Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/sjar

Spanish Journal of Agricultural Research 2010 8(1), 35-41
ISSN: 1695-971-X

The effects of melatonin on *in vitro* oocyte competence and embryo development in sheep

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Abstract

The aim of this study was to assess the effects of melatonin on the *in vitro* maturation and fertilization of sheep oocytes, and on the *in vitro* culture of the embryos. Oocytes from sheep ovaries collected at the slaughterhouse were divided into four groups, two of which were treated with either 10^{-5} M (M5) or 10^{-6} M (M6) melatonin, while the other two groups served as untreated controls (C5 and C6). After *in vitro* fertilization with fresh ram semen, the embryos produced in each group were divided into two sets, one cultured with melatonin (M5M, C5M, M6M and C6M), and the other without melatonin (M5C, C5C, M6C, and C6C). A melatonin concentration of 10^{-6} M increased maturation rate (82.5% *vs*. 73.7% for M6 and C6, respectively; $P < 0.05$) and tended to improve cleavage rate 36 hours after *in vitro* fertilization (79.4% *vs*. 72.6% for M6 and C6, respectively, $P = 0.08$). A higher melatonin concentration (10^{-5} M) did not have significant effects on those parameters. Blastocyst rates on day 8 did not differ significantly among groups.

Additional key words: *in vitro* fertilization, *in vitro* maturation.

Resumen

Efecto de la melatonina en la competencia del oocito y el desarrollo embrionario ovino *in vitro*

El objetivo de este estudio es determinar el efecto de la melatonina sobre la maduración y fecundación *in vitro* de ovocitos ovinos, y sobre el cultivo *in vitro* de los embriones obtenidos. Ovocitos extraídos de ovarios de ovejas obtenidos en matadero se dividieron en cuatro grupos, de los cuales dos fueron tratados con melatonina 10^{-5} M (M5) ó 10^{-6} M (M6), mientras que los otros dos sirvieron como grupos control (C5 y C6). Tras su fecundación *in vitro* con semen fresco de morueco, los embriones obtenidos en cada grupo se dividieron a su vez en otros dos grupos, de forma que la mitad se cultivaron con melatonina (M5M, C5M, M6M y C6M), y la otra mitad sin ella (M5C, C5C, M6C, y C6C). La concentración 10^{-6} M de melatonina aumentó el porcentaje de maduración de los ovocitos (82,5% *vs*. 73,7% en los grupos M6 y C6, respectivamente; $P < 0,05$) y tendió a mejorar el porcentaje de embriones divididos 36 horas tras la fecundación (79,4% *vs*. 72,6% en los grupos M6 y C6, respectivamente; $P = 0,08$). Una mayor concentración de melatonina (10^{-5} M) no tuvo efecto significativo sobre estos parámetros. No se encontraron diferencias significativas en los porcentajes de blastocistos tras ocho días de cultivo.

Palabras clave adicionales: fecundación *in vitro,* maduración *in vitro.*

Introduction

The use of melatonin implants to improve reproduction in sheep is widely spread in some Mediterranean countries. In the last few years some reports have revealed that melatonin not only modifies the perception of photoperiod by ewes at hypothalamic level but also can exert its influence at other targets of the reproductive system (Abecia *et al.*, 2008). Melatonin, an endogenous hormone produced by several organs,

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Received: 08-06-09; Accepted: 30-01-10.

Abbreviations used: DMSO (dimethyl sulfoxide), FSH (follicle-stimulating hormone), IVF (*in vitro* fertilization), IVM (*in vitro* maturation), LH (luteinizing hormone), PBS (phosphate buffered saline), SOF (synthetic oviductal fluid).

particularly the pineal gland (Huether, 1993), is essential for sleep and temperature regulation, circadian and seasonal rhythmic cycles, and reproductive physiology (Arendt, 1998; Arendt, 2003). Melatonin is used widely to advance the breeding season in sheep and is an effective method of inducing oestrous cycles, increasing lambing rates and prolificacy during the seasonal anoestrous (Haresign *et al.*, 1990; Haresign, 1992; Abecia *et al.*, 2007). Those effects are associated with an improvement in ovulation rate (Zúñiga *et al.*, 2002), luteal function (Durotoye *et al.*, 1997; Abecia *et al.*, 2002) and embryo viability (Forcada *et al.*, 2006). For a summary of the effects of exogenous melatonin on the ovary and early embryos in ewes, see Abecia *et al.* (2008).

The high amphilicity of melatonin promotes its rapid transfer into organs and fluids, and this pineal hormone can rapidly pass through cellular membranes. Reported increases in prolificacy subsequent to the administration of melatonin in sheep might be due to the direct effect of exogenous melatonin on the ovine oocyte, which has been observed in other species (Ishizuka *et al.*, 2000; Na *et al.*, 2005; Parka *et al*., 2006). Several studies have reported the presence of melatonin in human pre-ovulatory follicular fluid and the seasonal variation of its contents (Brzezinski *et al*., 1987; Yie *et al*., 1995), and melatonin receptors have been found on rat, mouse, and porcine ovarian cells (Soares *et al*., 2003; Na *et al*., 2005; Kang *et al*., 2008), which suggests that melatonin has a direct effect on oocyte competence.

In sheep, some studies have suggested that seasonal anoestrous can have a significant detrimental effect on the number of recovered oocytes per female, the competence of the oocytes, and the *in vitro* fertilization rate (Stenbak *et al*., 2001; Vázquez *et al*., 2009); however, information about the effects of melatonin treatments on oocyte quality and *in vitro* fertilization (IVF) during anoestrous is limited. In one study, after IVF of the oocytes recovered from superovulated ewes in the non-reproductive season, melatonin-treated and untreated ewes did not differ significantly in their fertilization rates (Luther *et al*., 2005). In another study, developmental competence of oocytes collected from ewes during the anoestrous was significantly improved by supplemental exogenous melatonin (Valasi *et al*., 2006). Although there are reports of the positive effects of adding melatonin in *in vitro* maturation (IVM) (Dimitriadis *et al*., 2005) and embryo culture media (Ishizuka *et al*., 2000; Na *et al*., 2005), the effect of melatonin in the IVM and IVF media on the developmental competence of ovine oocytes has not been evaluated thoroughly, which is needed to improve the efficiency of IVM, IVF, and *in vitro* embryo culture procedures.

The objective of this study was to evaluate the effects of two different melatonin concentrations on the *in vitro* maturation and fertilization of oocytes from sheep ovaries collected at the slaughterhouse, and on the *in vitro* culture of the embryos.

Material and methods

Oocyte collection and *in vitro* maturation

From November to February, the ovaries of mature Rasa Aragonesa ewes were collected at the slaughterhouse and transported to the laboratory in phosphate-buffered saline solution (PBS) supplemented with 100 IU mL^{-1} of penicillin-G and 100 μg mL^{-1} of streptomycin sulphate at 35-37°C. Except where indicated otherwise, all of the reagents for the preparation of the media were purchased from Sigma-Aldrich Co.

Oocytes were collected using a combination of puncture and slicing techniques (Wani *et al.*, 1999) in a Petri dish that contained handling medium (Hepes-buffered TCM-199 supplemented with 0.1% of polyvinyl alcohol, 0.04% of sodium bicarbonate, 25 IU mL^{-1} of heparin, 100 IU mL^{-1} of penicillin-G, and 100 μg mL^{-1} of streptomycin sulphate). Following Wani *et al*. (2000), oocytes were categorized based on their cumulus cells and cytoplasm morphology; only the oocytes that had several layers of cumulus cells and a uniform cytoplasm were selected for *in vitro* maturation. Those oocytes were randomly assigned either to one of two melatonin-treatment groups [10^{-5} M (M5) or 10^{-6} M (M6)] or to one of two untreated (control) groups [C5 (control group for embryos treated with 10^{-5} M melatonin) and C6 (treated with 10^{-6} M melatonin)]. and transferred into a maturation medium that contained bicarbonate-buffered TCM-199, supplemented with 10% (v/v) oestrus sheep serum, 10 μg mL^{-1} each of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), 100 μM of cysteamine, 0.3 mM of sodium piruvate, 100 IU mL^{-1} of penicillin G, and 100 μg mL^{-1} of streptomycin sulphate, covered with mineral oil, and incubated for 24 h at 39°C under 5% CO_2 and a saturated humidity.

Melatonin was dissolved in dimethyl sulfoxide (DMSO) and PBS to produce concentrations of 10^{-5} or

10^{-6} M, which was administered to the oocytes in the M5 and M6 groups, respectively. The final concentration of DMSO in the media was 0.01% v/v, with no effect on embryo development, as stated by Ishizuka *et al.* (2000).

In vitro fertilization and embryo culture

After the end of incubation, oocytes were freed from the cumulus cells and transferred to a fertilization medium that consisted of synthetic oviductal fluid (SOF) without glucose (Tervit and Whittingham, 1972), supplemented with 2% (v/v) of oestrous sheep serum, 10 μg mL^{-1} of heparin, and 1 μg mL^{-1} of hypotaurine. Again, melatonin was added to the fertilization medium of the M5 and M6 groups in the concentrations used in the maturation medium.

On the day of fertilization, fresh semen was collected from four Rasa Aragonesa rams, pooled, diluted 1:10 in a saline medium that contained 0.25 M sucrose, 10 mM Hepes, 2 mM KOH, 5 mM glucose, 0.5 M sodium phosphate monobasic, and 100 mM ethylene glycol tetraacetic acid (EGTA), and kept at 15°C until needed for fertilization. Highly motile spermatozoa were selected using the swim-up technique, added to the fertilization medium (350 μL) containing the oocytes at a final concentration of 1×10^6 spermatozoa mL^{-1}, covered with mineral oil, and incubated for 24 h, at 39°C in 5% CO_2.

After 24 and 36 h, the cleaved embryos in each group were randomly divided into two sets. Half of the embryos in each group were cultured in a media that contained melatonin at either a 10^{-5} M concentration (embryos from the M5 and C5 oocytes) or a 10^{-6} M concentration (embryos from the M6 and C6 oocytes), and the other half remained as untreated controls. The embryos from the eight groups (M5M, M5C, C5M, C5C, M6M, M6C, C6M, and C6C) were placed in a culture medium that contained SOF supplemented with essential and non-essential amino acids at oviductal concentrations (Walker *et al.*, 1996), 0.4% BSA (w/v), 1 mM of L-glutamine, 100 IU mL^{-1} of penicillin G, and 100 μg mL^{-1} of streptomycin sulphate, covered with mineral oil, and kept at 39ºC in a maximally humidified atmosphere, with 5% CO_2, 5% O_2, and 90% N_2 until the blastocyst stage (day 8). The number and developmental stage of blastocysts (young, expanded, hatching and hatched blastocysts) were recorded at 6, 7, and 8 days after fertilization.

Non-cleaved oocytes were observed under stereomicroscope to assess their maturation stage. Ooocytes showing the first polar body were considered matured, and oocytes with two polar bodies were considered fertilized, but not cleaved.

Statistical analysis

Maturation and cleavage rates were calculated over the number of selected oocytes, whereas fertilization rate was calculated over the number of matured oocytes, and blastocysts rates were calculated over the number of cleaved embryos.

Maturation, cleavage, and blastocyst rates of the experimental groups were compared by means of chi-square test (SPSS Software, v.14.0). When at least one value had an expected frecuency less than 5, Yates' correction for continuity was used.

Results and discussion

In this study, the lower of the two melatonin concentrations (10^{-6} M) increased significantly the maturation rate of Rasa Aragonesa oocytes ($P < 0.05$) and tended to increase the cleavage rate ($P < 0.1$); however, a 10^{-5} M concentration of melatonin in the media did not influence significantly the maturation, fertilization or cleavage (Table 1). The addition of melatonin to IVM and IVF media can improve *in vitro* oocyte maturation and cleavage rates in bovids (Dimitriadis *et al.*, 2005), mice (Na *et al.*, 2005) and humans (Parka *et al.*, 2006).

High concentrations of melatonin in follicular fluid (Brzezinski *et al.*, 1987), and the presence of receptors in granulose cells (Soares *et al.*, 2003; Na *et al.*, 2005; Kang *et al.*, 2008), suggest that melatonin might be important to ovarian function. At night, the melatonin concentrations in physiological plasma in Rasa Aragonesa ewes are about 4×10^{-7} M (Forcada *et al.*, 1995). Studies have shown that physiological melatonin concentrations in human follicular fluid are almost three times higher than they are in the serum (Brzezinski *et al.*, 1987; Ronnberg *et al.*, 1990). Thus, the melatonin concentration of 10^{-6} M could provide a basis for evaluating the benefits of exogenous melatonin at the gamete and early embryo stages. Furthermore, the beneficial effect of melatonin on early embryo development might be mediated through the reactive oxygen species (ROS) scavenger properties of the

Table 1. Maturation, fertilization, cleavage and blastocyst rate of oocytes from Rasa Aragonesa ewes matured and/or cultured with or without 10^{-5} or 10^{-6} mol L^{-1} melatonin

Oocyte group	Maturation	Fertilization	Cleavage	Embryo culture group	Blastocyst rate
M5	151/186 (81.2%)	146/151 (96.7%)	144/186 (77.4%)	M5M	23/69 (33.3%)
				M5C	20/75 (26.7%)
C5	147/191 (77.0%)	144/147 (98.0%)	144/191 (75.4%)	C5M	21/74 (28.4%)
				C5C	26/70 (37.1%)
M6	156/189 (82.5%)[a]	150/156 (96.2%)	150/189 (79.4%)[c]	M6M	27/74 (36.5%)
				M6C	29/76 (38.2%)
C6	132/179 (73.3%)[b]	131/132 (99.2%)	130/179 (72.6%)[d]	C6M	22/64 (34.4%)
				C6C	20/66 (30.3%)

Within columns, different letters (a, b) indicate differences of $P < 0.05$ at the same treatment dose. Within columns, different letters (c, d) indicate differences of $P = 0.08$ at the same treatment dose.

pineal hormone (Reiter *et al*., 2000). As a ROS scavenger, melatonin might be very valuable shortly after IVF because high concentrations of spermatozoa in small volumes of IVF medium lead to an increase in free radicals. Oxidative stress has toxic effects and melatonin provides protection against the oxidative stress (Tamura *et al*., 2008).

These results and those of others (Adriaens *et al*., 2006) suggest that the effect of melatonin on oocyte maturation is dose-dependent. Although melatonin toxicity is reported to be extremely low, oocyte maturation in female mice was significantly impaired by melatonin concentrations of 10^{-3} M or higher. In the early stages of maturation, melatonin has an even greater effect on the *in vitro* maturation of oocytes (Dimitriadis *et al*., 2005).

In this study, blastocyst rates did not differ significantly among groups at either of the two melatonin concentrations used (Table 1) but the highest dose added to the culture medium of the control group seemed to impair embryo development. When data were analyzed by culture day and blastocyst type, significant differences were found on hatching blastocyst rate in day 8 between 10^{-5} M treated and control groups, and on young blastocyst rate in day 8 derived from oocytes matured with or without 10^{-6} M melatonin and cultured without the pineal hormone (Table 2). Elsewhere, another study demonstrated a negative effect of melatonin on blastocyst rate when it was added to the maturation medium at 10^{-5} M concentration (Casao *et al*., 2007), but a concentration halfway between 10^{-6} and 10^{-5} M ($0.43 \cdot 10^{-5}$ M) in the culture medium appeared to increase the hatching rate of thawed ovine blastocysts (Abecia *et al.*, 2002).

In general, the effects of melatonin on *in vitro* preimplantational embryo development are unclear. Several studies have shown that enriching the culture medium with melatonin can improve embryo development, but blastocyst rates can vary widely depending on the melatonin concentrations (Ishizuka *et al*., 2000; Danilova *et al*., 2004; Manjunatha *et al*., 2008). When embryos are subjected to melatonin concentrations of 10^{-3} M or higher, cleavage rates can be impaired (Rodriguez-Osorio *et al*., 2007), but other studies covering a wide range of concentrations suggest that melatonin treatments do not influence the preimplantation development of embryos (McElhinny *et al*., 1996; Tsantarliotou *et al*., 2007).

In addition to its benefits to *in vitro* maturation and early embryo development, melatonin might have a beneficial effect on embryo preimplantation development because of its capacity as a radical scavenger (Chetsawang *et al*., 2006), to protect embryonic cells from oxidative stress. High levels of reactive oxygen species can damage spermatozoa, oocytes, and embryos (Agarwal *et al*., 2003), and *in vitro* melatonin treatments might protect them against oxidative stress. In a recent study, Papis *et al.* (2007) showed that the beneficial effects of melatonin on bovine embryo development were greater when embryos were cultured in the presence of high concentrations of atmospheric oxygen (20%) rather than at physiological concentrations (7%). In addition, melatonin can increase blastocyst rates and blastocyst total cell number concomitant to a significant decrease of apoptotic nuclei rate in preimplantational parthenogenetic porcine embryos (Choi *et al*., 2008).

Table 2. Blastocyst type rate of oocytes from Rasa Aragonesa ewes matured and/or cultured with or without 10^{-5} or 10^{-6} mol L^{-1} melatonin, on days 6, 7 and 8 after oocyte fertilization

	Day 6		Day 7				Day 8			
	Young	**Expanded**	**Young**	**Expanded**	**Hatching**	**Hathed**	**Young**	**Expanded**	**Hatching**	**Hatched**
M5M	7/69 (10.1%)	1/69 (1.4%)	11/69 (15.9%)[a]	9/69 (13.0%)	2/69 (2.9%)	1/69 (1.4%)	3/69 (4.3%)	12/69 (17.4%)	5/69 (7.2%)	3/69 (4.3%)
M5C	6/75 (8.0%)	1/75 (1.3%)	4/75 (5.3%)[b]	11/75 (14.6%)	3/75 (4.0%)	1/75 (1.3%)	3/75 (4.0%)	8/75 (10.6%)	1/75 (1.3%)[b]	8/75 (10.6%)
C5M	10/74 (13.6%)	2/74 (2.7%)	5/74 (6.8%)	11/74 (14.9%)	3/74 (4.1%)	2/74 (2.7%)	1/74 (1.4%)	10/74 (13.6%)	2/74 (2.7%)[b]	8/74 (10.8%)
C5C	7/70 (10.0%)	6/70 (8.6%)	6/70 (8.6%)	11/70 (15.7%)	6/70 (8.6%)	0/70 (0.0%)	7/70 (10.0%)	7/70 (10.0%)	10/70 (14.3%)[a]	2/70 (2.9%)
M6M	7/74 (9.5%)	4/74 (5.4%)	9/74 (12.2%)	11/74 (14.9%)	4/74 (5.4%)	0/69 (0.0%)	5/74 (6.8%)	11/74 (14.9%)	3/74 (4.1%)	8/69 (10.8%)
M6C	5/76 (6.6%)	6/76 (7.9%)	7/76 (9.2%)	15/76 (19.7%)	0/75 (0.0%)	3/76 (3.9%)	7/76 (9.2%)[a]	10/76 (13.2%)	3/76 (3.9%)	9/76 (11.8%)
C6M	4/64 (6.2%)	3/64 (3.1%)	9/64 (14.1%)	6/64 (9.4%)	1/64 (1.6%)	2/64 (3.1%)	5/64 (7.8%)	11/64 (17.2%)	1/64 (1.6%)	5/64 (7.8%)
C6C	4/66 (6.1%)	5/66 (7.6%)	7/66 (10.6%)	9/66 (13.6%)	4/66 (6.1%)	0/66 (0.0%)	0/66 (0.0%)[b]	7/66 (10.6%)	7/66 (10.6%)	6/66 (9.1%)

Within columns, different letters (a, b) indicate differences of $P < 0.05$ at the same treatment group.

In conclusion, this study demonstrated that adding 10^{-6} M melatonin to IVM and IVF media increased *in vitro* maturation and early cleavage rates of sheep oocytes. Although a higher concentration (10^{-5} M) of melatonin did not have the same effect, additional studies are needed to optimize the use of melatonin in IVM, IVF, and *in vitro* embryo culture procedures.

Acknowledgements

This study was supported by grants AGL2007-63822 from CICYT and A-26 from DGA.

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