracranial pressure.

Pinealectomy

Briefly, animals were placed in sternal recumbency with the head elevated no more than 30° above cardiac level with the tip of the nose tilted downwards and secured in a craniotomy head stand. A dorsal midline skin incision of ~5–7 cm in length was made beginning from the proximal attachment of the ligamentum nuchae with rostral direction. The subcutaneous fascia and the thin cutaneous muscles of the head (m. occipitalis and m. frontalis) were divided medially. The underlying muscles cervicospinal and parietoauricularis were medially incised, divided, elevated and retracted with Gelpi self-retractor to expose the underlying skull.

Craniectomy was then performed using an electrical drill with round burs. Pinealectomy was performed according to the procedure described by Dempsey *et al.* (1982). On opening the dura mater, the left parietooccipital cortex, the proximal portion of the tentorium cerebelli and, shielded by the edge of the bone opening, the dorsal sinus were exposed. The brain was bathed almost continuously in warm sterile saline. With a padded retractor inserted into the transversal cerebral fissure, the parietooccipital lobe was gently moved in rostral and lateral direction, thus allowing exposure of the deepest portion of the tentorium cerebelli. The vault of the quadrigeminal cistern was opened with a dissector and the cerebrospinal fluid was drained. Inserting the second spatula, the rostral colliculi were reached rostrally and dorsally to visualise the pineal gland. With the help of a dissector, the gland was delicately freed from the little vessels and nerves that surround it and, ventrally, from the internal and basal cerebral veins. The epiphysis was grasped and removed with forceps. Synthetic graft (Graftygen, Tecnos s.r.l., Torino, Italy) was sutured over the dural and the skull defects. The muscles were apposed on midline and the subcutaneous tissue and the skin were apposed routinely. The animals were given daily antibiotic (amoxicillin + clavulanic acid, 20 mg/kg per day for 10 days i.m.) and anti-inflammatory therapy for 5 days. The same procedure was performed on the animals in the sham–pinealectomised group, but the pineal gland was left *in situ*.

Morphometric evaluation of follicular and corpus luteal growth dynamics

Ovaries were examined by transrectal ultrasonography using a real-time B-mode scanner (Aloka SSD 500, Aloka Co., Tokyo, Japan) fitted to a 7.5 MHz linear array probe. Scanning was performed as described previously (Gonzalez-Bulnes *et al.*

2010). In brief, after introducing a hydrosoluble contact gel into the rectum, the transducer was introduced perpendicular to the abdomen wall. When the urinary bladder was surpassed and the uterine horns were located, the probe was rotated laterally 90° clockwise and 180° anticlockwise to observe both ovaries and their structures.

Each ovary was scanned several times from different angles in order to ensure that all follicles ≥ 2 mm (during the follicular phase) and corpora lutea (during the luteal phase) were seen. The largest diameter of each follicle and the area of the corpora lutea were measured using the electronic callipers of the ultrasound machine. The position of the largest follicles was recorded on a diagram of each ovary. In those corpora lutea bearing a cavity, its area was calculated and subtracted for obtaining the correct measurement of the area occupied by the luteal tissue.

Hormone assay

For the evaluation of plasma melatonin concentrations, two blood samples were taken from the jugular vein using vacuum blood evacuation tubes containing EDTA (Vacutainer Systems Europe, Becton Dickinson, Meylan Cedex, France). The samples were withdrawn in the afternoon at 1200 h and at night at 2400 h in the dark, immediately centrifuged and preserved at -80°C until assayed. Melatonin concentration was determined as described previously (Zarazaga *et al.* 2010). In short, melatonin was assayed by a direct RIA method with 2-[125I]iodomelatonin as a tracer (Vakkuri *et al.* 1984) and a previously described anti-melatonin antiserum (Tillet *et al.* 1986) (melatonin Fluka-63610). This direct assay, originally described by Fraser *et al.* (1983), was performed according to the modifications of Webley *et al.* (1985) and Ravault *et al.* (1989). Briefly, 100 μl rabbit anti-melatonin antiserum (final dilution: 1/400 000) and 300 μl 2-[125I]iodomelatonin (15 000 c.p.m./tube) were added to 100 μl assay buffer (tricine) or undiluted plasma. After 16–18 h of incubation at 4°C , the antigen–antibody complexes formed were precipitated by addition of 1 ml sheep anti-rabbit antiserum (INRA). After 1 h of incubation, the samples were centrifuged (2800 *g* for 30 min at 4°C), then the supernatant was discarded and the pellet counted on a Gamma counter. A standard curve consisting of 4–1000 pg/ml melatonin in pinealectomised sheep plasma was prepared. The value of coefficient of variation (CV) was estimated by assaying three plasma pools (low, medium and high concentrations) in duplicate for every 50 unknown samples. All samples were analysed in the same assay and the intra-assay CV value was 12.8%.

The luteal cell functionality was assessed by determining plasma concentrations of P_4 in samples obtained, coincidentally with ultrasound scanning, using vacuum blood evacuation tubes containing lithium heparin (Vacutainer Systems Europe, Becton Dickinson). Immediately after recovery, blood samples were centrifuged at 1500 *g* for 10 min, and plasma was removed and stored at -20°C until assayed. P_4 levels were measured in duplicate using a commercial ELISA Kit (DRG Instruments GmbH, Marburg, Germany), which is a solid-phase ELISA, based on the principle of competitive binding. The microtiter wells were coated with a polyclonal antibody

directed towards an antigenic site on the P_4 molecule. Endogenous P_4 of a sample competes with a P_4 -HRP conjugate for binding to the coated antibody. After incubation, the unbound conjugate is washed off. The amount of bound enzyme conjugate is visualised by the addition of the substrate solution (3,3',5,5'-tetramethylbenzidine) and incubation that leads to the development of a blue colour. The substrate reaction is stopped by the addition of sulphuric acid. The colour intensity is measured photometrically at 450 nm and is inversely proportional to the P_4 concentration in the sample. All kit reagents, controls and stored samples to be analysed were brought to room temperature (25 °C) at the beginning of the test. All assays were performed using the Personal Lab Adaltis (Adaltis srl, Rome, Italy), which is a tool that performs automated ELISA protocols. The analytical sensitivity was 0.045 ng/ml and the intra- and interassay CV values were <10%.

Determination of Trolox equivalent antioxidant capacity in plasma

Samples of blood plasma (EDTA) were processed rapidly and kept frozen at -80 °C until assayed. Plasma Trolox equivalent antioxidant capacity (TEAC) was determined using the method described by Re *et al.* (1999) and modified by Lewinska *et al.* (2007). Briefly, a fresh solution was prepared by dissolving 19.5 mg 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS)) and 3.3 mg potassium persulphate in 7 ml of 0.1 mol/l phosphate buffer, pH 7.4. This solution was stored in the dark for 12 h for completion of the reaction. ABTS+ solution was diluted (usually ~1:80) in 0.1 mol/l phosphate buffer, pH 7.4, to give an absorbance reading of 1.0 at 734 nm and mixed thoroughly. The absorbance of the mixture was measured twice in a spectrophotometer (Thermo Electron Corporation Genesys 10 u.v., Madison, WI, USA), at 734 nm for 3 min after mixing a sample with the ABTS+ solution. The extent of ABTS+ bleaching (decrease in absorbance, corrected for a small decrease in absorbance of ABTS+ solution alone) is proportional to the activity of antioxidants in a given sample. The antioxidant capacity was expressed as TEAC, that is, the concentration (amount) of Trolox producing the same effect as the sample studied. Calculations were made on the basis of standard curves obtained for a Trolox solution (5–20 µM Trolox).

Statistical analyses

To analyse differences in growth patterns, data for the number of follicles and functionality of corpora lutea were normalised with respect to the day in which ovulation was detected. First, ultrasonographic data during the induced oestrous cycle were summarised to characterise patterns of ovarian follicular development. All follicles detected by ultrasonography were classified by their largest diameter and, thereafter, were grouped as total (≥ 2 mm), large (≥ 4 mm) or small follicles (< 4 mm). Secondly, ultrasonographic and plasma hormonal data during luteal phase were summarised to characterise patterns of growth dynamics and functionality of corpora lutea. Data on individual growth of dominant follicles were used to

characterise follicular waves on the basis of: i) wave onset (emergence), the day on which follicles were first detected at 3 mm; ii) growth phase (length), the time taken by a single follicle to grow from 3 mm to its maximum diameter; iii) regression phase, the time taken by a single follicle to regress from its maximum size until the day on which it reached its smallest size; iv) wave end, the day when the dominant follicle ended its regressing phase and v) wave duration (total length), the time taken by a single follicle to grow from 3 mm to its maximum diameter and to completely regress to its minimum size. Effects of day and wave of follicular development on individual characteristics of dominant follicle throughout the study were assessed by ANOVA. Effects of melatonin deprivation on the time length of the reproductive season, number and size of follicles, number of ovulations per cycle, size of corpora lutea, hormone concentrations and TEAC values were analysed by a split-plot ANOVA, followed by a Kruskal–Wallis test, when Levene's test showed non-homogeneous variables. Duncan's multiple range tests were used for significant interactions.

Statistical analysis was performed using the statistical software program Statgraphics Centurion XV (version 15.2.06 for Windows; StatPoint, Inc., Herndon, VA, USA) and a probability of $P \geq 0.05$ was considered to be the minimum level of significance. All results were expressed as the mean $\pm$ S.E.M.

Results

Determination of melatonin plasma levels confirmed the surgical removal of the pineal gland in pinealectomised ewes (Fig. 1). Melatonin concentration during the day did not differ in the two groups, being < 4 pg/ml. After the surgery, the night values did not rise in pinealectomised ewes and remained significantly lower than that in sham-operated ewes ($P < 0.01$).

Reproductive seasonality was affected by melatonin deprivation. Pinealectomised ewes had a longer reproductive season when compared with sham-operated ewes

Figure 1 Night values of melatonin (blood sampling was performed at 2400 h) in sham-operated ($n=6$) and pinealectomised ewes ($n=6$) before and after the surgery (2 and 12 months). *Significant difference between sham-operated and pinealectomised ewes (ANOVA, $P < 0.05$).

Figure 2 Example of wave-like pattern of follicle dynamics in sham-operated and pinealectomised ewes during an induced oestrous cycle in the breeding season.

(310 ± 24.7 vs 217.5 ± 24.7 days respectively; $P < 0.05$). Two pinealectomised ewes cycled year round.

The time length of the induced oestrous cycle (17 ± 1.6 vs 18.2 ± 1.4 days in sham-operated and pinealectomised ewes respectively) and the ovulation rate (1.4 ± 0.2 vs 1.1 ± 0.2 in sham-operated and pinealectomised ewes respectively) did not differ between the two experimental groups.

The ultrasonographic study indicated that both pinealectomised and sham-operated ewes showed a well-defined wave-like pattern of follicle dynamics (Fig. 2); however, there were some significant differences between groups in the characteristics of the follicular waves (Table 1).

Pinealectomy resulted in fewer waves during the oestrous cycle (4.3 ± 0.2 vs 5.2 ± 0.2 ; $P < 0.05$), because waves were 1 day longer when compared with the controls ($P < 0.05$). Analysing one by one the different follicular waves through the oestrous cycles, it was observed that the longest waves in pinealectomised ewes were the third (8.2 ± 0.8 days) and the fourth (8.3 ± 0.8 days; Fig. 3) waves. In particular, they lasted longer when compared with both the ovulatory wave (5.2 ± 0.5 days; $P < 0.05$) and the respective follicular waves in sham-operated ewes (third wave, 6 ± 0.9 days and fourth wave, 5.8 ± 0.6 days; $P < 0.05$). In addition, the regression phase of the first follicular wave lasted longer in pinealectomised ewes compared with control ones (2 ± 0.2 vs 1.1 ± 0.2 respectively; $P < 0.05$). In contrast to pinealectomised ewes, where no differences emerged among the length of the dominant follicle growth phase through the oestrous cycle, in sham-operated ewes ovulatory follicles took longer (5.5 ± 0.4 days) to reach their maximum diameter when compared with the

other follicles which developed during the luteal phase (first wave, 3.3 ± 0.5 days; second wave, 4.3 ± 0.5 ; third wave, 3.6 ± 0.5 and fourth wave, 3.5 ± 0.5 ; $P < 0.05$). No differences were recorded in ovulatory wave characteristics between the two experimental groups.

The number of small (< 4 mm in diameter), large (≥ 4 mm) and total follicles (≥ 2 mm) varies across the oestrous cycle in both groups (Fig. 4). In particular, in sham-operated ewes, the number of small, large and total follicles increased during the luteal phase, peaked approximately within days 10 and 15 of the oestrous cycle and then dropped again in the follicular phase ($P < 0.05$). In pinealectomised ewes, the pattern of follicle development was not homogeneous among the different follicle categories. Small follicles peaked in the very first days of the luteal phase, then rose again at the end of the luteal phase (days 9–15) and in the first days of the follicular phase ($P < 0.01$). In the same way, no precise pattern of follicle development could be depicted for large follicles. The same pattern was followed approximately by the number of total follicles. In addition, the mean number of follicles belonging to the different categories differed between the two experimental groups. The number of small follicles was higher in pinealectomised ewes compared with sham-operated ones (13.5 ± 0.3 vs 12.2 ± 0.3 ; $P < 0.05$). Conversely, the number of large follicles was higher in sham-operated ewes compared with pinealectomised ones (2.7 ± 0.1 vs 2.3 ± 0.1 ; $P < 0.05$). No difference was recorded in the mean number of total follicles.

The mean area of the corpora lutea recorded by ultrasonography was significantly smaller in pinealectomised ewes compared with sham-operated ones (65.4 ± 5.9 vs 105.4 ± 5.9 mm²; $P < 0.0001$). These differences were confirmed by the analysis of plasma P₄ level (Fig. 5), whose mean values were lower in pinealectomised ewes compared with sham-operated ones (4.9 ± 0.6 vs 7.1 ± 0.7 ng/ml; $P < 0.05$).

TEAC values were significantly lower in pinealectomised ewes compared with control ones ($P < 0.01$; Fig. 6A). No difference was observed in the two

Table 1 Characteristics of the follicular waves in pinealectomised ($n = 6$) and sham-operated ewes ($n = 6$) during an induced oestrous cycle in the breeding season.

	Sham-operated ewes (mean $\pm$ S.E.M.)	Pinealectomised ewes (mean $\pm$ S.E.M.)
Number of follicular waves	5.2 ± 0.2^a	4.3 ± 0.2^b
Follicular wavelength (days)	6.1 ± 0.3^a	7.2 ± 0.3^b
Maximum diameter reached by DF (mm)	5.8 ± 0.1	5.9 ± 0.1
DF growth rates (mm/day)	0.74 ± 0.05	0.69 ± 0.05
DF regression rates (mm/day)	1.4 ± 0.1	1.4 ± 0.1
DF growth phase (days)	3.9 ± 0.2	4.5 ± 0.2
DF regression phase (days)	1.8 ± 0.2	2.4 ± 0.2

In the same row, different superscripts indicate statistical difference (ANOVA $a \neq b$, $P < 0.05$). DF, dominant follicles.

Figure 3 Differences in the characteristics of the follicular waves in sham-operated ($n=6$) and pinealectomised ewes ($n=6$) during an induced oestrous cycle in the breeding season. *Difference in regression rates between experimental groups (ANOVA, $P<0.01$). **Difference in wavelength between experimental groups (ANOVA, $P<0.05$). ***Difference in both wavelength and growth rates between experimental groups (ANOVA, $P<0.05$). Different letters indicate differences among wave characteristics in the same group: lower case letters in sham-operated ewes indicate differences in maximum diameter of follicles and upper case letters in pinealectomised group indicate differences in wavelength (ANOVA, $P<0.05$).

experimental groups when data were analysed on a monthly basis (Fig. 6B).

Discussion

Three main conclusions can be drawn from our study. First, we are reporting for the first time that melatonin deprivation affects follicular wave growth pattern during the oestrous cycle. Secondly, it also affects corpus luteal

growth and function, in terms of P_4 production. Thirdly, we are confirming previous studies reporting that pinealectomy alters reproductive seasonality in seasonal breeders (Dardente 2012).

Melatonin plays a deterministic role in the regulation of seasonal reproduction in mammals. Classical studies in sheep, and other mammalian species expressing circannual rhythms, indicated that elimination of the circadian secretion of melatonin by removal of the

Figure 4 Number of small (<4 mm in diameter), large (≥4 mm) and total follicles (≥2 mm) in sham-operated ($n=6$) and pinealectomised ewes ($n=6$) during an induced oestrous cycle in the breeding season. *Significant difference between sham-operated and pinealectomised ewes (ANOVA, $P<0.05$).

Figure 5 Mean area of corpora lutea (A) and plasma progesterone levels (B) in sham-operated ($n=6$) and pinealectomised ewes ($n=6$) during an induced oestrous cycle in the breeding season. Day 0, oestrus onset. *Significant difference between sham-operated and pinealectomised ewes (ANOVA, $P<0.05$). ** indicates values which tended to be significantly different between sham-operated and pinealectomised ewes (ANOVA, $P<0.056$)

pineal gland disrupts photoperiodic responsiveness and causes circannual rhythms to free-run (Herbert *et al.* 1978, Zucker 1985, Woodfill *et al.* 1994). It has been described that pinealectomy abolishes seasonal responses, while melatonin administration restores them (Reiter *et al.* 1980, Woodfill *et al.* 1994, Arendt 1998). This study demonstrated a significant extension of the reproductive season in pinealectomised ewes, with two ewes cycling all year round.

At all stages of the breeding season and throughout seasonal anoestrus, the growth of antral ovarian follicles exhibits a distinct wave-like pattern in ewes (Bartlewski *et al.* 1998, Evans *et al.* 2000). During the breeding season, in both prolific and non-prolific breeds, there are typically three or four waves of follicle emergence per interovulatory interval (for review, see Bartlewski *et al.* (2011)). This pattern of antral follicular development is closely associated with periodic elevations in serum concentrations of follicle-stimulating hormone (FSH); peaks of transient increases in daily FSH concentrations occur just prior to follicle wave emergence (Bartlewski *et al.* 2000).

In this study, pinealectomy did not modify the pattern of follicular development, as pinealectomised ewes showed a well-defined wave-like pattern of follicular dynamics. On the other hand, follicular development was altered by melatonin deprivation, as evidenced by the lower number of follicular waves and by their longer length in pinealectomised ewes. This result confirms previous findings in goats that melatonin treatment during the anoestrous season increases the turnover of

dominant follicles by reducing their period of emergence, thus shortening the time length of the follicular waves (Berlinguer *et al.* 2009). Melatonin has been proposed to regulate the pulsatile secretion of gonadotropin-releasing hormone from the hypothalamus, thereby influencing FSH/luteinising hormone (LH) secretion (Bronson 1995) and favouring follicular development. Recent data suggest that, in seasonal breeders, melatonin could act on the KiSS1 cells to modulate reproductive activity (for review, see Revel *et al.* (2007)). In the ewe, which is a short-day breeder and so responds to a long melatonin secretion period, there is evidence that the pattern of melatonin secretion governs *KISS1* mRNA expression in the arcuate nucleus (Smith 2012). Considering that kisspeptin potently elicits LH/FSH secretion (Thompson *et al.* 2004, Messenger *et al.* 2005), the activation of this endocrine pathway may create a more suitable hormonal milieu for follicle growth, and this may explain the slower rate of follicular development in pinealectomised ewes.

It should be pointed out that gonadotrophins work in concert with locally produced growth factors, such as the insulin-like growth factors (IGFs), to promote and regulate follicular development. IGFs are mitogenic and antiapoptotic peptides produced by granulosa and theca cells during follicular development (Poretsky *et al.* 1999). These factors stimulate DNA synthesis and oestradiol and P_4 secretion by human granulosa and granulosa-luteal cells (Poretsky *et al.* 1999). It has been demonstrated that melatonin stimulates IGF1 production

Figure 6 Trolox equivalent antioxidant capacity (TEAC) values of blood from pinealectomised ($n=6$) and sham-operated ewes ($n=6$): (A) mean values and (B) monthly values. *Significant difference between pinealectomised and sham-operated ewes (ANOVA, $P<0.01$).

by cultured human granulosa cells (Schaeffer & Sirotkin 1997). Thus, a disturbance in IGF1 action may also have contributed to the slower rate of follicular development in pinealectomised ewes.

In this study, the alteration in follicular growth pattern observed in pinealectomised ewes was not accompanied by alterations in the process of ovulation, as the ovulation rate did not differ between pinealectomised and sham-operated ewes. In rats, pinealectomy resulted in the absence of ovulation (Maganhin *et al.* 2013), while in other species no changes in ovulation rates were observed after the surgical removal of the pineal gland (wolf, Grubaugh *et al.* (1982) and pony mare, Asa *et al.* (1987)). A previous study reported that pinealectomised ewes can have a normal pregnancy and deliver a healthy newborn (Kennaway *et al.* 1985). More recent studies suggest that melatonin have a role in the process of ovulation (Tamura *et al.* 2009), considering the presence of both types of membrane melatonin receptors in granulosa cells and that melatonin can up-regulate *LHCGR* mRNA (Woo *et al.* 2001). The LH is essential for the initiation of luteinisation. In addition, elevated melatonin in preovulatory follicles is likely to protect granulosa cells and the oocyte from free radicals that are induced during ovulation (Tamura *et al.* 2009). Thus, further studies are needed to investigate the exact role of melatonin in the ovulatory process.

Poor follicular development may result in production of an inadequate corpus luteum (Vasconcelos *et al.* 2001, Jimenez-Krassel *et al.* 2009). As a matter of fact, ovulation in pinealectomised ewes led to the formation of smaller corpora lutea, which produced less P_4 when compared with sham-operated ewes. The poor corpus luteum function may also be explained by the indirect and direct actions of melatonin on luteal cells. As described above, melatonin stimulates LH/FSH secretion. LH regulates a variety of ovarian functions, although its main effect may be to stimulate P_4 secretion by the corpus luteum. In fact, LH strongly stimulates P_4 production by cultured bovine luteal cells (Juengel & Niswender 1999) and plays some luteoprotective roles by increasing luteal cell viability (Kawaguchi *et al.* 2013).

Melatonin also exerts a direct action on the corpus luteum. Melatonin-binding sites have been detected in granulosa–luteal cells in humans (Yie *et al.* 1995, Woo *et al.* 2001) and in cattle (Wang *et al.* 2012). Previous studies demonstrated that melatonin significantly increased P_4 production in *in vitro*-cultured human and bovine granulosa cells (Webley & Luck 1986). Similarly, this effect has been described in many other species, including rats and sheep (Durotoye *et al.* 1997, Adriaens *et al.* 2006). On the contrary, some reports showed no effects or negative effects of melatonin on P_4 production in the growing and luteinised granulosa cells (Sirotkin 1994, Murayama *et al.* 1997, Schaeffer & Sirotkin 1997, Bodis *et al.* 2001, Nakamura *et al.* 2003). The interpretation of studies on the *in vitro* effects of

melatonin on steroidogenesis is complicated and seems to depend on cell types (theca or granulosa cells), duration of treatment (acute or long-term response), experimental model (cell culture or follicle culture), species, dose and different culture media (Tamura *et al.* 2009). *In vivo* studies showed that melatonin stimulates P_4 production both in sheep (Durotoye *et al.* 1997, Vázquez *et al.* 2010) and in rats (Dair *et al.* 2008, Romeu *et al.* 2011, Maganhin *et al.* 2013). These findings are confirmed by results of this study, showing that both P_4 production and corpus luteum growth were impaired in pinealectomised ewes. This result may be explained taking into account that melatonin remarkably increases mRNA expression of the *LHCGR* (but not *FSHR*) in human granulosa–luteal cells (Woo *et al.* 2001).

In addition, thanks to its well-known antioxidant properties (Tamura *et al.* 2013), melatonin, together with several of its derivatives, also functions as a direct free radical scavenger to reduce oxidative stress at the level of the ovary; this beneficial action is carried out without an interaction with a receptor. Additional antioxidant functions of melatonin are achieved when the indole stimulates enzymes (superoxide dismutase, glutathione peroxidase and catalase), which metabolise free radicals to less toxic products in thecal cells, granulosa cells and in the follicular fluid (Reiter *et al.* 2013). Melatonin reduces free radical damage and maintains the follicle in an optimally functional state via these actions (Reiter *et al.* 2013). This study revealed a lower total antioxidant capacity in the plasma of pinealectomised ewes when compared with sham-operated ones and confirmed previous studies reporting a direct relationship between circulating melatonin levels and total antioxidant capacity in blood (Benot *et al.* 1998, 1999, Reiter *et al.* 2005). This finding may also explain the impaired follicular and corpora luteal function found in pinealectomised ewes.

In particular, melatonin antioxidant action has a key role at the moment of ovulation and corpus luteum formation (Tamura *et al.* 2013). Melatonin is likely to protect the corpus luteum from reactive oxygen species (ROS) and has an important role in maintaining its function. ROS have been reported to inhibit P_4 production by luteal cells through the inhibition of steroidogenic enzymes (Behrman *et al.* 2001) and intracellular carrier proteins involving transport of cholesterol to mitochondria (Behrman & Aten 1991). ROS also disrupt the plasma membrane of luteal cells because of lipid peroxidation and, as a consequence, membrane damage is often seen in the regressing corpus luteum (Gatzuli *et al.* 1991, Vega *et al.* 1995). In this way, melatonin prevented apoptosis in *in vitro*-cultured bovine granulosa cells (Wang *et al.* 2012).

Pineal-derived melatonin is not a conventional hormone, as it has both receptor-mediated and receptor-independent actions and, thanks to its highly lipophilic nature, virtually all cells are its targets whether

or not they possess receptors for the indolamine. In addition, recent findings strongly suggest that also its metabolites exert a biological action (for review, see [Hardeland et al. \(2009\)](#)). Disturbances of the melatonin rhythm, which are a reflection of generalised chronodisruption, have a variety of potential consequences ([Korkmaz et al. 2009](#)). It has been long known that pinealectomy causes hypertension ([Simko et al. 2013](#)) and decreases daily secretion of insulin stimulated by glucose intake ([Lima et al. 1998](#)). An alteration in the blood flow of the corpus luteum has been associated with smaller size and low plasma P_4 concentrations ([Lüttgenau et al. 2011](#)). A disturbance in carbohydrate homeostasis may alter the development of both the follicle and the corpus luteum ([Robinson et al. 2006](#)). Thus, both the metabolic and the cardiovascular actions of melatonin could have contributed to the slower follicular development and to development of smaller corpora lutea observed in pinealectomised ewes in this study.

In conclusion, the ultrasonographic scanning in pinealectomised ewes was a useful experimental model in the study of melatonin action on the reproductive axis. It allowed observation of both its classical effect on the regulation of seasonal reproduction in short-day breeders and its action on the ovarian functional structures, i.e. the follicle and the corpus luteum. Reported results indicate that melatonin deficiency causes a decrease in plasma antioxidant capacity and alters both follicle and corpus luteum growth dynamics, leading to a decrease in the number of follicular waves/cycle and in the mean area of corpora lutea. It also negatively affected P_4 production by luteal cells. These data suggest that melatonin, besides exerting its well-known role in the synchronisation of seasonal reproductive fluctuations, also influences the growth pattern of the follicles and the steroidogenic capacity of the corpus luteum.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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