Undernutrition and Exogenous Melatonin Can Affect the In Vitro Developmental Competence of Ovine Oocytes on a Seasonal Basis

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Contents
This study evaluated the effects of exogenous melatonin and level of nutrition on oocyte competence, in vitro fertilization (IVF), and early embryonic development in sheep during seasonal anoestrus (SA) and the reproductive season (RS). Adult Rasa Aragonesa ewes were assigned randomly to one of four treatment groups in two experiments based on a 2 x 2 factorial design. Individuals were treated (+MEL) or not treated (–MEL) with a subcutaneous implant of melatonin for 42 days and then were fed 1.5 (Control, C) or 0.5 (Low, L) times the daily maintenance requirements for 20 days. Ewes were synchronized and mated at oestrus (Day = 0). On Day 5, ovaries were collected and oocytes were used for IVF. Season had a significant (p < 0.01) effect on the number of oocytes recovered (RS: 19.6 ± 1.8; SA: 14.5 ± 1.0) and the number of healthy oocytes (RS: 13.9 ± 0.7; SA: 9.0 ± 0.7). In the RS, neither nutrition nor melatonin had a significant effect on the evaluated oocytes quality parameters although melatonin implants appeared to reduce the number of unhealthy oocytes in the undernourished group (p < 0.05). During SA, in undernourished ewes exogenous melatonin tended to increase the number of healthy (L+MEL: 9.4 ± 1.0; L–MEL: 7.6 ± 1.4; p < 0.1), and significantly improved both cleaved oocytes (L+MEL: 7.0 ± 0.7; L–MEL: 4.1 ± 0.9; p < 0.05) and blastocyst rate (L+MEL: 37.2; L–MEL: 21.9%; p < 0.05). In conclusion, oocyte competence in ewes was affected by season, and melatonin implants appeared to improve developmental competence in the seasonal anoestrous period, particularly in experimentally undernourished ewes.

Introduction
Season and nutritional condition can have a significant effect on reproduction in sheep, particularly in the Mediterranean region, where the availability of nutritional resources are highly seasonally variable. Embryo production and viability are the main factors that reflect female reproductive success, and both are affected by follicular development and oocyte quality. Season appears to influence the fertilization and viability rates of sheep embryos (Vázquez et al. 2008), and can affect some of the characteristics of the embryos (Mitchell et al. 2002). Mitchell et al. (2002) observed that the numbers of fertilized ova and embryos that had <16 cells impacted embryo quality, which was lower during anoestrus than during the reproductive season (RS). In addition, Stenbak et al. (2001) found the in vitro fertilization (IVF) rates of oocytes recovered from superovulated ewes were higher in the RS than they were during anoestrus.

Melatonin implants can be an effective method of inducing oestrous cycles, increasing lambing rates, and prolificacy during anoestrus (Haresign et al. 1990; Robinson et al. 1991; Haresign 1992; Abecia et al. 2007). Abecia et al. (2008) summarized the effects of exogenous melatonin on the ovary and early embryos in ewes. Although melatonin treatments during anoestrus did not improve fertilization rates in superovulated ewes (McEvoy et al. 1998; Forcada et al. 2006), this pineal hormone can improve the viability of embryos (Forcada et al. 2006). Although information about the effects of melatonin on oocyte quality and IVF during anoestrus is limited, Luther et al. (2005) did not observe a significant difference between melatonin-treated and untreated ewes in their fertilization rates after IVF of the oocytes recovered from superovulated ewes in the non-reproductive season.

Nutrition can have a significant effect on numerous aspects of reproduction, including hormone release, fertilization, and early embryonic development (Boland et al. 2001). Nutritional condition can be correlated with embryo survival and is a key factor in the efficiency of assisted reproductive technologies (Webb et al. 2004). Borowczyk et al. (2006) found that the oocytes derived from underfed ewes yielded fewer blastocysts and had lower rates of cleavage and blastocyst formation than did control ewes. Lonazo et al. (2003) observed lower cleavage rates in underfed ewes than in overfed ewes; in addition, undernutrition can impair embryo viability (Vázquez et al. 2008). To our knowledge, yet, there are no published studies on the competence of oocytes recovered from the ovaries of undernourished ewes.

As melatonin can increase embryo survival and IVF rates (Valasi et al. 2006), we hypothesized that this hormone might override the effects of undernutrition and season on oocyte competence, which is the ability of an oocyte to be fertilized and develop to the blastocyst stage. Thus, the aim of this study was to evaluate the effects of melatonin and undernutrition on oocyte competence, IVF and early embryonic development during the anoestrus and the RS in ewes.

Materials and Methods
All procedures used in this study were approved by the Ethics Committee of the University of Zaragoza, Spain, performed at the Experimental Farm of the University of Zaragoza, Spain (41°41’N), and met the requirements of the European Union for Scientific Procedure Establishments.
Animals and experimental design

The study included multiparous, non-pregnant Rasa Aragonesa ewes. In the RS, 42 cycling ewes with a mean (± SEM) live weight (LW) of 64.1 ± 1.7 kg and mean body condition (BC) (Russell et al. 1969) of 3.1 ± 0.1 were used. To confirm ovarian cyclicity, 7 days and 14 days before the insertion of melatonin implants, blood samples were collected and plasma progesterone concentrations were measured. All of the ewes had progesterone concentrations > 1 ng/ml in at least one of the samples, which indicated ovarian activity. On 8th December, 21 ewes received a subcutaneous melatonin implant (18 mg melatonin, Melovine®, CEVA Salud Animal S.A., Barcelona, Spain) at the base of the ear and 21 others did not. Forty-two days after implantation, all of the ewes were synchronized after a 14-day treatment with intravaginal prostogestagen (30 mg Flurogestone Acetate; Sincropart®, CEVA Salud Animal S.A.). When the pessaries were removed, the ewes were treated i.v. with 400 IU of equine chorionic gonadotrophin (eCG) (Sincropart® PMSG, CEVA Salud Animal S.A.). During the seasonal anoestrus (SA), at 7 and 14 days before the start of the experiment, blood samples were collected from 42 ewes and their plasma progesterone concentrations were measured. To create a group of anoestrous ewes, those that had progesterone levels >1 ng/ml in at least one of their samples were considered cyclic, and were excluded from the study. Consequently, the experiment included 36 adult, non-cycling, non-pregnant Rasa Aragonesa ewes that had a mean (± SEM) LW of 59.2 ± 1.3 kg and BC of 3.2 ± 0.06. On 26 March, 17 ewes received a subcutaneous implant of melatonin, while another 19 did not. Synchronization with intravaginal prostogestagen pessaries occurred 42 days after implantation. When the pessaries were removed, the ewes were treated i.v. with 480 IU of equine chorionic gonadotrophin (eCG).

In both seasons, from the date of insertion of the pessaries until slaughter (Day 5 post-oestrus), the ewes were fed a diet that provided either 1.5x M (control, C groups) or 0.5x M (low, L groups) the daily maintenance requirements (Agricultural and Food Research Council 1993) and had unlimited access to water. Ewes fed the C diet received 0.60 kg of pellets and 1 kg of barley straw per day, which provided 12.4 MJ of metabolizable energy (ME) and 9.3% crude protein (CP). The L diet comprised 0.20 kg of pellets and 0.35 kg of barley straw per day, which provided 4.1 MJ of ME and 9.1% CP. The pellets consisted of barley (73%), soybean (22%), and a mineral supplement (5%). Live weight and BC were recorded at the time of pessary insertion, pessary withdrawal, and at slaughter. A 1.5x M diet ensures the maintenance of LW and BC, whereas a 0.5x M diet leads to a 12% reduction in LW and BC after 20 days. Thus, the four groups in the study were: group C+MEL, ewes that were offered the C diet and did not receive a melatonin implant; group C+MEL, ewes that were offered the C diet and received a melatonin implant; group L+MEL, ewes that were offered the L diet and did not receive a melatonin implant; and group L+MEL, ewes that were offered the L diet and received a melatonin implant.

From 24 h after pessary withdrawal, every 8 h intact rams wearing harnesses with marking crayons were used to monitor oestrus (Day 0). To verify the functionality of the corpora lutea at Day 5 (slaughter), jugular blood samples were collected in evacuated heparinized tubes. To test the effectiveness of the melatonin implants, one diurnal blood sample was collected from each of the melatonin-implanted ewes 45 days after implantation. The samples were centrifuged within 15 min (1000 × g for 10 min) and the plasma was stored at −20°C.

Collection and in vitro maturation of oocytes

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 10 ml of sodium thiopental (20 mg/ml) (Thiobarbital Braun Medical, Jaén, Spain) administered by i.v. injection 5 min later. Uterine horns were flushed with pre-warmed (36°C) phosphate-buffered saline (PBS) and embryos were collected by mid-ventral laparotomy. The effects of exogenous melatonin and level of nutrition on the viability of embryos are described elsewhere (Vázquez et al. 2008).

After embryo collection, ewes were euthanized using an i.v. injection of sodium thiopental (T-61®, Intervet, Salamanca, Spain). Ovaries were collected 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 processed. Except where indicated otherwise, all of the reagents were from Sigma-Aldrich Co., St. Louis, MO, USA. A combination of puncture and slicing techniques (Wani et al. 1999) were used to collect oocytes in a Petri dish and partially covered with a handling medium (Hepes-buffered TCM-199 supplemented with 0.1% polysvinyl alcohol (PVA), 0.04% sodium bicarbonate, 25 IU/ml of heparin, 100 IU/ml of penicillin-G, and 100 μg/ml of streptomycin sulphate). Following Wani et al. (2000), oocytes were classified, based on their cumulus cells and cytoplasm morphology, as: Good, included all oocytes with a lot of complete layers of granulose cells and homogeneous cytoplasm; Fair, included all oocytes with few or incomplete layers of granulose cells and homogeneous cytoplasm; and Poor, included oocytes with few or absence of granulose cells and non-homogeneous cytoplasm. Only the oocytes that had several layers of cumulus cells and a uniform cytoplasm (called healthy oocytes) were selected for in vitro maturation (IVM).

Healthy oocytes were transferred into a maturation medium that contained bicarbonate-buffered TCM-199 supplemented with 10% (v : v) oestrous sheep serum, 0.1 μg/ml each of FSH and LH, 100 μM of cysteamine, 0.3 μM of sodium pyruvate, 100 IU/ml of penicillin G, and 100 μg/ml of streptomycin sulphate, which was covered with mineral oil and incubated at 39°C under 5% CO₂ and saturated humidity for 24 h.

In vitro fertilization (IVF) and embryo cultures

At the end of IVM, oocytes were freed from the cumulus cells and transferred to the fertilization medium, which
consisted of synthetic oviductal fluid (SOF) without glucose (Tervit and Whittingham 1972) and supplemented with 2% (v : v) of oestrous sheep serum (Hopper et al. 1993; Huneau et al. 1994; Li et al. 2006), 10 μg/ml of heparin (Cox and Saravia 1992), and 1 μg/ml of hypotaurine.

On the same day as fertilization, semen was collected from four Rasa Aragonesa rams, pooled, diluted 1:10 in a saline medium with 0.25 mol/l of sucrose, 10 mmol/l of Hepes, 2 mmol/l of potassium hydroxide, 5 mmol/l of glucose, 0.5 mol/l of sodium phosphate monobasic, and 100 mmol/l of ethylene glycol tetra-acetic acid (EGTA), and kept at 15°C until fertilization. Highly motile spermatozoa were selected using the swim-up technique (Wani et al. 2000; Luther et al. 2005). The fertilization dose was 1 × 10⁶ spermatozoa/ml sperm were added to the fertilization medium that contained the oocytes, covered with mineral oil, and incubated for 24 h at 39°C in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂.

At 24 h and 36 h after fertilization, presumptive zygotes were placed in a culture medium that contained SOF supplemented with essential and non-essential amino acids at oviductal concentration (Walker et al. 1996), 0.4% bovine serum albumine (wt/vol), 1 mM of t-glutamine, 100 IU/ml of penicillin G, and 100 μg/ml of streptomycin sulphate, and covered with mineral oil and kept at 39°C in a maximally humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ for 8 days, to blastocyst stage.

The cleaved embryos were added to the embryo wells, and uncleaved oocytes were examined under a stereomicroscope to assess their stage of maturation. Oocytes that exhibited the first polar body were considered mature, and oocytes that had two polar bodies were considered fertilized, but not cleaved.

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 per ewe), number of recovered oocytes (included all recovered oocytes), number of healthy oocytes (included only such of the recovered oocytes that were rated as good and fair), healthy oocytes rate (number of healthy oocytes/number of recovered oocytes), number of non-healthy oocytes, number of cleaved oocytes, cleavage rate (number of cleaved oocytes/number of healthy oocytes), number of blastocysts, and blastocyst rate (number of blastocysts/number of cleaved oocytes). All values were expressed per ewe and all rates were expressed as percentages.

Hormone assays
Plasma melatonin concentrations were measured by a direct, solid-phase radioimmunoassay (RIA) using a commercially available kit (Bühlmann RK-MDI; Bühlmann Lab, Schönbuch, Switzerland) within a single assay. The sensitivity of the assay was 1.3 pg/ml and the intra-assay CV was 10.9% for low control concentrations (3.3 pg/ml) and 4.9% for high control concentrations (20.6 pg/ml).

Plasma progesterone concentrations were measured by a direct, solid-phase RIA using commercially available kits (Count-A-Count TKPG; DPC) (Meikle et al. 1997). The RIA had a sensitivity of 0.02 ng/ml. The intra-assay CV was 14% for low control concentrations (3 ng/ml), 8.5% for medium concentrations (15 ng/ml), and 7.5% for high concentrations (30 ng/ml). The interassay CV was <15% for all of the standard concentrations.

Statistical analyses
The experimental was based on a 2 × 2 × 2 factorial design in which nutritional level, melatonin treatment, and season were fixed effects. The effects of the treatments on the development and quality of oocytes and blastocyst were evaluated statistically using the PROC GENMOD (SAS 1999) with the Poisson distribution specified in a model that included season (reproductive or anoestrus), nutrition level (low or control), and melatonin treatment (with or without melatonin implant), and their interactions. The values expressed as percentages were arcsine-transformed before being compared using the chi-squared test. The probability level for statistical significance was set to p < 0.05 and the results are expressed as mean ± SEM.

Results
Live weight and body condition of Rasa Aragonesa ewes
In the RS, during the period of the experimental diets (20 days), the LW of ewes in groups C—MEL and C+MEL did not change significantly, but ewes in groups L—MEL and L+MEL experienced significant (p < 0.001) weight loss (on average, in the two groups, 7.5 and 9.3 kg, respectively) (Fig. 1). In addition, ewes in groups L—MEL and L+MEL experienced significant (p < 0.001) reductions in BC (on average, in the two groups, 0.3 and 0.5 respectively), but ewes in groups C—MEL and C+MEL did not (Fig. 1). In the SA, ewes in groups C—MEL and C+MEL maintained their LW and BC, but the ewes in groups L—MEL and L+MEL experienced significant (p < 0.001) reductions in LW (in the two groups, average losses of 2.5 and 3.8 kg, respectively) and BC (average losses of 0.2 and 0.3, respectively; p < 0.05) in the 20-day period of the experimental diets (Fig. 1). In both seasons, after pessary withdrawal (14 days after the start of the experimental diets), mean LW and BC were significantly (p < 0.01) lower among the ewes in the Low groups than among the ewes in the Control groups.

Circulating hormones
In both seasons, all melatonin implants induced high plasma melatonin concentrations during daylight hours at Day 45 after implantation (mean = 53.6 ± 4.2 pg/ml and 62.6 ± 4.9 pg/ml in January and May, respectively), which indicated that the implants released melatonin properly. Plasma progesterone concentrations indicated that all of the ewes exhibited functional corpora lutea at slaughter on Day 5 after oestrus. During the RS, mean progesterone levels ranged from 3.7 ± 0.5 ng/ml (group C—MEL) to 4.7 ± 0.7 ng/ml (group L—MEL) and, during the SA, they ranged from...
2.6 ± 0.6 ng/ml (group L–MEL) to 3.8 ± 0.5 ng/ml (group C+MEL).

Ovulation rate
Ovulation rates did not differ significantly among treatments or between seasons (Tables 1 and 2).

Oocyte quality and IVF
The number of recovered oocytes per ewe was significantly (p < 0.01) higher in the RS (19.6 ± 1.0) than it was in the SA (14.5 ± 1.0). In addition, the competence of the oocytes was influenced by season; the number of healthy oocytes that could be used for IVF and the healthy oocyte rate were significantly (p < 0.01) higher in the RS than they were in the SA (13.9 ± 0.7 and 71.4% vs 9.0 ± 0.7 and 62.3% respectively). In contrast, cleavage rate tended (p < 0.1) to be higher in the SA (65.0%) than in the RS (54.2%).

Reproductive season
Neither nutrition nor treatment with melatonin implants appeared to have a significant effect on the number of recovered, healthy, or cleaved oocytes, or on the healthy oocyte and cleavage rates (Table 1); yet, diet and supplemental melatonin had a significant (p < 0.05) interaction effect on the number of non-healthy oocytes, which suggests that melatonin implants had a negative effect on this parameter in the C+MEL group and a positive effect in the L+MEL group.

Anoestrous season
Undernutrition tended (p < 0.1) to reduce the number of recovered oocytes per ewe (C: 15.6 ± 1.4, L: 13.3 ± 1.8) (Table 2). In the groups that did not receive melatonin implants, undernutrition induced a significant (p < 0.05) decrease in the number of cleaved oocytes per ewe (C= 7.2 ± 0.8, L=MEL: 4.1 ± 0.9).

Table 1. Results of the in vitro fertilization (IVF) of oocytes collected from Rasa Aragonesa ewes that were fed to provide 1.5× (C) or 0.5× (L) the maintenance requirements and treated (+MEL) or not (−MEL) with melatonin implants at the beginning (black bars) and the end (open bars) of the 20-days experimental diet period in the reproductive (left panel) and anoestrus (right panel) seasons. Bars with different superscripts (a, b) among groups differ at p < 0.01. Asterisks within groups indicate p < 0.001 (**) or p < 0.05 (*).

<table>
<thead>
<tr>
<th>Reproductive season</th>
<th>C–MEL</th>
<th>C+MEL</th>
<th>L–MEL</th>
<th>L+MEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproductive season</td>
<td></td>
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<tr>
<td>No. of ovulating ewes with functional corpus luteum</td>
<td>8/10</td>
<td>9/11</td>
<td>9/11</td>
<td>9/10</td>
</tr>
<tr>
<td>Ovulation rate</td>
<td>1.9 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td>2.2 ± 0.4</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>No. of recovered oocytes</td>
<td>17.1 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.2 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.3 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.8 ± 2.2</td>
</tr>
<tr>
<td>No. of healthy oocytes used for IVF</td>
<td>13.3 ± 1.4</td>
<td>15.3 ± 1.3</td>
<td>14.1 ± 1.0</td>
<td>13.0 ± 1.6</td>
</tr>
<tr>
<td>Healthy oocytes rate (%)</td>
<td>77.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.2</td>
<td>73.0</td>
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<tr>
<td>No. of non-healthy oocytes</td>
<td>3.9 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.9 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.2 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.8 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>No. of oocytes cleaved</td>
<td>6.1 ± 0.9</td>
<td>9.3 ± 1.2</td>
<td>8.1 ± 1.0</td>
<td>6.8 ± 1.3</td>
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<td>Cleavage rate (%)</td>
<td>45.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57.5</td>
<td>52.3</td>
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<tr>
<td>No. of blastocysts</td>
<td>0.8 ± 0.5</td>
<td>1.2 ± 0.3</td>
<td>2.6 ± 0.6</td>
<td>0.8 ± 0.5</td>
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<tr>
<td>Rate of blastocysts (%)</td>
<td>13.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.9</td>
<td>32.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are expressed per ewe.
Different superscripts (a, b) in the same row mean indicate significant differences (p < 0.05).
Different superscripts (c, d) in the same row indicate significant differences (p < 0.1).
Table 2. Results of the in vitro fertilization (IVF) of oocytes collected from Rasa Aragonesa ewes that were fed to provide 1.5x (C) or 0.5x (L) the maintenance requirements and treated (+MEL) or not treated (−MEL) with melatonin during the anoestrous period

<table>
<thead>
<tr>
<th></th>
<th>C−MEL</th>
<th>C+MEL</th>
<th>L−MEL</th>
<th>L+MEL</th>
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<tr>
<td>No. of ovulating ewes with functional corpus luteum</td>
<td>11</td>
<td>9</td>
<td>8</td>
<td>8</td>
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<td>Ovulation rate</td>
<td>2.1 ± 0.4</td>
<td>2.4 ± 0.4</td>
<td>2.5 ± 0.5</td>
<td>1.9 ± 0.5</td>
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<td>No. of recovered oocytes</td>
<td>16.1 ± 1.8</td>
<td>15.2 ± 1.9</td>
<td>12.0 ± 2.1</td>
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<td>No. of healthy oocytes used for IVF</td>
<td>9.6 ± 0.4a</td>
<td>9.4 ± 0.8</td>
<td>7.6 ± 1.4a</td>
<td>9.4 ± 1.0a</td>
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<td>Healthy oocytes rate (%)</td>
<td>59.6</td>
<td>61.8</td>
<td>63.3</td>
<td>64.4</td>
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<tr>
<td>No. of non-healthy oocytes</td>
<td>6.5 ± 0.9</td>
<td>5.8 ± 1.0</td>
<td>4.4 ± 1.1</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>No. of oocytes cleaved</td>
<td>7.2 ± 0.8b</td>
<td>5.3 ± 0.9</td>
<td>4.1 ± 0.9b</td>
<td>7.0 ± 0.7b</td>
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<tr>
<td>Cleavage rate (%)</td>
<td>75.0</td>
<td>56.4</td>
<td>53.9b</td>
<td>74.9b</td>
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<tr>
<td>No. of blastocysts</td>
<td>0.8 ± 0.4</td>
<td>1.4 ± 0.5</td>
<td>0.9 ± 0.6</td>
<td>2.6 ± 0.8</td>
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<tr>
<td>Rate of blastocysts (%)</td>
<td>11.3a</td>
<td>26.4abcd</td>
<td>21.9b</td>
<td>37.2abcd</td>
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</table>

All values are expressed per ewe.
Different superscripts (a, b, c) in the same row mean indicate significant differences (p < 0.05).
Different superscripts (d, e) in the same row indicate significant differences (p < 0.01).

Discussion

This study investigated the effects of exogenous melatonin and undernutrition on oocyte quality, IVF, and early embryonic development in Rasa Aragonesa ewes in the reproductive and anoestrous seasons. We hypothesized that the known beneficial effects of exogenous melatonin – both in vivo and in vitro – on embryo survival would compensate for the negative effects of undernutrition and season on oocyte developmental competence.

In our experiments, undernourished ewes exhibited a significant decrease in LW and BC. Although the extent of the decrease in the LW of ewes in the L groups was lower during the seasonal anestrous period, the nutritional treatments appeared to be effective because of the significant (p < 0.01) differences in the LW and BC of the ewes in the control groups and the low groups at slaughter. In previous studies, we found that a level of undernutrition similar to that of the present study for 3–4 weeks induced a significant reduction in the LW and BC of Rasa Aragonesa ewes in the breeding season (Abecia et al. 1997, 1999) and during anoestrus (Sosa et al. 2006). In addition, underfed ewes exhibited increased lipolytic activity (Sosa et al. 2006). The results of this study are consistent with the observations that short-term undernutrition associated with a progestagen-synchronized oestrus does not appear to impair ovulation rate in the RS (Abecia et al. 1997, 1999; Borowczyk et al. 2006) or the anoestrous season (Sosa et al. 2006).

In our study, SA had a significant detrimental effect on the number of recovered oocytes per ewe, the competence of the oocytes, and the fertilization rate. Anoestrus is a period of infrequent pulses of LH, whereas, in the breeding season, the frequency and amplitude of LH release is appropriate for controlling the luteal and follicular phases of the oestrous cycle (Goodman 1988). Oussaid et al. (1999) showed that a temporary suppression of LH release during the follicular phase impaired oocyte developmental competence in cyclic ewes. Even in humans, reduced pre-ovulatory levels of LH are associated with impaired in vivo oocyte fertilization (Verpoest et al. 2000). Stenbak et al. (2001) reported that IVF of oocytes recovered from superovulated ewes was higher in the breeding season than during the anoestrous. In addition, season influences in vivo fertilization rate, and some studies have shown a greater number of fertilized oocytes recovered from superovulated ewes in the breeding season than during the anoestrous (Mitchell et al. 2002; González-Bulnes et al. 2003). Changes in semen quality might contribute to the seasonal differences in IVF, but low seasonal variations in the volume and quality of the ejaculates from Rasa Aragonesa rams (Marti et al. 2007) suggest that the lower cleavage rates in the anoestrous season might be mainly caused by lower oocyte quality.

Although exogenous melatonin did not have a significant effect on most of the parameters evaluated in our study, treatment with this pineal hormone during anoestrus significantly improved the number of oocytes cleaved in undernourished ewes and also the blastocyst rate independently of the level of nutrition. In some studies, no significant positive effect of melatonin was found either on the number of visible follicles or the number of oocytes recovered from both anoestrous FSH-supervoluted ewes (Luther et al. 2005) and goats (Berlinguer et al. 2007); yet, treatment with exogenous melatonin in anoestrus seems to improve the developmental competence (IVF) of oocytes recovered from progestagen and eCG-treated ewes (Valasi et al. 2006) and the cleavage and blastocyst rates after IVF of oocytes collected from superovulated goats (Valasi et al. 2006) and the cleavage and blastocyst rates after IVF of oocytes collected from superovulated ewes (Berlinguer et al. 2007). Our recent experiments have shown that supplemental melatonin can reduce significantly the number and rate of non-viable (degenerated and retarded) recovered embryos from superovulated ewes during anoestrus (Forcada et al. 2006). The luteotrophic effect of the hormone in vivo (Durotoye et al. 1997) 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 during anoestrus. Furthermore, in humans and rats, granulose cells express specific melatonin-binding sites (Yie et al. 1995; Clemmens et al. 2001) but there are no reports in sheep. On the other hand, the radical scavenger properties of melatonin could have exerted a beneficial effect on the early embryo development, protecting embryonic cells from oxidative stress during in vitro maturation, as reported by Chetsawang et al. (2006). During the IVF, high concentrations of spermatozoa in the small volumes of IVF medium result in increased levels of free radicals but toxic effects caused by the oxidative stress were reduced with the protective radical scavenger properties of melatonin (Tamura et al. 2008). However, the mechanism by which exogenous melatonin improves the developmental competence of oocytes is still unclear, although it might be exerted both at ovarian level and at early cleavage stages.

In our study, although undernutrition did not influence significantly oocyte competence during the RS, undernourished ewes showed a reduced number of recovered and cleaved oocytes in the SA, which indicated that, in Mediterranean breeds of sheep, nutrition can, at least, partially compensate for the negative effects of season on the hypothalamic-hypophysal-ovarian axis (Forcada et al. 1992; Lindsay 1996). Impaired in vitro developmental competence of oocytes in undernourished ewes has been observed in superovulated ewes (Papadopoulos et al. 2001; Borowczyk et al. 2006). Scaramuzzi et al. (2006) summarized the effects of nutrition on the regulation of folliculogenesis in sheep. Undernutrition is associated with an increase in the secretion of oestradiol in the early follicular phase, which enhances the negative feedback at the hypothalamic-hypophyseal level and, therefore, impairs follicular competence. The effect of undernutrition on the release of oestradiol is maintained during the 2 weeks after oestrus (Sosa et al. 2006) and, in our experiment, probably affected the follicular population and in consequence the oocyte quality at Day 5. In addition, undernutrition is associated with a reduction in ovarian and endometrial sensitivity to ovarian steroids (Sosa et al. 2006, 2008), which compromises early embryo development (Vázquez et al. 2008).

In our study, the melatonin implants appeared to improve the low developmental competence of oocytes induced by undernutrition during anoestrus, and the beneficial effect of melatonin on nutrition-impaired folliculogenesis during anoestrus has been observed elsewhere (Robinson et al. 1991; Forcada et al. 1995). These studies clearly showed that the ability of exogenous melatonin to improve ovulation rates was more pronounced in ewes that were on a low, rather than a high, level of feed intake; although the mechanisms involved in that response remain to be elucidated.

In conclusion, the results of our study indicate that seasonal anoestrus and undernutrition can impair the quality of oocytes recovered from Rasa Aragonesa, a Mediterranean breed of sheep. Furthermore, supplemental melatonin appeared to improve oocyte developmental competence during the seasonal anoestrous period, especially in undernourished ewes. Those results underscore the importance of understanding the nature of nutrition-melatonin interactions and their mechanisms, particularly at the ovarian and early embryo development levels and during the non-reproductive season.

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Author contributions
Drs Forcada and Abecia have made contributions on the main aspects of this publication (as research design, analysis and interpretation of data and writing; drafting and revising the manuscript critically). Drs Sosa and Palacin participated in the acquisition of data; Dr Casao was the responsible for the IVF laboratory. Miss Vazquez was involved in the acquisition, analysis and interpretation of data and writing; drafting and revising the manuscript.

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