Effects of Melatonin and Controlled Internal Drug Release (CIDR) Device on Follicular Development and Oocyte Quality in the Anestrous Ewes Treated with Follicle Stimulating Hormone

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ABSTRACT

Administration of exogenous melatonin (MEL) and progesterone (P_4) in conjunction with follicle stimulating hormone (FSH) affects the number of developing follicles and oocyte quality in the anestrus ewe. Crossbred Rambioullet x Targhee Western range ewes (n=25) were randomly assigned to four treatment groups in a 2 x 2 factorial design [+/-MEL and +/- CIDR device; MEL/CIDR, CIDR, MEL, and Control (no treatment), respectively]. MEL/CIDR and MEL ewes (n=14) received an 18 mg Melovine® (melatonin) implant for 42 days before oocyte collection. MEL/CIDR and CIDR ewes (n=11) were vaginally implanted with CIDR (Type G) devices (intravaginal pessaries containing P_4) for five days before oocyte collection. Two days before oocyte collection all ewes received FSH injections twice daily. At slaughter, ovaries were removed, all visible follicles were counted, and oocytes were collected, matured and fertilized *in vitro*. The average number of follicles was greater ($P<0.08$) for MEL/CIDR ewes than Control ewes (37.3$\pm$5.5 and 22.6$\pm$5.5, respectively), but not different from MEL (31.3±5.5) and CIDR (25.8±5.5) ewes. Percentage of oocytes recovered from follicles was similar ($P>0.10$) for all treatments (overall 89.9±7.1%). Additionally, the rates of maturation of oocytes were similar ($P>0.10$) across treatments (overall 78.6±11.0%). Oocytes collected from CIDR treated ewes (CIDR and MEL/CIDR treatment groups), had lower ($P<0.02$) fertilization rates than ewes not treated with CIDR (MEL and Control; 10.3±2.0 and 10.1±2.0 vs. 18.5±2.0 and 20.0±2.0%, respectively). These data indicate that melatonin and P_4 increases the number of developing follicles, and although P_4 assists in the recruitment of more follicles, it decreases fertilization rates.

INTRODUCTION

The ewe is seasonally polyestrous and will stop cycling during late winter in response to the increasing photoperiod (Robinson et al., 1993; Bittman, 1984). Low fertilization rates during seasonal anestrous may be caused by an altered endocrine status when compared to the normal breeding season in the ewe (Stenbak et al., 2001). Numerous studies have focused on developing hormonal treatments to improve follicular development and induce estrus in ewes during seasonal anestrus (Robinson et al., 1991 and 1993; Gordon, 1997; Carlson, 2000; Knights et al., 2000, 2001). The main focus of these previous studies was to improve pregnancy rates and maximize reproductive performance in vivo. However, limited data are available concerning the effects of exogenous hormones, such as melatonin and progesterone, on oocyte quality for in vitro fertilization (IVF) during seasonal anestrus.

Many studies have been conducted during seasonal anestrus to evaluate the effectiveness of different melatonin treatments on reproductive performance in vivo. Melatonin treatment has been shown to be an effective method to induce estrous cycles, increase ovulation rates, and increase lambing rates during seasonal anestrous (Waller, 1988; Haresign, 1990 and 1992; Robionson 1991 and 1993; Bister, 1999; Carlson, 2000). It has also been demonstrated that melatonin affects oocyte development and support fertilization and early embryonic development following IVF in rats and mice (Fernandez, 1995; Ishizuka, 2000).

Another common method of inducing fertile estrus in the seasonal anestrous ewe is through the use of progesterone-based therapies (Robbinson et al., 1991; Jabbar et al., 1994; Knights et al., 2000, 2001). An improvement in fertility of ewes synchronized with higher doses of progesterone is due to an increase in sperm transport (Hawk, 1971), synchrony in the onset of estrus in relationship to the luteinizing hormone surge (Van Cleeff, 1998), and/or patterns of follicular development (Johnson, 1996). Knights et al. (2001) demonstrated that a 5-day treatment with progesterone, in combination with follicle stimulating hormone (FSH), stimulated a fertile estrus as effectively as a 12-day progesterone treatment with FSH. In addition, this resulted in prolificacy comparable to that observed during the normal breeding season. However, data concerning the effects of melatonin and/or progestagen treatment on quality of ovine oocytes are not available at present. The aim of this study was to evaluate the effects of exogenous melatonin and progesterone on follicular development and oocyte quality in FSH-treated ewes. Oocyte quality was measured by the rate of maturation, fertilization, and morula and blastocyst formation following *in vitro* fertilization procedures.

MATERIALS AND METHODS

Animals and Experimental Design

Seasonally anestrous, crossbred Rambioullet/Targhee Western range ewes (n=25) were randomly assigned to four treatment groups (n=4-7/group) in a 2x2 factorial design [+/- melatonin (MEL) and +/-CIDR device]. Ewes received an 18 mg Melovine® (melatonin; Sanofi Sante Nutrition Animal, La Ballastiere, France) implant for 42 days before oocyte collection and were vaginally implanted with CIDR devices for five days before slaughter (day of oocyte collection). Two days before slaughter all ewes received FSH injections as described by Stenbak et al. (2001). This study was conducted during the period from March to May

Follicular Evaluation and Oocyte Collection

Ovaries were removed at slaughter and placed in phosphate buffer solution (PBS) containing penicillin/streptomycin (Gibco, Gaithesburg, MD) at 39° C. The number of visible follicles on each ovary were counted. Oocytes were collected using a no. 15 scalpel and a Pasteur pipette in a petri dish containing oocyte collection media (Stenbak et al., 2001). Each follicle was cut with a scalpel and washed/flushed two or three times. Oocytes were then evaluated based on morphology and categorized as healthy or atretic according to Thompson et al. (1995). All oocytes were washed three times before being transferred into maturation medium containing epidermal growth factor (EGF; Choi et al., 2001; Grazul-Bilska et al., 2001; Stenbak et al., 2001) stabilized under mineral oil.

In Vitro Maturation

Oocytes were matured for 21-24 h at 39☜ C, 5% CO_2, and 95% air, and then oocytes were evaluated again for health based on morphology (Thompson et al., 1995). Only healthy-looking oocytes were used for IVF. The cumulus cells were removed by using 0.1% hyaluronidase (Type I-S; Sigma) treatment (Stenbak et al., 2001). Following cumulus cell removal oocytes were transferred to stabilized fertilization medium, consisting of synthetic oviductal fluid (SOF; O'Brien et al., 1997; Tervit et al., 1992; Walker et al., 1996; Wang et al., 1998; Stenbak et al., 2001) and 2% heat inactivated sheep serum collected on day 0 of the estrous cycle.

In Vitro Fertilization and Culture

Frozen semen, pooled from 4 Hampshire rams, was thawed and viable sperm were separated using the swim-up technique in modified sperm washing medium (Irvine Scientific, Santa Ana, CA; Yovich, 1995; Stenbak et al., 2001). The oocytes were fertilized with 0.5-1.0 x 10^6 viable sperm/mL (up to 20 oocytes/500 💻L well). The oocytes were incubated with the sperm for 17 to 20 h at 39☜ C, 5% CO_2, 5% O_2, and 90 N_2. Then zygotes

were cultured in SOF medium without glucose (Catt et al., 1997, Wang, 1998; Stenbak et al., 2001). The dishes were evaluated approximately 48 h after adding sperm to determine the rate of fertilization based on the number of cleaved oocytes.

Oocyte Staining to Determine Maturation Status

Oocytes that failed to fertilize were fixed in methanol and then stained with 0.1 µg/ml of 4,6-diamino-2-phenylindole (DAPI; Molecular Probes Inc., Eugene, OR, USA) in methanol for 15 minutes and mounted on slides (Jablonka-Shariff and Olson, 2000). The evaluation of nuclear status was done by epifluorescence microscopy (Gardner et al., 1997). Oocytes in the germinal vesicle stage, containing diplotene chromatin were considered to be immature. Mature oocytes demonstrated exclusion of the first polar body and therefore, were found to be in Metaphase II (Gaudet et al., 1997).

Statistical Analysis

Numbers of follicles and oocytes collected, and numbers and percentages of matured oocytes and cleaved zygotes were analyzed by using the general linear models procedure of the Statistical Analysis System (User's Guide, 1985). When the F-test was significant, differences between specific means were evaluated using the least square differences test (Kirk, 1982). Rates of oocyte maturation and fertilization were analyzed by using the Chi-Squared procedure of the Statistical Analysis System (User's Guide, 1985).

RESULTS

Figure 1 presents the average number of visible follicles on the ovaries of Control, CIDR, MEL, and MEL/CIDR ewes.

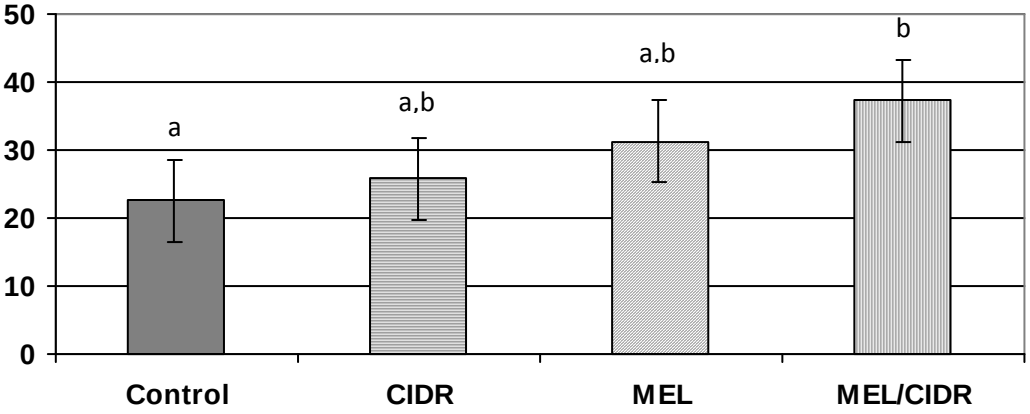

Figure 1. Average number of visible follicles for Control, CIDR, MEL, and MEL/CIDR ewes. [a,b] values are different; P<0.08. Data (mean ±SEM) are expressed per ewe.

The average number of follicles was greater (P<0.08) for MEL/CIDR ewes than Control ewes (37.3±5.5 and 22.6±5.5, respectively), but similar to MEL (31 and CIDR (.5) ewes.

Table 1 pr the number centage of oocytes (nu f healthy oocytes/total number of oocytes re d X 100) re from Cont R, MEL, a /CIDR ewes.

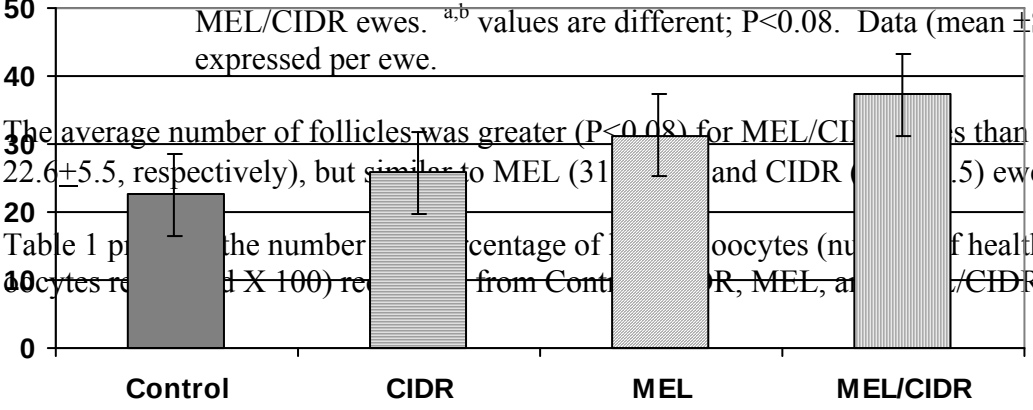

Table 1. Total number of oocytes and healthy oocytes recovered from Control, CIDR, MEL, and MEL/CIDR ewes.

		Recovered Oocytes		Healthy Oocytes		Healthy Oocytes (%)
Treatment	n	Per Ewe	Total	Per Ewe	Total	Total
Control	7	21.6±5.3	151	19.9±0.7	139	92.1
CIDR	4	21.8±7.1	87	21.5±7.4	86	98.9
MEL	7	27.3±5.3	191	26.3±0.7	184	96.3
MEL/CIDR	7	34.7±5.3	243	33.0±0.7	231	95.1
Overall	25	28.6±7.1	672	25.2±7.4	640	95.2

n=number of ewes

Total number of oocytes, healthy ooctyes and percentage of healthy oocytes from Control, CIDR, MEL, and MEL/CIDR ewes was similar (P>0.10) across treatments.

Table 2 presents the total number of oocytes analyzed for maturation status (fertilized oocytes and non-fertilized oocytes analyzed with DAPI staining) and the maturation rate (number of matured oocytes/total number of oocytes analyzed for maturation status X 100) of oocytes collected from Control, CIDR, MEL, and MEL/CIDR ewes.

Table 2. Total number of oocytes analyzed for maturation status and the rate of maturation (%) for oocytes collected from Control, CIDR, MEL, and MEL/CIDR ewes.

		Oocytes Analyzed for Maturation		Matured Oocytes		Maturation Rate (%)
Treatment	n	Per Ewe	Total	Per Ewe	Total	Total
Control	7	13.6±3.5	95	9.8±2.9	69	72.6
CIDR	4	13.8±4.2	55	11.1±3.6	44	80.0
MEL	7	16.3±3.5	114	13.8±2.9	97	85.1
MEL/CIDR	7	18.4±3.5	129	33.0±2.9	99	76.7
Overall	25	15.5±4.2	393	12.2±3.6	309	78.6

n=number of ewes

Maturation rates of oocytes were similar (P>0.10) across treatment groups, ranging from 72.6-85.1% (Table 2).

Table 3 presents the total number of healthy oocytes used for the IVF portion of this studay and their corresponding fertilization rates (number of fertilized oocytes/number of oocytes used for IVF X 100) in Control, CIDR, MEL, and MEL/CIDR treated ewes.

Table 3. Number of oocytes used for IVF and their corresponding fertilization rates (%) in Control, CIDR, MEL, and MEL/CIDR treated ewes.

		Oocytes for IVF		Oocytes Fertilized		Fertilization Rate (%)
Treatment	n	Per Ewe	Total	Per Ewe	Total	Total
Control	7	18.6±6.1	130	3.7±1.5	26	20.0[a]
CIDR	4	21.8±8.0	87	2.3±2.0	9	10.3[b]
MEL	7	25.4±6.1	178	4.7±1.5	33	18.5[a]
MEL/CIDR	7	34.0±6.1	238	3.4±1.5	24	10.1[b]
Overall	25	25.0±8.0	633	3.5±2.0	92	14.5

n=number of ewes
[a,b] values are different with in a column; $P<0.02$.

Oocytes collected from P_4 treated ewes (CIDR and MEL/CIDR treatment groups), had lower ($P<0.02$) fertilization rates than MEL and Control ewes (10.3±2.0 and 10.1±2.0 vs. 18.5±2.0 and 20.0±2.0, respectively).

DISCUSSION

The number of follicles and the number of oocytes and embryos obtained from animals must be optimized to maintain high efficiency of assisted reproductive technologies (ART). Numerous studies have focused on hormonal treatments to optimize follicular development in ewes during seasonal anestrus for successful embryo production and subsequent pregnancy rates (Gordon, 1997). As shown in this study and previously, administration of exogenous FSH (Gordon, 1997; Reynolds et al., 1998; Stenbak et al., 2001), melatonin (Rajkumar, 1989; Wigzell et al., 1986), and progestagens (Waller et al., 1988; Rajkumar et al., 1989; Wheaton et al., 1990; Safranski et al., 1992) have been shown to promote ovarian activity and succeeding follicular development in the seasonally anestrus ewe.

Follicle stimulating hormone (FSH) has been shown to promote a large number of follicles on each ovary when injected into ewes for two or more days at regular intervals during the breeding season and seasonal anestrus (Gordon, 1997; Reynolds et al., 1998; Stenbak et al., 2001). Additionally, several studies have examined the effects of exogenous melatonin and progestagen administration on ovarian activity and follicular development in vivo (Wheaton et al., 1990; Waller, 1988; Carlson, 2000). A major role of melatonin is to coordinate seasonal changes in reproductive activity (Hazlerigg, 2001). Melatonin has been shown to increase ovulation rate and litter size in the seasonally anestrus ewe (Rajkumar, 1989; Haresign, 1992). The use of progestagens have also been shown to promote ovarian activity by increasing the number of follicles and rate of ovulation during seasonal anestrous in the ewe (Waller et al., 1988; Rajkumar et al., 1989; Wheaton et al., 1990; Safranski et al., 1992; Leyva, 1998; Knights et al., 2001). Progestagens-treatment has been used in conjunction with high levels of FSH to promote the development of a large number of follicles during seasonal anestrus (Reynolds et al., 1998; Stenbak et al., 2001) and during the natural breeding season (Gordon, 1997; Stenbak et al., 2001).

In addition to hormonal treatment, the 'ram effect' has been shown to stimulate an earlier start of the breeding season in the ewe. Introduction of the ram among ewes during seasonal anestrus has been shown to be an effective method at inducing ovulation and subsequent estrus (Gordon, 1997; Faller and Berg, 2001). Additionally, the 'ram effect' has been reported to be an important and integral part of melatonin treatment strategies (Oldham and Martin, 1978). The principal role of melatonin in previous studies has been to advance

the period of sensitivity to the 'ram effect' and then to use ram introduction to promote a greater degree of synchrony in mating (Haresign, 1992). The role of the 'ram effect' on oocyte quality during seasonal anestrus remains unclear and requires further research.

In agreement to previous in vivo studies, which used exogenous melatonin and progestagen administration during seasonal anestrus (Rajkumar, 1989; Haresign, 1992), the current study demonstrated that the greatest number of follicles was achieved by administration of a slow-release melatonin implant in combination with a progesterone-releasing device and FSH-administration. Therefore, this hormonal treatment allows for the generation of a greater number of oocytes to be used for IVF procedures.

In numerous studies a fertile estrus was induced during the non-breeding season with the use of progestagens (Waller et al., 1988; Rajkumar et al., 1989; Wheaton et al., 1990; Safranski et al., 1992; Leyva, 1998; Knights et al., 2001). In a study by Knights et al. (2001), ewes treated with P_4 and low levels of FSH demonstrated an increase in the number of follicles and a greater portion of P_4 treated ewes lambed to the first service period when compared to non-P_4 treated controls. Treatment with P_4 for 5 days was shown to be as effective as for 12 d to induce a fertile estrus in FSH-treated anestrous ewes.

Previous studies have shown that exposure of oocytes to various hormones in vivo causes maturational changes that are necessary for proper development to occur (Cheng, 1985; Pugh et al., 1991; Armstrong et al., 1994; Assey et al., 1994; Fernandez et al., 1995; Ishizuka et al., 2000; Stenbak et al., 2001). Optimal levels of exogenous gonadotropins shoud be used to promote proper oocyte development. Depending on the regime of gonadotropin treatment, positive or negative effects on oocyte maturation and fertilization were observed (Evans and Armstrong, 1984; Pugh et al., 1991; Assey et al., 1994; Greve et al., 1995; Stenbak et al., 2001). In the current study, maturation rates were relatively high and similar across treatment groups, ranging from 72.6-85.1%. Therefore, exogenous melatonin and progesterone administration does not appear to affect maturational ability of oocytes during seasonal anestrus in FSH treated ewes.

In the present study, oocytes collected from P_4 treated ewes (CIDR and MEL/CIDR treatment groups) had lower fertilization rates than ewes that were not treated with P_4. In a study by Stenbak et al. (2001), similar rates of fertilization were achieved following a 14-day progestagen (SMB) and two-day FSH treatment. However, the fertilization rates following administration of SMB twice for 14 days appear to be greater than a five-day CIDR treatment (27%; Stenbak et al., 2001). Although the use of progesterone-releasing devices appeared to decrease fertilization rates in the current study, different results may be revealed if used twice (Stenbak et al., 2001). However, the mechanisms for promoting an increased rate of fertilization following a treatment with SMB twice for 14 days are not fully understood.

In contrast, Pugh et al. (1991) reported relatively high IVF rates (about 50-60%) for oocytes matured in the presence of granulosa cells for FSH-treated and non-treated ewes during the non-breeding season. These differences may be due to hormonal treatment, culture conditions, breed and location.

Data concerning the effects of melatonin on IVF rates in larger animals are not available. In the present experiment, we did not observe any melatonin effects on the rates of maturation or fertilization. However, for rats and mice, melatonin has been shown to affect oocyte development and sustain fertilization and early embryo development after IVF (Fernandez et al., 1995; Ishizuka et al., 2000).

During seasonal anestrus the rates of IVF (about 10-30%) are much lower than during the normal reproductive season (about 70-80%; Watson et al., 1994; Ledda et al., 1997; O'Brien et al., 1997; Choi et al., 2001; Grazul-Bilska et al., 2001; Stenbak et al., 2001). Therefore, additional studies need to be conducted in order to provide more optimal hormonal treatments for seasonal anestrous sheep to mimic the hormonal environment of sheep during the breeding season.

In conclusion, administration of melatonin and CIDR devices in conjunction with FSH to the anestrus ewe increases the number of developing follicles. However, CIDR-treatment decreased the rates of IVF. These data indicate that further research involving the use of hormonal treatment is needed to improve in vitro fertilization techniques for the seasonally anestrus ewe. The role of melatonin in the oocyte maturation and fertilization processes remains unclear and requires additional studies.

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