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Effect of exogenous melatonin on the ovary, the embryo and the establishment of pregnancy in sheep

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Administration of melatonin to advance the breeding season in sheep has been widely used, since this hormone conveys the photoperiodic signal to the reproductive neuroendocrine axis. An increased lambing percentage has been reported following such treatment during anoestrus, which could be mediated through a higher rate of embryonic survival, either by an improvement in luteal function or by a reduction in the antiluteolytic mechanisms. The aim of this article is to review the body of knowledge on the effect of melatonin on the ovine ovary, the embryo and the establishment of pregnancy. Some studies using synchronized ewes have found that melatonin treatments during anoestrus do not improve ovulation rate by modifying the timing of follicle emergence, but increasing the number of ovulatory follicles by decreasing the atresia of medium and large follicles. On the other hand, the addition of melatonin to the in vitro *maturation medium does not improve oocyte maturation rate in oocytes from sheep ovaries recovered either in anoestrus or in the breeding season. However, a luteotrophic effect of melatonin at either short or medium term has been reported. We have recently observed that melatonin implants tend to improve the survival of embryos collected from ewes after superovulation in anoestrus. More specifically, melatonin induced a significant reduction of the number and rate of non-viable (degenerate and retarded) embryos. Preliminary data from our laboratory suggest that the uterine sensitivity to progesterone – in terms of progesterone receptor expression – of superovulated ewes could be reduced by melatonin treatment. It can be concluded that the success of exogenous melatonin as a means to improve lamb production of sheep is due, at least in part, to an improvement of luteal support and embryonic survival.*

Keywords: embryo, melatonin, ovary, pregnancy

Introduction

Administration of melatonin to advance the breeding season in sheep has been widely used, since this hormone conveys the photoperiodic signal – which in turn mediates seasonality in sheep (Yeates, 1949) – to the reproductive neuroendocrine axis (Bittman *et al*., 1983). An increased lambing percentage has been reported following such treatment during anoestrus (Haresign *et al*., 1990; Chemineau *et al*., 1991), which could be mediated through a higher rate of embryonic survival, either by an improvement in luteal function or by a reduction in the antiluteolytic mechanisms.

In ruminants, the onset and maintenance of pregnancy is established from signalling by the embryo to the maternal system, and progesterone secreted from the corpus luteum is essential in the initial stages of pregnancy. The maternal recognition of pregnancy has been defined as the process through which the regression of the corpus luteum (luteolysis) is blocked in early pregnancy (Short, 1969). The luteolytic mechanism in ruminants includes a positive feedback system involving the endometrial prostaglandin (PG) $F_{2\alpha}$, and oxytocin. On the other hand, oestradiol and progesterone are the main modulators of uterine function, and both of them are implicated in the timing of luteolysis via their nuclear receptors (Lamming *et al*., 1995; Meikle *et al*., 2004). The embryo generates a signal (interferon-tau, IFNτ) into the uterine horns during elongation, which alters uterine gene expression, modifying the episodic $PGF_{2\alpha}$ release, which is responsible for the luteal regression (review, Spencer *et al*. (2004)).

In this context, works describing the effect of exogenous melatonin on the establishment of pregnancy and on embryo development are scarce. For instance, Gimeno *et al*. (1980) reported that melatonin blocks *in vitro* generation of $PGF_{2\alpha}$ by the uterus, and it has been found that melatonin can modify PG biosynthesis in the hypothalamus (Bojanowska and Forsling, 1997). On the other hand, an effect of melatonin

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on progesterone secretion by the corpus luteum has been reported in sheep (Durotoye *et al.*, 1997), cattle (Webley and Luck, 1986) and humans (Webley and Luck, 1986; Webley *et al.*, 1988), and the presence of melatonin receptors has been demonstrated in human granulosa cells (Yie *et al.*, 1995) and in the uterine endometrium of oestrous rats (Zhao *et al.*, 2000).

Little is known about the direct effect of melatonin on the embryo. McEvoy *et al.* (1998) did not observe differences at medium term in embryo production between melatonin-treated (April) or non-treated donor ewes, and in the ability of transferable embryos to survive the transfer. There is evidence of melatonin receptors in the embryonic retina of chicks (Iuvone and Gan, 1994) and of melatonin content in the retina of chick embryos from the 7th day of embryonic age (Espinar *et al.*, 1994), but there is no record about the presence of melatonin or its receptors in sheep blastocysts.

The aim of this article is to review the body of knowledge on the effect of melatonin on the ovine ovary, the embryo and the establishment of pregnancy.

Effect of melatonin on the ovine ovary

Ovulation rate

It is widely known that the highest ovulation rate is achieved a few weeks after the onset of the breeding season, and lower ovulation rates have been reported in both seasonal anoestrus (for reduced seasonality breeds) and at the beginning and at the end of the breeding season (Forcada *et al.*, 1992; Rondon *et al.*, 1996). Therefore, most of the references in the literature deal with the effects at medium term of melatonin treatments during seasonal anoestrus. Several authors have reported an increased ovulation rate after treatment with melatonin implants in both seasonal (Robinson *et al.*, 1991; Haresign, 1992) and Mediterranean ewes (Forcada *et al.*, 1995; Zuniga *et al.*, 2002). Some studies using synchronized ewes have found that a medium-term treatment with melatonin does not improve ovulation rate by modifying the timing of follicle emergence, but increasing the number of ovulatory follicles by decreasing the atresia of medium and large follicles (Bister *et al.*, 1999; Noel *et al.*, 1999). However, melatonin treatments do not induce significant increases in ovulation rate following a superovulatory treatment, either in anoestrus (Forcada *et al.*, 2006) or during the breeding season (McEvoy *et al.*, 1998), suggesting that a protocol of several FSH injections probably recruits all the follicles independently of atresia mechanisms (McEvoy *et al.*, 1998). Likewise, no differences have been reported between ovulation rates consecutive to superovulatory treatments performed in anoestrus or the reproductive season (Mitchell *et al.*, 2002; Gonzalez-Bulnes *et al.*, 2003).

Oocyte production and competence

No differences in the number and *in vitro* maturation (IVM) rates of oocytes from ovaries recovered from slaughtered ewes were found between the anoestrus and the breeding season (Rao *et al.*, 2002). Moreover, Luther *et al.* (2005) reported that melatonin did not improve both parameters in implanted ewes when compared with controls during the seasonal anestrous period. Previous results from our laboratory show that the addition of melatonin to the IVM medium does not improve maturation rate in oocytes from sheep ovaries recovered either in anoestrus or in the breeding season (Casao *et al.*, unpublished results). In contrast, Dimitriadis *et al.* (2005) reported a positive effect of supplementation with melatonin in the IVM medium on the number of bovine oocytes reaching the metaphase-II stage.

Luteal activity

The premature demise of the corpora lutea after exogenous gonadotropin stimulation is a poorly understood cause of embryo recovery failure that is reported following gonadotropin-releasing hormone (GnRH)-induced ovulation in anestrous ewes (McLeod *et al.*, 1982), although progestagen priming before ovulation induction eliminates premature luteolysis in those females (McLeod *et al.*, 1984; Southee *et al.*, 1988). Inadequate luteal function has also been reported in superovulated ewes following oestrous synchronization using a dual prostaglandin injection treatment (Trounson *et al.*, 1976; Schiewe *et al.*, 1991). In embryo donor ewes, the incidence of premature luteal regression after a progestagen protocol and superovulation seems to be higher in the seasonal anoestrus than in the breeding season, even at temperate latitudes (Jabbour *et al.*, 1991; Ryan *et al.*, 1991). Corpora lutea from ewes undergoing premature luteolysis have a reduced population of small luteal cells compared to normal corpora lutea (Schiewe *et al.*, 1991). Subnormal luteal function is a result of the premature release of $PGF_{2\alpha}$ from the uterus because of the presence of endometrial oxytocin receptors, which in turn is up-regulated by high levels of oestradiol (Ishwar and Memon, 1996). The incidence of ewes with regressed corpora lutea seems to be related to excessive levels of oestrogen, bringing about luteolysis and to a lower endometrial concentration of progesterone receptors (which down-regulate oestrogen endometrial receptors expression in anestrous ewes (Robinson *et al.*, 1991) than in normal cycles (Bister *et al.*, 1999).

An acute luteotrophic effect of melatonin infusion during either anoestrus or the breeding season has been reported (Durotoye *et al.*, 1997), leading to an increase in plasma progesterone concentrations. These results were confirmed *in vivo* in another experiment (Abecia *et al.*, 2002); during the luteal phase of a synchronized sexual cycle (7 and 15 days after pessary removal), five ewes were challenged with an intravenous melatonin injection (3 μg/kg $BW^{0.75}$), and the response of the corpus luteum, in terms of plasma progesterone concentration after the challenge, was measured. All the ewes on day 7 and three of five on day 10 showed a progesterone response at some time during the hour following the melatonin challenge (Figure 1).

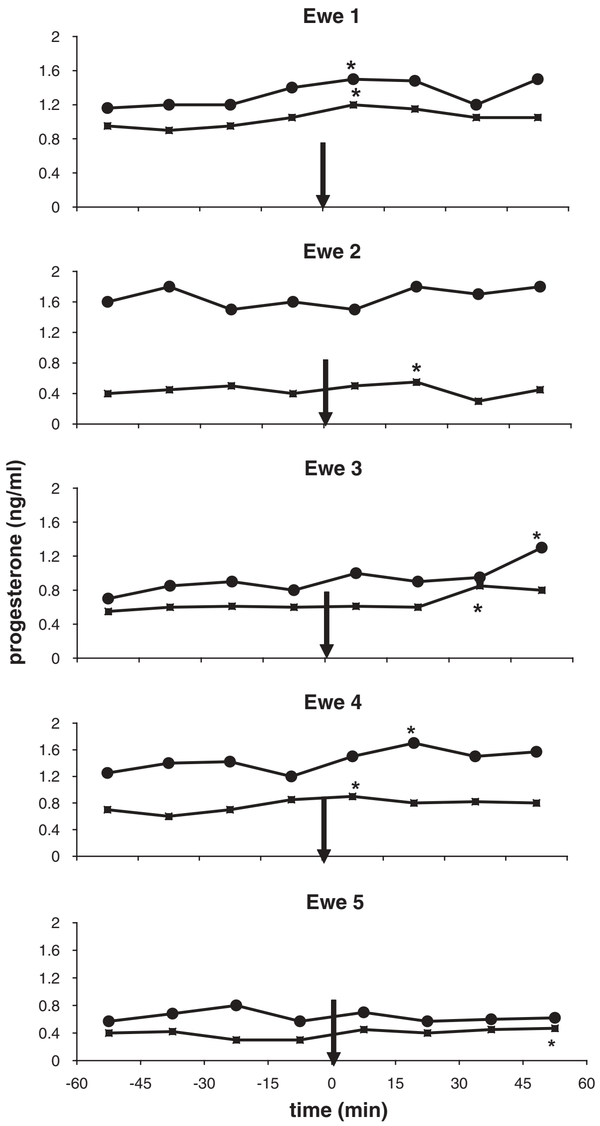

Figure 1 Profiles of mean plasma progesterone response on day 7 (■) and day 10 (●) after pessary withdrawal. The time of melatonin injection (3 μg/kg $BW^{0.75}$) is indicated by arrow. *Indicates a progesterone response, defined as an increase at two points during the post-treatment period above the mean ± 2 s.d. of the values in the pre-treatment period (adapted from Abecia *et al.*, 2002).

The paired *t*-test revealed that melatonin increased the overall plasma progesterone concentrations, both on day 7 (pre, 0.61 ± 0.11; post, 0.73 ± 0.13 ng/ml; $P < 0.01$) and on day 10 (pre, 1.16 ± 0.19; post, 1.30 ± 0.20 ng/ml; $P < 0.01$). These results provided evidence for an effect of melatonin on progesterone secretion *in vivo*. Since the experimental procedures were performed at a very short term, this effect cannot be attributed to the endogenous rhythm of melatonin secretion.

Those acute luteotrophic effects of melatonin seem to be coincident with the results reported after medium-term treatments during anoestrus to advance the breeding season and to improve the ovulation rate. Wallace *et al.* (1988) observed that melatonin administered daily improved progesterone concentrations in the latter half of the luteal phase. Although melatonin implants during the anoestrus associated with a male-effect management did not modify the secretion of luteinising hormone (LH) before or following the introduction of rams (Gómez Brunet *et al.*, 1995), results from our laboratory have reported that the pineal hormone induces a significant decrease of the proportion of ewes exhibiting a short cycle in response to the ram effect (Abecia *et al.*, 2006). This short cycle is the consequence of a subnormal corpus luteum that has a short life span and regresses around day 7 after ovulation. However, no effect of melatonin implants on luteal function has been elucidated after superovulation during the seasonal anoestrous period (Forcada *et al.*, 2006).

Effect of melatonin on the embryo

Fertilization rate

Season has an effect on fertilization rate, and some authors have reported a higher number of fertilized oocytes recovered from superovulated ewes in the breeding season than during the anoestrus (Mitchell *et al.*, 2002; Gonzalez-Bulnes *et al.*, 2003). However, the treatment with melatonin in anoestrus did not improve fertilization rate in superovulated ewes when compared with controls (McEvoy *et al.*, 1998; Forcada *et al.*, 2006). Likewise, Stenbak *et al.* (2001) reported a higher *in vitro* fertilization (IVF) of oocytes recovered from superovulated ewes in the breeding season than during the anoestrus, whereas no differences in fertilization rate were elucidated after IVF of oocytes recovered from superovulated ewes treated or not with melatonin in anoestrus (Luther *et al.*, 2005).

It would be advisable to highlight that the ram is the other agent involved in the fertilization process. Although the effects of melatonin on semen production and quality are not fully known, it seems that the treatment with pineal hormone can improve sperm motility (Kaya *et al.*, 2000) and even modify semen plasma characteristics (Garde López-Brea *et al.*, 1996).

Embryo survival and development

Some studies have suggested that there is no effect of season on the number of recovered embryos from superovulated ewes, particularly in Mediterranean genotypes (Lopez Sebastian *et al.*, 1990; Gonzalez-Bulnes *et al.*, 2003). However, embryos from superovulated ewes during the seasonal anoestrus seem to have a lower viability, as indicated by a higher percentage of retarded and degenerated embryos when compared with those recovered during the reproductive season (Mitchell *et al.*, 2002). Daily treatment with oral melatonin for 45 days to superovulated ewes in anoestrus does not seem to modify embryo production or survival (McEvoy *et al.*, 1998); however, Forcada *et al.* (2006) recently reported that melatonin implants tended to improve the survival of embryos collected from Rasa Aragonesa ewes after superovulation in anoestrus. More specifically, melatonin induced a significant reduction

Table 1 *Percentage of embryos in the different stages of development when incubated with (M+) or without (M−) melatonin (1 μg/ml) in the culture medium (adapted from Abecia* et al.*, 2002)*

	Start culture as morulae				Start culture as blastocysts			
	24 h		48 h		24 h		48 h	
	M+	M−	M+	M−	M+	M−	M+	M−
n	23	23			22	23		
Compact morula	78.3	69.6	4.3	17.4				
Blastocyst – expanded blastocyst	13.0	30.4	21.7	8.7	40.9	52.2	27.3	4.3
Hatched blastocyst	8.7	–	13.0	34.8	54.5^{a}	17.4^{b}	59.1	52.2
Degenerated	0	0	60.9	34.8	4.5^{a}	30.4^{b}	13.6^{c}	43.5^{d}

$^{a,b}P < 0.01$.
$^{c,d}P < 0.05$.

in the number and rate of non-viable (degenerate and retarded) embryos.

In order to determine whether melatonin could improve embryo development *in vitro*, 91 thawed sheep embryos (46 morulae and 45 blastocysts) were cultured with or without melatonin (Abecia *et al.*, 2002). If the embryos were blastocysts when the culture started, melatonin increased the percentage that had hatched after 24 h of culture ($P < 0.01$), and there was a lower percentage of degenerated embryos at the end of the incubation period ($P < 0.05$). However, melatonin seemed to exert no effect on the *in vitro* development of morulae, a similar percentage of embryos reaching the stage of blastocyst or hatched blastocyst when compared with controls after 24 or 48 h of culture (Table 1). As we were aware, this was the first report of a direct effect of melatonin on embryonic development, using an *in vitro* model. Further research in our laboratory has recently elucidated a lower percentage of degenerated embryos after an *in vitro* culture of fertilized ova associated with the presence of melatonin in the culture medium when compared with controls, cultured in the absence of melatonin after fertilization (Casao *et al.*, unpublished results).

It remains to determine whether melatonin is able to exert effects at the uterine level, thus modifying embryo environment *in vivo*. Some preliminary data from our laboratory (Sosa, unpublished results) suggest that the uterine sensitivity to progesterone – in terms of progesterone receptor expresssion – of superovulated ewes could be reduced by melatonin treatment. We have recently demonstrated that melatonin-treated ewes during anoestrus had higher plasma concentrations of progesterone during the male effect-induced luteal phase than untreated ewes (Abecia *et al.*, 2006), and this could have been reflected in a stronger down-regulation of progesterone receptor (PR) in the melatonin group. Taking into account the results of that preliminary trial, and the fact that steroid-modulated melatonin receptors have been found in rat uterus (Zhao *et al.*, 2000 and 2002), a role of melatonin in the endometrial physiology could be envisioned, although there is still a wide field to explore.

Effect of melatonin on maternal pregnancy recognition mechanisms

Abecia *et al.* (1999), in a study involving the effect of both dietary energy and melatonin on the *in vitro* production of $PGF_{2\alpha}$ by endometrial tissue on days 9 and 15 of pregnancy, observed that melatonin affected $PGF_{2\alpha}$ secretion *in vitro* only in undernourished ewes on day 15 of pregnancy, when the signals of maternal recognition of pregnancy are being established. Neither the plane of nutrition nor the inclusion of melatonin in the culture medium modified progesterone production on day 9 of pregnancy. On day 15 of pregnancy, the corpora lutea of undernourished ewes tended to secrete

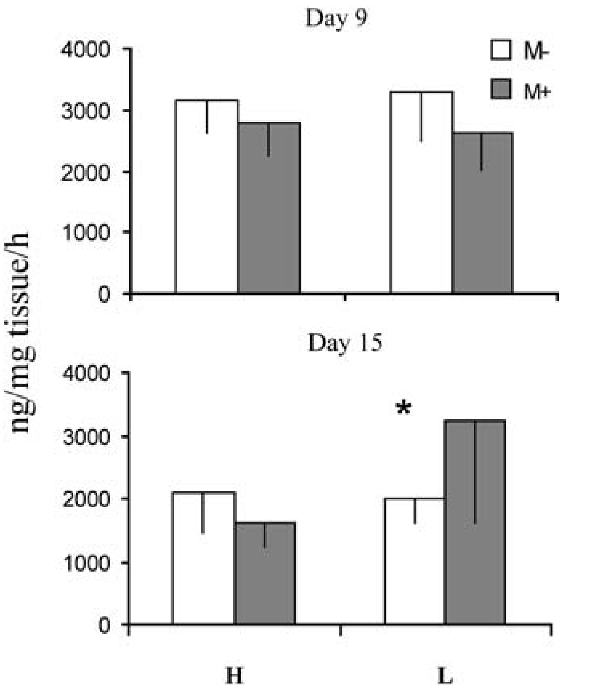

Figure 2 Mean (±s.e.) progesterone secretion *in vitro* (ng/mg tissue per h) by endometrial tissue, with (M+) or without (M−) melatonin in the culture medium (10 ng/ml), on days 9 and 15 of pregnancy, in ewes fed 1.5 × (H) and 0.5 × (L) maintenance requirements (adapted from Abecia *et al.*, 1999).

more progesterone in the presence of melatonin than in its absence (Figure 2). These ewes had a lower pregnancy rate and higher secretion of $PGF_{2\alpha}$, and this apparent reduction in endometrial luteolytic hormone suggested that melatonin treatment might improve embryonic survival in undernourished ewes through a reduction of the luteolytic signals.

Regarding an indirect effect of melatonin on embryo development Cagnacci *et al*. (1997), in a study on the effect of melatonin on the core body temperature in humans, postulated that this relationship is modified in the luteal menstrual phase; this modification may represent a mechanism for keeping the core body temperature higher at night to promote better embryonic implantation and survival. Moreover, McElhinny *et al*. (1996) concluded that although melatonin is administered to treat a range of conditions in a mouse strain, it would not adversely affect the embryo.

Overall, results of these reviewed experiments seem to indicate a direct effect of melatonin on the ovine ovary, the embryo and some of the mechanisms involved in the establishment of pregnancy. Although the improvement of ovulation rate seems to be related to its seasonal pattern, an acute, non-seasonal direct effect of melatonin on luteal functionality and embryo development has been demonstrated. Thus, it can be concluded that the success of exogenous melatonin as a means to improve lamb production of sheep is due, at least in part, to an improvement of luteal support and embryonic survival.

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