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Effect of exogenous melatonin on embryo viability and uterine environment in undernourished ewes

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ABSTRACT

The effect of exogenous melatonin on embryo viability in undernourished ewes was investigated. At lambing, 24 ewes were treated (+MEL) or not (−MEL) with a melatonin implant. After 45 days, both groups were fed to provide 1.5 (Control, C) or 0.5 (Low, L) times daily maintenance requirements, so that experimental groups were: C−MEL, C+MEL, L−MEL and L+MEL. Ewes were mated (Day 0) and on Day 5 embryos were recovered and classified according to their developmental stage and morphology. Ovaries were used for *in vitro* fertilization and uterine horns were processed to study progesterone and oestrogen receptor (PR and ERα) expression by inmunohistochemistry. After 21 days, groups L−MEL and L+MEL had an average weight loss of 10 kg ($P < 0.001$). Number of viable embryos per CL from L+MEL (0.50 ± 0.2) was higher than from other groups ($P < 0.05$). Overall, the melatonin effect was particularly evident in undernourished ewes, increasing both viability (L+MEL: 65%; L−MEL: 25%; $P < 0.05$) and pregnancy rates (L+MEL: 66.6%; L−MEL: 16.6%; $P < 0.05$). Neither nutrition and melatonin nor their interaction had a significant effect on the *in vitro* oocyte development. Melatonin treatment tended to increase the percentage of positive cells to PR in deep glandular epithelium, independently of diet ($P = 0.09$), and the greatest staining intensity of PR was observed in the luminal and superficial glandular epithelia ($P < 0.0001$). In conclusion, melatonin implants at lambing during the breeding season improve the viability of embryos recovered from undernourished ewes, although this effect seems not to be mediated at the oocyte competence level.

1. Introduction

Reproductive seasonality and nutritional condition are the main factors that influence embryo production in sheep, particularly in the Mediterranean region, where the availability of food is highly seasonal. The effects of nutrition on reproduction have been widely reviewed (Boland et al., 2001; Forcada and Abecia, 2006; Robinson, 1996). In general, nutrition has a significant effect on several aspects of reproduction including hormone production, fertilization, and early embryonic development (Boland et al., 2001; Boland and Lonergan, 2005). In sheep, the relationship between nutrition and embryo outcomes has not been established conclusively, but undernutrition can compromise follicle-oocyte competence (O'Callagan et al., 2000),

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luteal function (Jabbour et al., 1991; Sosa et al., 2004), embryo development (for review, Abecia et al., 2006), oocyte and embryo quality (Ashworth et al., 2009) and uterine protein expression and secretion (Sosa et al., 2004, 2009).

On the other hand, seasonality in sheep is mediated by photoperiod, which is conveyed to the reproductive neuroendocrine axis by melatonin (Bittman et al., 1983). Melatonin is released under conditions of darkness and acts in the mediobasal hypothalamus to modulate the pulsatile secretion of GnRH (Karsch et al., 1984; Robinson et al., 1985). Subcutaneous implants of melatonin are widely used to advance the breeding season and to improve reproductive performance during anestrus both in highly seasonal (Haresign et al., 1990) and in Mediterranean ewes (Chemineau et al., 1996; Zuñiga et al., 2002). Subcutaneous implants cause a short-day length-like response without suppressing endogenous secretion (Malpaux et al., 1997; O'Callagan et al., 1991). In general, melatonin treatment increases fertility and prolificacy in ewes (Palacin et al., 2011); but particularly, it can have a positive effect on embryo survival in ewes (Abecia et al., 2002; Durotoye et al., 1997; Vázquez et al., 2010b) even after superovulation (Forcada et al., 2006) due to its luteotrophic effects (Abecia et al., 2002; Durotoye et al., 1997; Forcada et al., 2006). However, the effects of exogenous melatonin on the *in vitro* process of oocytes are less well established (Vázquez et al., 2010a). Stenbak et al. (2001) reported that the fertilization rates of oocytes recovered from superovulated ewes were higher in the reproductive season than they were in anestrus. A previous study of our group demonstrated that treatment with exogenous melatonin at lambing improves the viability of ovine embryos from undernourished ewes during the anoestrous period, although the effects of exogenous melatonin on the ovary and early embryos remain unclear (Abecia et al., 2008; Vázquez et al., 2010b).

The establishment of pregnancy depends on a finely tuned dialogue established between the embryo and the endometrium. The alteration of this system results in retarded or accelerated embryo development and growth, failure of implantation or/and early embryonic loss (Barnes, 2000). The increase of plasma progesterone (P4) concentrations after ovulation plays a major role in determining the stage of development of the uterus (Spencer et al., 2004; Wilmut and Sales, 1981) since it has the ability to modify the embryo–maternal relationship by stimulating changes in the physiological state of uterus sufficiently to influence embryo survival (Lawson and Cahill, 1983). The action of steroid hormones (oestrogens and P4) in the uterus is mediated through interactions with their intracellular receptors (ER and PR) (Clark et al., 1992) and cellular responsiveness to steroid hormones is dependent on receptor amounts. Thus, mechanisms that modify receptor concentration may control the action of steroid hormones, altering the uterine environment. Undernutrition provokes a reduction in the sensitivity of the endometrium to steroids, as demonstrated by a lesser expression of PR and ERα (Sosa et al., 2004, 2006). To our knowledge, there is no information about the effect of exogenous melatonin or/and the effect of the nutrition–melatonin interactions on the uterine sex steroid receptor expression.

Thus, taking into account that melatonin treatment increases fertility and prolificacy in sheep (Palacin et al., 2011) and has a positive effect on embryo viability in undernourished ewes (Vázquez et al., 2010b), it can be hypothesized that melatonin could improve oocyte fertilization capacity and/or embryo development. Moreover, since post-partum and/or undernutrition could be limiting to embryo growth in the uterus, we postulate that melatonin may also increase embryo survival in sheep production systems during those critical periods. Thus, the aim of this study was to investigate the effect of melatonin implants on early embryo development, both *in vivo* and *in vitro*, and on the endometrial expression of PR and ERα in post-partum undernourished ewes during the reproductive season.

2. Materials and methods

The experiment was conducted at the Experimental Farm of the University of Zaragoza, Spain (41°41′N). All procedures were approved by the in-house Ethics Committee for Animal Experiments, of the University of Zaragoza. The care and use of the animals followed the Spanish Policy for Animal Protection RD1201/05, which meets the requirements of the European Union Directive 86/609 on the Protection of Animals used for Experimental and Other Scientific Purposes.

2.1. Animals and experimental design

The experiment involved 24 adult, post-partum Rasa Aragonesa ewes, with a mean (±SEM) live weight (LW) of 61.0 ± 1.4 kg and a mean body condition (BC) score of 2.90 ± 0.07 (Russell et al., 1969). At parturition (mid-November), ewes received a subcutaneous melatonin implant (18 mg melatonin, Melovine®, CEVA Salud Animal, Spain) (+MEL, $n = 12$) or not (−MEL, $n = 12$). In mid-January, 50 days after melatonin implantation, ewes received intravaginal progestagen pessaries (30 mg of fluorogestone, Sincropart®, Ceva Salud Animal S.A., Spain) for 14 days and 400 IU eCG (Sincropart® PMSG, Ceva Salud Animal S.A., Spain) at pessary withdrawal. From 24 h after pessary withdrawal and every 8 h, the onset of estrus (Day 0) was monitored using teaser rams. As estrus was detected, ewes were mated with certified fertile rams at least twice.

From parturition until weaning, ewes suckled single lambs for 45 days and, during lactation, they received a diet that provided the daily requirements for maintenance (M) plus the lactation of one lamb (AFRC, 1993). Lactation diet included 0.8 kg of commercial pellets (Ovinanta-Triplex®, Nanta, España), 1 kg of alfalfa hay, and 0.3 kg of barley straw per day, which provided 4.0 Mcal of metabolizable energy (ME). From weaning (Day −20) to the beginning of the experimental diets (pessary insertion), all ewes received 0.45 kg of pellets and 0.55 kg of barley straw per day, which provided 2.0 Mcal of ME and 12% crude protein (CP). From the day of pessary insertion to slaughter (Day 5 after estrus), ewes were offered collectively one of two experimental diets: 1.5× (control, C) or 0.5× (low, L) M (AFRC,

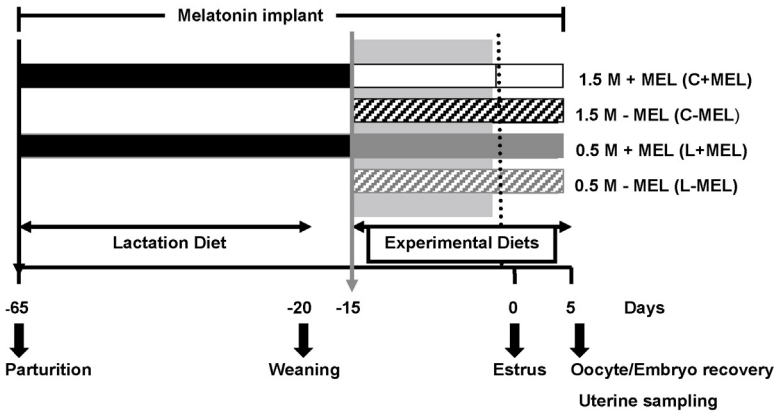

Fig. 1. Experimental design. Black bars indicate +MEL ewes. Vertical grey arrow indicates the beginning of nutritional treatment (Day −15). Ewes were offered either 1.5 (control, C) (solid white and grey bars) or 0.5 (low, L) (stripped white and grey bars) times the daily maintenance (M) requirements. Grey shade indicates estrus synchronization treatment. Vertical dotted line indicates estrus (Day 0).

1993). The C diet consisted of 0.7 kg of pellets and 0.85 kg of barley straw per ewe per day, which provided 3.0 Mcal of ME and 12% CP (1.5 M). The L diet comprised 0.25 kg of pellets and 0.30 kg of barley straw per ewe per day (1.0 Mcal of ME and 11% CP) (0.5 M). Pellets contained barley (65%), soybean (30%), and a mineral supplement (5%). Live weight and BC were recorded at parturition, onset of the nutritional treatment, pessary withdrawal, and slaughter.

Thus, considering both melatonin and nutritional treatments, ewes were divided into four groups: C+MEL (C diet, implanted with melatonin); C−MEL (C diet, non-implanted); L+MEL (L diet, implanted) and L−MEL (L diet, non implanted) (Fig. 1).

To quantify plasma P4 concentrations, jugular blood was collected in heparinised tubes on Days −1, 0, 3, and 5 after estrus. In addition, to test the effectiveness of the melatonin implants, one diurnal (noon) blood sample was collected from +MEL ewes 40 days after implantation (Day −25). From the start of the experimental diets until slaughter, plasma samples were collected every 2 days, to determine non-esterified fatty acids (NEFA). Blood samples were centrifuged within 15 min of collection (1000 g, 10 min) and plasma was stored at −20 °C until analysis.

2.2. Embryo recovery

On Day 5, 20 days after the start of the experimental diets, ewes were anaesthetized using an i.m. injection of 0.4 mL 2% xylazine (Xilagesic 2%®, Calier, Barcelona, Spain) and an i.v. injection of 10 mL of sodium thiopental (20 mg/mL) (Thiobarbital®, Braun Medical, Jaén, Spain). After a mid-ventral laparotomy, the uterine horns were flushed with pre-warmed (36 °C) phosphate-buffered saline (PBS) to collect the embryos. The ovarian response was assessed by the number of CL that was morphologically sound and consistent with an active luteal phase as determined through plasma P4 concentrations. Ova and embryos were examined and classified (Winterberger-Torres and Sevellec, 1987). Morulae and compacted morulae were considered viable embryos, according to the expected for day 5 of pregnancy (Winterberger-Torres and Sevellec, 1987). For each ewe, the number of corpora lutea (CL), number of recovered ova (oocytes + embryos), number of total embryos per CL, and number of viable embryos per CL, were recorded. Fertilization rate is the number of embryos divided by the number of ova recovered. Viability rate is the number of viable embryos divided by the number of ova recovered. Pregnancy rate is the number of ewes with viable embryos on Day 5. All rates are expressed as proportions (%).

2.3. Collection of oocytes and in vitro embryo production

After embryos were collected, ewes were euthanized using an i.v. injection of sodium thiopental (T-61®; Intervet, Salamanca, Spain). Ovaries were recovered and placed in PBS supplemented with 100 IU/mL of penicillin-G and 100 μg/mL of streptomycin sulphate at 39 °C until they were analyzed. All of the reagents were from Sigma–Aldrich Co., St. Louis, MO, USA. *In vitro* embryo production was performed following procedures explained in our previous studies (Vázquez et al., 2010a,b). The following information was recorded for each animal in each experimental group: ovulation rate (expressed as the number of corpora lutea observed at Day 5), number of recovered oocytes (including all recovered oocytes), number of healthy oocytes (including only those recovered oocytes that were rated as Good or Fair), healthy oocyte rate (number of healthy oocytes divided by the number of recovered oocytes), number of non-healthy oocytes, number of cleaved embryos, cleavage rate (number of cleaved embryos divided by the number of healthy oocytes), number of blastocysts, and blastocyst rate (number of blastocysts divided by the number of cleaved embryos). All values are expressed per ewe and all rates are expressed as proportions (%).

2.4. Hormone and metabolite assays

Plasma melatonin concentrations were measured by a direct, solid-phase radioimmunoassay (RIA) using a commercially available kit (Melatonin direct RIA; IBL

International GMBH, Hamburg, Germany) within a single assay. The sensitivity of the assay was 0.9 pg/mL and the intra-assay CV was 5.5% for low (9.0 pg/mL) and 9.5% for high (110.0 pg/mL) control concentrations.

Plasma P4 concentrations were measured using a direct, solid-phase RIA kit (Count-A-Count TKPG; Siemens, Los Angeles, USA). The RIA had a sensitivity of 0.02 ng/mL. The intra-assay CV was 9.3% for low (3 ng/mL), 5.8% for medium (15 ng/mL), and 4.2% for high (30 ng/mL) control concentrations.

Plasma NEFA were determined using an autoanalyzer (Gernonstar®, Transasia, Bombay, India), and were quantified using commercially available kits (NEFA®, Randox Laboratories Ltd., Crumlin, UK). The intra- and inter-assay CVs were 6.7% and 8.0%, respectively.

2.5. Receptor protein localization and abundance

After embryo recovery, sections from the middle third of the uterine horn ipsilateral to the CL were fixed in 4% w/v paraformaldehyde in PBS and embedded in paraffin for inmunohistochemistry.

Inmunoreactive PR and ERα were visualized in transverse 3-μm sections from uterine horns ipsilateral to the CL using an avidin–biotin–peroxidase immunohistochemical technique (Meikle et al., 2000). Monoclonal mouse antibodies were used as primary antibodies: anti-PR (Zymed, South San Francisco, CA, USA) and anti-ERα (Santa Cruz, Santa Cruz, CA, USA), diluted 1:100 and 1:25 in phosphate buffered saline (PBS), respectively. Negative controls for each receptor were obtained by replacing the primary antibody with non-immune mouse IgG at an equivalent concentration (Santa Cruz, Santa Cruz, CA, USA). After primary antibody binding, sections were incubated with a biotinylated horse anti-mouse IgG (Vectastain; Vector Laboratories, Burlingame, CA, USA) diluted in normal horse serum. Thereafter, tissue sections were incubated with a horseradish peroxidase–avidin–biotin complex (Vectastain Elite; Vector Laboratories, Burlingame, CA, USA). The location of the bound enzyme was visualized by 3,3-diaminobenzidine in H_2O_2 (DAB kit; Vector Laboratories, Burlingame, CA, USA) and sections were counterstained with haematoxylin and dehydrated before they were mounted. For each receptor, all samples were analyzed in the same immunohistochemical assay.

2.6. Image analysis

The amount of PR and ERα in different cell types was estimated subjectively by two independent observers who were blinded to the treatment groups from which the tissues originated (Sosa et al., 2004). Both receptors were evaluated in five endometrial compartments: luminal epithelium (LE), glandular epithelium (arbitrarily divided in two portions, superficial glandular epithelium (sGE) next to the uterine lumen and deep glandular epithelium (dGE) next to the myometrium) and intercaruncular stroma (classified as superficial (sS) and deep (dS) following the same criteria). Ten fields were analyzed for each cell type at a magnification of 1000× in all ewes. The staining of the nuclei was scored as being negative (−), faint (+), moderate (++) or intense (+++) and the extent of staining of each cell type was expressed in proportion on a scale of 0–10 (Thatcher et al., 2003). The average staining was calculated as $1 \times n1 + 2 \times n2 + 3 \times n3$, where n is the proportion of cells per field exhibiting faint ($n1$), moderate ($n2$) and intense ($n3$) staining (Boos et al., 1996). The estimated proportion of total positive cells per field was also recorded.

2.7. Statistical analyses

The experiment was based on a 2×2 factorial design in which nutritional level and melatonin treatment were fixed effects. The effects of the treatments on the development and quality of *in vivo* embryos and *in vitro* oocytes and blastocysts were evaluated statistically using the PROC GENMOD (SAS, 1999) with the Poisson distribution specified in a model that included nutrition level (low or control), melatonin treatment (with or without melatonin implant), and their interactions. Statistical comparisons of proportional values were based on the Chi-squared Test. For the analysis of repeated measurements (hormones and NEFA levels), the covariance structure was modelled to consider the correlation between sequential observations on the same animal (Littell et al., 2000). For the analysis of hormones, BC and LW, NEFA, measurements of the period before treatment were included in the model as covariables. The variables studied in the analysis of receptor localization were the proportion of total positive cells and the average staining of the 10 fields. The statistical model included the effects of observer, melatonin treatment, nutrition treatment, cell type (luminal epithelium, glandular epithelium and stroma), localization (superficial and deep) and their interactions. The results are expressed as mean ± SEM, and the level for statistical significance was set to $P < 0.05$.

3. Results

3.1. Live weight and body condition

No melatonin effect was observed on both LW and BC, during the period from parturition to the beginning of nutritional treatments. But an effect of nutrition–melatonin interactions was observed on LW, but not on BC ($P < 0.05$). In the control group, melatonin implants increased LW, although it decreased LW in undernourished ewes (Table 1, $P < 0.05$). During the period of the experimental diets (21 days), mean LW of C ewes did not change, but in L ewes there was a decrease, on average more than 14% of their initial LW (Table 1, $P < 0.01$). Accordingly, L ewes experienced a significant reduction in their BC in comparison with C ewes ($P < 0.01$; Table 1). After pessary withdrawal (14 days after the onset of the experimental diets), mean LW and BC of L ewes were significantly lower than those of C ewes ($P < 0.01$). In parallel with the reductions in live weight among ewes in the undernourished groups, in the course of the experimental diets, plasma NEFA concentrations increased rapidly in the L groups, which were three to four times higher than they were in the C−MEL and C+MEL groups (C: 0.14 ± 0.02 mmol/L *vs.*

Table 1
Mean (±SEM) live weight (LW), body condition (BC) and non-esterified fatty acids (NEFA) in post-partum Rasa Aragonesa ewes, fed either 1.5× (C) and 0.5× (L) the maintenance requirements and treated (+MEL) or not (−MEL) with melatonin implant after parturition during the reproductive season.

	Live weight			
	C−MEL	C+MEL	L −MEL	L+MEL
At parturition (Day −65)	65.7 ± 2.8	66.2 ± 2.3	65.7 ± 2.8	66.2 ± 2.3
At onset of nutritional treatment (Day −15)	62.2 ± 1.6	70.7 ± 2.5	70.2 ± 2.2[a]	62.2 ± 2.8[a]
At pessary withdrawal (Day −1)	60.0 ± 2.3	66.8 ± 2.3	62.2 ± 2.7[b]	53.8 ± 3.4[b]
At slaughter (Day 5)	60.2 ± 2.2	67.0 ± 2.1	60.6 ± 2.8[b]	51.6 ± 3.0[b]
	Body condition			
	C−MEL	C+MEL	L −MEL	L+MEL
At parturition (Day −65)	2.7 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	2.8 ± 0.1
At onset of nutritional treatment (Day −15)	2.9 ± 0.1	3.0 ± 0.1	3.1 ± 0.2[a]	3.0 ± 0.1[a]
At pessary withdrawal (Day −1)	3.0 ± 0.1	2.9 ± 0.1	2.9 ± 0.1[b]	2.7 ± 0.1[b]
At slaughter (Day 5)	2.9 ± 0.1	2.9 ± 0.1	2.8 ± 0.1[b]	2.7 ± 0.1[b]
	Non-esterified fatty acids			
	C−MEL	C+MEL	L −MEL	L+MEL
During the experimental diets period (mmol/L)	0.13 ± 0.2[x]	0.15 ± 0.2[x]	0.43 ± 0.1[z]	0.44 ± 0.1[z]

Different superscripts in the same column indicate significant differences: $P < 0.01$ (a and b) and $P < 0.001$ (x and z).

L: 0.44 ± 0.01 mmol/L; $P < 0.001$). Exogenous melatonin did not have a noticeable effect on plasma NEFA levels (Table 1).

3.2. Circulating progesterone and ovulation rate

Plasma P4 concentrations increased gradually after estrus in the four experimental groups, confirming that all ewes presented an active luteal phase on Day 5 (slaughter). Progesterone concentrations on Day 5 tended to be higher in the +MEL than in the −MEL ewes (Fig. 2, $P = 0.09$); this was particularly evident in undernourished ewes ($P < 0.05$). Ovulation rate did not differ among treatments (Table 2).

3.3. Embryo recovery

Neither exogenous melatonin nor nutritional treatment affected the number of recovered ova per CL (Table 2), although exogenous melatonin tended to increase the number of total recovered embryos per ewe (−MEL: 0.75 ± 0.3; +MEL: 1.50 ± 0.3; $P = 0.09$). This melatonin effect was particularly evident in undernourished ewes (Table 2, $P < 0.05$).

Undernutrition decreased significantly the viability rate of non-melatonin treated ewes (Table 2, $P < 0.05$), but neither the nutrition treatment nor the nutrition–melatonin interaction treatments had a significant effect on most of the parameters evaluated (Table 2).

3.4. In vitro embryo development

Neither nutrition nor exogenous melatonin nor the interaction between nutrition and melatonin treatments had a significant effect on any of the parameters evaluated, except on the number of cleaved embryos and blastocysts rate (Table 3). In the undernourished group, melatonin treatment tended to increase the number of cleaved embryos ($P < 0.1$), although it decreased blastocyst rate ($P < 0.05$). In addition, nutritional treatment tended to improve the number of cleaved embryos (C: 8.1 ± 0.9; L: 5.4 ± 0.9; $P = 0.09$), and consequently, the number of

Table 2
Ovarian response and embryo production of Rasa Aragonesa ewes fed to provided 1.5 (C) or 0.5 (L) times the maintenance requirements and treated (+MEL) or not (−MEL) with melatonin 70 days before oocyte/embryo recovery during the reproductive season (mean ± SEM).

	Group			
	C−MEL	C+MEL	L−MEL	L+MEL
No. of ewes	6	6	6	6
No. of ewes in estrus	6/6	6/6	6/6	6/6
Ovulation rate	2.17 ± 0.3	2.17 ± 0.3	1.50 ± 0.3	2.0 ± 0.3
No. of recovered ova/CL	0.30 ± 0.2	0.60 ± 0.3	0.50 ± 0.2	0.83 ± 0.2
No. of total embryos/CL	0.30 ± 0.2[c]	0.30 ± 0.2[c]	0.50 ± 0.2[c]	0.75 ± 0.2[d]
Fertilization rate (%)	100[c]	50[d]	100[c]	90[c]
No. of viable embryos/CL	0.30 ± 0.2	0.20 ± 0.2	0.12 ± 0.1[a]	0.50 ± 0.2[b]
Viability rate (%)	100[a,c]	37.5[d]	25[b]	65[a]
Pregnancy rate (%)	50.0 (3/6)	33.3 (2/6)	16.6 (1/6)[a]	66.6 (4/6)[b]

Different superscripts in the same row indicate significant differences: $P < 0.05$ (a and b) and $P < 0.1$ (c and d).

Table 3
In vitro embryo production from recovered oocytes of Rasa Aragonesa ewes fed to provide 1.5 (C) or 0.5 (L) times the maintenance requirements and treated (+MEL) or not (−MEL) with melatonin 70 days before oocyte/embryo recovery during the reproductive season (mean ± SEM).

	Group			
	C−MEL	C+MEL	L−MEL	L+MEL
No. of ovulating ewes with functional CL	6	6	6	6
Ovulation rate	2.2 ± 0.3	2.2 ± 0.3	1.50 ± 0.3	2.0 ± 0.3
No. of recovered oocytes	25.3 ± 5.5	25.5 ± 5.5	21.2 ± 5.4	27.3 ± 5.4
No. of healthy oocytes used for IVF	15.8 ± 3.4	13.5 ± 3.4	14.3 ± 3.4	15.0 ± 3.4
Healthy oocytes rate (%)	62.5	52.9	67.5	54.9
No. of non-healthy oocytes	9.5 ± 2.8	12.0 ± 2.8	6.8 ± 2.8	12.3 ± 2.8
No. of cleaved embryos	8.2 ± 1.3c	8.0 ± 1.3c	3.9 ± 1.3d	6.8 ± 1.3c
Cleavage rate (%)	51.9a	59.3a	27.3b	45.3a
No. of blastocysts	3.6 ± 1.0	3.3 ± 1.0	1.5 ± 1.0	1.1 ± 1.0
Blastocysts rate (%)	43.9a	41.3a	38.5a	16.2b

All values are expressed per ewe. Different superscripts in the same row indicate $P < 0.05$ (a and b) and $P < 0.1$ (c and d).

blastocyts after the *in vitro* process (C: 3.5 ± 0.7; L: 1.1 ± 0.7; $P = 0.09$).

3.5. Expression of progesterone and oestrogen receptors in the endometrium

Progesterone and oestrogen receptors were localized into the nuclei of the endometrial cell types studied. When specific antibodies were substituted with a non-immune IgG, the absence of staining confirmed the high specificity of immunostaining (Fig. 3).

Melatonin treatment tended to increase the percentage of positive cells to PR in dGE, independently of diet treatment ($P = 0.09$), and an interaction of melatonin and nutritional treatment was observed for the staining intensity of PR in the dGE: L+MEL ewes had higher intensity of PR than L−MEL ($P < 0.05$), whereas no changes were observed in the control groups (Fig. 4). On the other hand, melatonin increased ERα in the sS of the C group ($P < 0.01$); while it decreased the expression of ERα in the dS of the L group ($P < 0.01$).

An effect of the localization of PR and ERα was observed ($P < 0.0001$): the greatest staining intensity of PR was mainly observed in the LE and sGE ($P < 0.0001$). No differences between sS and dS were observed, whereas in the sGE the content of PR was 2-fold greater than in the dGE ($P < 0.0001$). Regarding ERα, the lesser abundance was observed in the LE. Overall, the expression of ERα was greater in the sS than in the dS ($P < 0.001$), and lesser in the sGE than in the dGE ($P < 0.001$).

4. Discussion

This study investigated the effects of exogenous melatonin on *in vivo* and *in vitro* embryo development and on uterine environment in undernourished post-partum Rasa Aragonesa ewes lambing during the reproductive season. Exogenous melatonin had a beneficial effect on *in vivo* embryo viability, particularly in undernourished ewes, but not on *in vitro* oocyte competence. In addition, an increment of the expression of PR in the deep glandular epithelium of undernourished melatonin implanted ewes was observed, although no effects of nutrition or melatonin on the expression of PR and ERα in the endometrium were observed.

Ovulation rate in the present study is consistent with the observations indicating that short-term undernutrition associated with a progestagen-synchronized estrus does not appear to impair this parameter in the breeding season (Abecia et al., 1997, 1999; Borowczyk et al., 2006; Vázquez et al., 2009, 2010b).

Although in our experiment, nutritional restrictions reduced significantly mean LW and BC, and increased significantly NEFA concentrations in undernourished ewes,

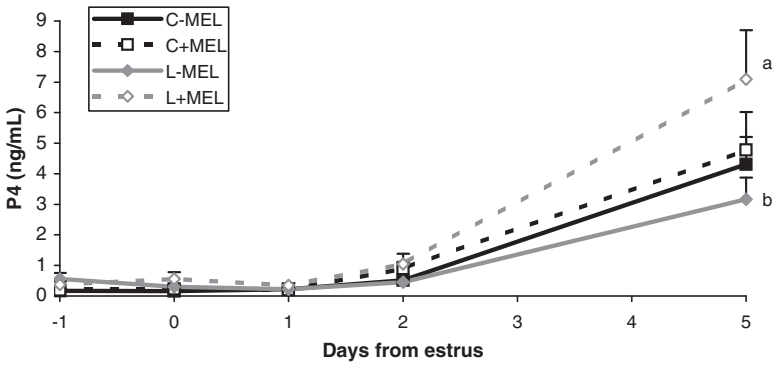

Fig. 2. Plasma progesterone (P4) concentrations from pessary withdrawal to embryo collection (Day 5) in post-partum Rasa Aragonesa ewes fed either 1.5× (C) and 0.5× (L) the maintenance requirements and treated (+MEL) or not (−MEL) with melatonin implant during the reproductive season. Different letters on Day 5 indicate $P < 0.05$ between L−MEL and L+MEL.

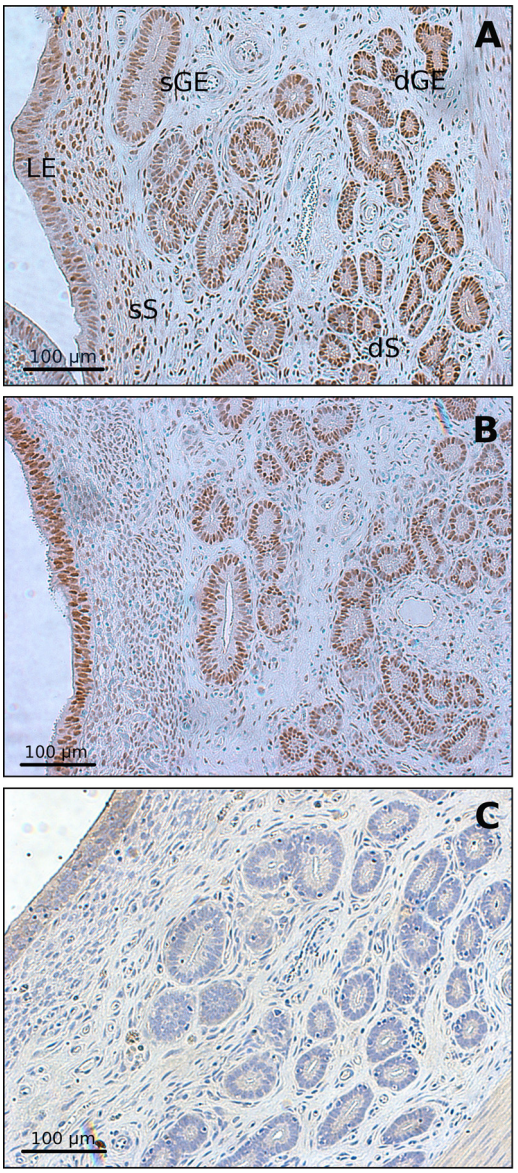

Fig. 3. Immunohistochemical localization of oestrogen receptor α (A) and progesterone receptor (B). The negative control (C) shows absence of specific staining. Magnification: 200×.

fact that reflected an increase in lipolytic activity. Our previous studies (Abecia et al., 1997, 1999; Sosa et al., 2006, 2009; Vázquez et al., 2009, 2010a,b) demonstrated that mature ewes subjected to a degree of undernutrition similar to that of the present experiment for 3–4 weeks exhibited a similar significant reduction in LW and BC. Unexpectedly, and in contrast with those works, the present study had no remarkable effect of undernutrition on the majority of the variables studied, except for a decrease of viability rate in recovered embryos at Day 5 after estrus. One possible explanation could be that the initial LW and BC of the ewes was above the observed in works where there was an evident effect of undernutrition (Abecia et al., 1999; Lozano et al., 1998; Sosa et al., 2009; Vázquez et al., 2009, 2010b). In this respect, Blanc et al. (2006) concluded that the impact of a given level of underfeeding will differ according to the animal's physiological state and according to its body condition when it undergoes food restriction. Indeed, responses to changes in metabolic status could depend on recent (feeding level) or more ancient (body reserves) metabolic history, a concept termed "metabolic memory" (Blache et al., 2006). Thus, despite higher losses of body reserves, it is possible that the final LW and BC reached in the undernourished group of the present study were not low enough to translate into notable deleterious effects on embryos, oocytes and uterine physiology.

In our study, nutritional treatments *per se* did not have a significant effect on most of the reproductive parameters evaluated, but exogenous melatonin significantly improved the viability of *in vivo* recovered embryos from undernourished ewes. Thus, pregnancy rates on Day 5 after estrus were significantly higher in the melatonin-implanted undernourished ewes than in the non-implanted undernourished group. Previous studies have shown that supplemental melatonin in the seasonal anestrus can improve embryo viability in superovulated (Forcada et al., 2006) and non-superovulated ewes (Vázquez et al., 2009). The luteotrophic effect of the pineal hormone observed *in vivo* (Durotoye et al., 1997; Vázquez et al., 2010b) and *in vitro* (Abecia et al., 2002), and the effects of melatonin at the hypothalamic-hypophyseal level (Malpaux et al., 1997) might be involved in the melatonin-induced improvement in embryo viability of undernourished ewes during reproductive season. Our study appears to provide additional evidence of those luteotrophic effects because melatonin-implanted ewes exhibited a trend towards higher P4 concentrations than non-implanted animals. The high P4 levels at Day 5 after estrus might be responsible, at least in part, for the greater embryo viability and pregnancy rates in the melatonin-implanted undernourished ewes in the present study and also in our previous study during the anestrus season (Vázquez et al., 2010a). In the present work, the beneficial effects of melatonin were greater in the undernourished ewes. Undernutrition is common in extensive pasture-dependent and intensive-accelerated reproductive sheep farms, and impairs the expression of endometrial steroid receptors and binding capacity in the early luteal phase, which compromises embryo survival (Sosa et al., 2004, 2006).

Undernutrition affected embryo *in vitro* development, being detrimental for the IVF competence of oocytes. This is consistent with several studies that have reported impaired *in vitro* developmental competence of oocytes from superovulated (Borowczyk et al., 2006; Papadopoulos et al., 2001) and non-superovulated undernourished ewes (Vázquez et al., 2010a). As expected (Vázquez et al., 2010a), melatonin implants increased the number of *in vitro* cleaved embryos of undernourished ewes. Consistently, our previous studies with a similar experimental design with non-pregnant Rasa Aragonesa ewes showed that supplemental melatonin during the anestrus increased the number of cleaved embryos and blastocyst rates in undernourished ewes (Vázquez et al., 2010b). Moreover, treatments with exogenous melatonin seemed to improve

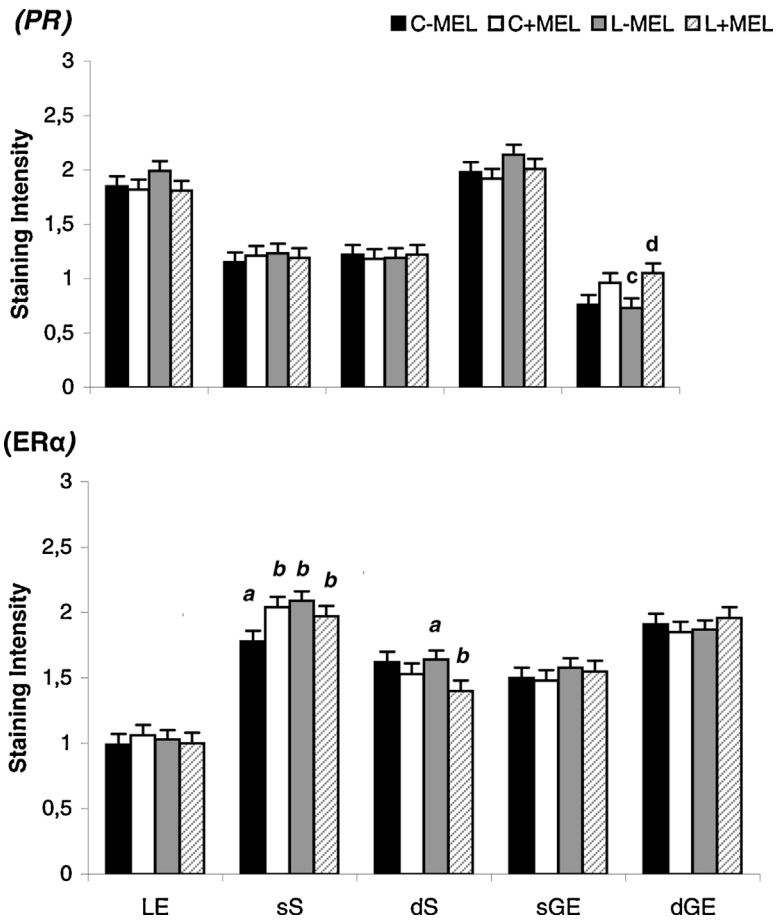

Fig. 4. Staining intensity of PR (upper panel) and ERα (lower panel) in the endometrium of Rasa Aragonesa ewes with 45 days of post-partum, fed to provide 1.5 (C) or 0.5·(L) the maintenance requirements and treated (+MEL) or not treated (−MEL) with melatonin at parturition day during the reproductive season. LE = luminal epithelium, sS = superficial stroma, dS = deep stroma, sGE = superficial glandular epithelium, dGE= deep glandular epithelium. Different letters within the same cell type indicate $P < 0.01$ (a, b) and $P < 0.05$ (c, d).

the developmental competence for the IVF of recovered oocytes from progestagen and eCG treated ewes (Valasi et al., 2006) and superovulated goats (Berlinguer et al., 2007, 2009).

To our knowledge, this is the first report on the effect of exogenous melatonin or/and on the effect of the nutrition–melatonin interactions in endometrial protein expression of sheep or other species. In general, no nutritional effects were observed on the expression of PR and ERα in the endometrium, but an increment of the expression of PR in the deep glandular epithelium and a decrease of ER in the deep stroma of undernourished melatonin-implanted ewes was observed. Although the elucidation of the biological relevance of the change in PR expression in the deep glands is not straightforward, it can be speculated that melatonin compensates the adverse effects of undernutrition by increasing sensitivity to P4, since this hormone enhances the differentiation of the epithelia and thereby, the secretory function of the endometrium (Cunha et al., 2004). On the other hand, melatonin decreased ERα expression in deep stroma of undernourished ewes; E2-ERα complex acts as a luteolytic agent, thus a decrease in oestrogen sensitivity (ERα content) in deep stroma is consistent with the possible beneficial effects of melatonin in the uterine preparation for pregnancy as observed for PR receptor. Indeed, Abd-Allah et al. (2003) have proposed melatonin as a modulator of uterine ERα and PR in rats, as melatonin treatment markedly reduces the number of uterine ERα with a proportional concomitant increase in the progesterone receptors. The mechanisms by which melatonin may influence the expression of the uterine steroid receptors are not easy to understand since no references about a direct action of melatonin at the uterine level through specific receptors in the reproductive tract of the sheep have been reported. However, Zhao et al. (2002) characterized the melatonin receptor (MT_1) transcript in rat endometrium during oestrous cycle, and more recently, Chuffa et al. (2011) described that the expression of MT_1 is up-regulated by the melatonin treatment and is associated with the down-regulation of the ER-beta and PRB in the reproductive tissue of rats.

In conclusion, our data demonstrate that the treatment with melatonin implants at lambing improves the viability of embryos of undernourished ewes during the reproductive season, although the effect of melatonin seems not to be mediated at the oocyte competence level. Moreover,

melatonin induces changes in the endometrial sensitivity of steroids in undernourished ewes. Thus, the use of melatonin implants at parturition, even during the breeding season, could be a helpful tool, particularly when embryo development is affected by negative factors as undernutrition or the post-partum period.

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