Improved Methods for the Production of Embryos in Seasonally Anestrous Ewes

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Abstract

In a recent study from our laboratory, a combination of melatonin implants and controlled internal drug release devices increased the number of developing follicles in follicle stimulating hormone-treated anestrous ewes; however, CIDR treatment decreased the rate of *in vitro* fertilization (IVF). In the current study, effects of alternative CIDR treatments on follicular development and rate of IVF in anestrous ewes was evaluated. On d -60 from oocyte collection (d 0) all ewes (1CIDR and 2CIDR treatment groups; n=6/group) received a melatonin implant. In addition, CIDR's were implanted on d -22 and removed on d -17. On d -10 2CIDR ewes received a second CIDR for 8 d (removed on d -2). All ewes received FSH injections twice daily on d -2 and d -1. On d 0, ovaries were removed and oocytes were aspirated from follicles, matured *in vitro* for 17-24 h and then subjected to IVF on d 1 of culture. 1CIDR and 2CIDR ewes had a similar ($P > 0.10$) number of follicles $\geq$ 1 mm in diameter (26.7 ± 8.6 and 24.3 ± 13.3, respectively) and rate of oocyte recovery (91.3 ± 3.5 and 99.3 ± 1.6%, respectively). On d 3 of culture, the rate of oocyte maturation (sum of fertilized and matured unfertilized oocytes [determined by DAPI staining]) was similar ($P > 0.10$) for 1CIDR and 2CIDR ewes (89.7 ± 7.4 and 87.6 ± 6.3%, respectively). However, oocytes collected from 2CIDR ewes had lower ($P < 0.01$) rates of IVF than oocytes collected from 1CIDR ewes (30.2 vs. 58.0%, respectively). Thus, IVF rates were adversely affected by an additional 8 d CIDR treatment. These data indicate that the interval between CIDR treatment and oocyte collection affects IVF rates in melatonin and FSH-treated ewes during anestrous. Therefore, progestogen treatment protocols used in ovine IVF programs should be carefully designed to minimize adverse effects on fertilization rates.

Introduction

It has been suggested that low fertilization rates during the non-breeding season may be caused by an altered endocrine status when compared with ewes during the normal breeding season (Stenbak et al., 2001). Numerous studies have focused on developing hormonal treatments to improve follicular development and induce a fertile estrus in ewes during the non-breeding season (Robinson et al., 1991 and 1993; Gordon, 1997; Carlson, 2000; Knights et al., 2000, 2001). The main focus of previous studies has been to improve pregnancy rates and maximize reproductive performance *in vivo*. However in a recent *in vitro* fertilization (IVF) study from our laboratory (Luther et al., 2002), a combination of melatonin implants and CIDR devices increased the number of developing follicles in FSH-treated anestrous ewes. In addition, treatment with CIDR devices decreased the rates of IVF. Limited data are available concerning the effects of exogenous hormones, such as progesterone, on oocyte quality for *in vitro* fertilization (IVF) during the non-breeding season in adult ewes.

Progesterone-based therapies have been widely used for inducing a fertile estrus in seasonally anestrous ewes (Robbinson et al., 1991; Jabbar et al., 1994; Knights et al., 2000, 2001). Knights et al. (2001) demonstrated that a 5-day treatment with progesterone, in

combination with FSH, stimulated a fertile estrus just as effectively as a 12-day progesterone treatment; and that prolificacy was comparable to that observed during the normal breeding season. In FSH-treated pre-pubertal ewe lambs, Armstrong et al. (1994) found an increase in the number of follicles, and oocyte recovery and maturation rates when progestogen pre-treatment was withdrawn 2 days before oocyte collection as apposed to a continuous progestogen pre-treatment.

The aim of the current study was to evaluate the effects of exogenous progesterone treatment on follicular development and oocyte quality in melatonin and FSH-treated seasonally anestrous ewes. Oocyte quality was measured by the rates of maturation and *in vitro* fertilization.

Material and Methods

Animals and Experimental Design

Crossbred Rambioullet/Targhee Western U.S. range ewes were used during the non-breeding season from March to May. The treatment protocols were as follows:

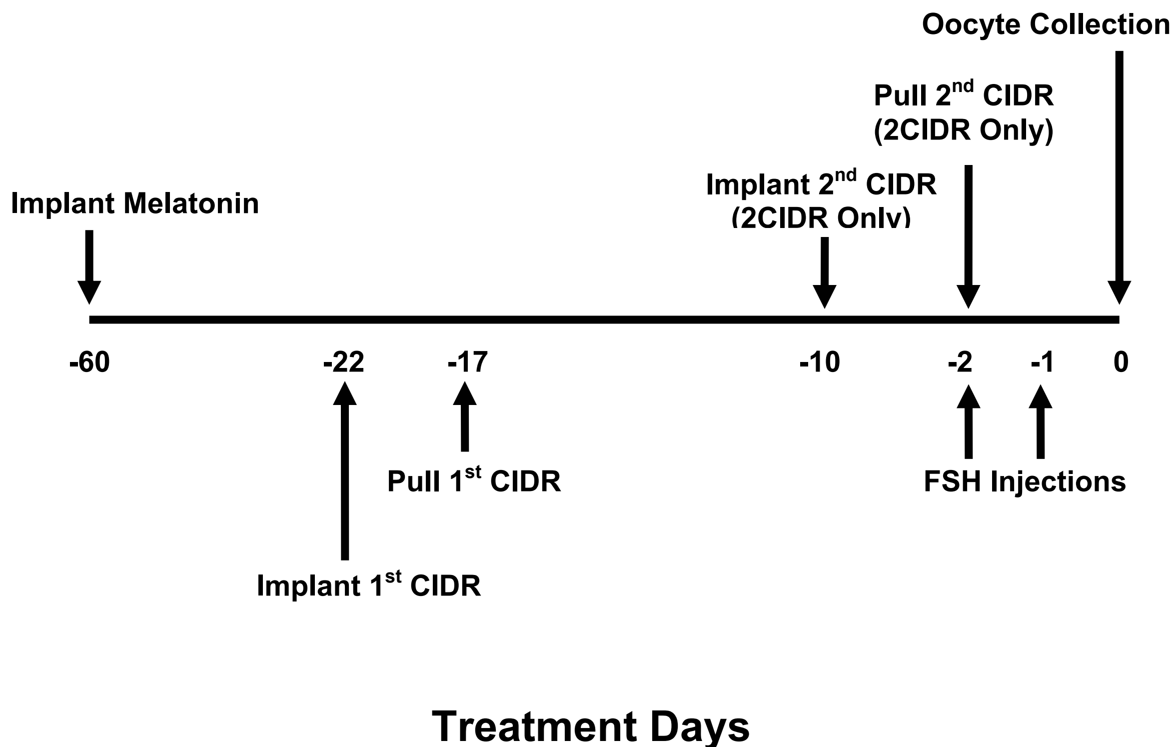

Figure 1. Treatment protocol for the 1CIDR and 2CIDR groups.

On d -60 from oocyte collection (d 0) all ewes (1CIDR and 2CIDR treatment groups; n=6/group) received a melatonin (Melovine®; 18 mg melatonin, Sanofi Sante Nutrition Animal, La Ballastiere, France) implant. In addition, ewes were implanted with CIDR (CIDR-Type G; 300 mg progesterone, Inter Ag, Hamilton, New Zealand) devices on d -22 and devices were removed on d -17. On d -10 2CIDR ewes received a second CIDR for 8 d (removed on d -2). All ewes received FSH (FSH-P with 10% luteinizing hormone; Sioux Biochemical, Sioux Center, IA, USA) injections twice daily on d -2 and d -1. On d 0, oocytes were aspirated from follicles and

then subjected to IVF procedures. Animal care and use procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of North Dakota State University.

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. To collect oocytes, each follicle was cut with a scalpel and washed/flushed with collection medium 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; Sigma, St. Louis, MO; Grazul-Bilska et al., 2003) 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). Denuded oocytes were transferred to stabilized fertilization medium, consisting of synthetic oviductal fluid (SOF; Stenbak et al., 2001; Grazul-Bilska et al., 2003) 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 exposed to 0.5-1.0 x 10^6 viable sperm/mL (up to 20 oocytes/500 : L/well) in fertilization medium (Stenbak et al., 2001). The oocytes were then incubated with the sperm for 17 to 20 h at 39E C, 5% CO_2, 5% O_2, and 90 N_2. Then zygotes were cultured in SOF medium without glucose (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 (Fig. 1; 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

The mean number of visible follicles was similar (P>0.10) for 1CIDR and 2CIDR ewes (30.3±3.5 and 24.3±5.4, respectively).

The number of recovered oocytes, healthy ooctyes and percentage of healthy oocytes was similar (P>0.10) for 1CIDR and 2CIDR ewes (Table 1).

Table 1. Number of oocytes and healthy oocytes recovered from ewes.*

		Recovered Oocytes		Healthy Oocytes		Healthy Oocytes (%)	
Treatment	n	Per Ewe	Total	Per Ewe	Total	Per Ewe	Total
1CIDR	6	24.3±3.2	146	24.0±3.0	144	99.0±1.0	98.6
2CIDR	6	24.2±5.5	145	22.3±5.1	134	92.3±2.1	92.4
Overall	12	24.3±3.0	291	23.3±2.9	278	95.7±1.5	91.1

*Data are expressed as means ± S.E.
n=number of ewes

The maturation rate of oocytes was similar (P>0.10) for 1CIDR and 2CIDR ewes (Table 2).

Table 2. Number of oocytes analyzed for maturation status and the maturation rate (%) for oocytes collected from ewes.*

		Oocytes Analyzed for Maturation		Matured Oocytes		Maturation Rate (%)	
Treatment	n	Per Ewe	Total	Per Ewe	Total	Per Ewe	Total
1CIDR	6	19.5±2.2	117	17.5±3.2	105	91.8±3.0	89.7
2CIDR	6	18.8±3.8	113	16.5±.3.7	109	88.5±2.6	96.5
Overall	12	19.2±2.4	230	17.0±2.3	214	90.2±1.9	93.0

*Data are expressed as means ± S.E.
n=number of ewes

Fertilization rates were lower (P<0.01) for ewes withdrawn from P4 treatment two days before oocyte collection (2CIDR) than for ewes withdrawan from P4 treatment seventeen days before oocyte collection (1CIDR; Table 3).

Table 3. Number of oocytes used for IVF and the corresponding fertilization rates (%) in 1CIDR and 2CIDR ewes.*

		Oocytes for IVF		Oocytes Fertilized		Fertilization Rate (%)	
Treatment	n	Per Ewe	Total	Per Ewe	Total	Per Ewe	Total
1CIDR	6	23.8±2.9	143	13.8±2.6	83	56.0±9.0	58.0[a]
2CIDR	6	21.0±5.0	126	6.3±1.1	38	36.2±11.1	30.2[b]
Overall	12	22.4±2.8	269	10.5±1.8	121	46.1±7.5	45.0

*Data are expressed as means ± S.E.
n=number of ewes.
[a,b] P<0.02; values are different with in a column.

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 the non-breeding season for successful embryo production and subsequent pregnancy rates (Gordon, 1997). Previous studies have shown that administration of exogenous FSH (Gordon, 1997; Reynolds et al., 1998; Stenbak et al., 2001), melatonin (Rajkumar, 1989; Wigzell et al., 1986), and progestogens (Waller et al., 1988; Rajkumar et al., 1989; Wheaton et al., 1990; Safranski et al., 1992) promote ovarian activity and succeeding follicular development during the non-breeding season of the ewe.

Follicle stimulating hormone (FSH) has been shown to induce development of multiple follicles on each ovary when injected into ewes for two or more days at regular intervals during the normal breeding season and non-breeding season (Gordon, 1997; Reynolds et al., 1998; Stenbak et al., 2001). Additionally, several studies have examined the effects of exogenous melatonin and progestogen 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 ewe during the non-breeding season (Rajkumar, 1989; Haresign, 1992). The use of progestogens have also been shown to promote ovarian activity by increasing the number of follicles and rate of ovulation (Waller et al., 1988; Rajkumar et al., 1989; Wheaton et al., 1990; Safranski et al., 1992; Leyva, 1998; Knights et al., 2001). Treatment with progestogens has been used in conjunction with high levels of FSH to promote the development of a large number of follicles during the non-breeding season (Reynolds et al., 1998; Stenbak et al., 2001) and during the normal breeding season (Gordon, 1997; Stenbak et al., 2001).

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 should be used to promote proper oocyte development and depending on the regime of gonadotropin treatment, positive or negative effects on oocyte maturation and fertilization have been 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 89.7 to 96.5%. Therefore, administration of exogenous P4 does not appear to affect the maturational ability of oocytes during the non-breeding season in FSH-treated ewes.

In numerous studies a fertile estrus was induced during the non-breeding season with the use of progestogens (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 P4 for 5 days and low levels of FSH demonstrated an increase in the number of follicles and a greater portion of P4 treated ewes lambed to the first service period when compared to non-treated controls. Additionally, treatment with P4 for 5 days was shown to be as effective as a 12 day treatment for inducing a fertile estrus in FSH-treated ewes during the non-breeding season (Knights et al., 2001).

In our experiment, P4-treatment regimen had no effect on the number of follicles. However, termination of an 8 day CIDR-treatment only 2 days before oocyte collection appears to decrease IVF rates in anestrous ewes. In a study by Stenbak et al. (2001), IVF rates following

administration of a Synchro-Mate B (SMB) implant twice for 14 days appeared to be greater than treatment with a single SMB implant withdrawn 2 days before oocyte collection. This indicates that prolonged P4 treatment and/or induction of ovulation before P4 treatment affects oocyte quality. The mechanisms for promoting these increases in rates of IVF during the non-breeding season are not fully understood at present and require further investigation.

According to Senger (1999) the onset of seasonal cyclicity is similar to the onset of puberty. The seasonal anetrous period of the ewe and pre-puberty can be identified by hypothalamic dormancy with regard to GnRH (gonadotropin releasing hormone) pulse frequency and release. Before the seasonally anestrous adult ewe and pre-pubertal ewe lamb can enter a normal breeding season the hypothalamus must release GnRH in sufficient quantities to generate a response by the anterior pituitary (Senger, 1999). Therefore, the data obtained from studies with seasonally anestrous ewes may have some implications for studies using juvenile ewe lambs and vice versa.

The use of juvenile donors in embryo transfer and IVF programs offers substantial potential for accelerated genetic gain through a reduction in the generation interval. A more recent review has indicated that IVF rates of lamb ooctyes does not differ significantly whether matured *in vivo* or *in vitro*, and rates of further embryonic in culture have shown to be similar to the development observed for embryos derived from adult donors (Armstrong, 1997). In a study by Armstrong et al. (1994), 6-8 week old donor ewe lambs were used to determine the effects of withdrawal time of a progestogen pre-treatment on follicular development and oocyte maturation. In this experiment, all lambs received an injection of GnRH 18-24 h before oocyte collection, which was intended for inducing oocyte maturation. The follicular development was considerably enhanced by progestogen withdrawal two days before oocyte collection and a greater portion of the recovered oocytes had commenced maturation as indicated by expanded cumulus cells when compared to continuous or lack of a progestogen pre-treatment. This data would also indicate the ability of progesterone priming to sensitize the hypothalamic-pituitary LH-surge system allowing for a possible higher rate of oocyte maturation. The presence of fewer follicles in the continued presence of progestogens suggests an action of progesterone in retarding gonadotropin release and subsequent follicular growth. Unfortunately, Armstrong et al. (1994) did not evaluate the rates of IVF between progestogen pretreatment groups and therefore, the effect of progestogen pretreatment withdrawal time on oocyte quality remains unclear. In the current study, 1CIDR ewes were withdrawn from progesterone 17 d before oocyte collection and 2CIDR ewes were withdrawn from progesterone treatment 2 d before oocyte collection; the rate of oocyte maturation was similar between the two treatment groups.

In summary, IVF rates were adversely affected by an additional 8 d CIDR treatment. These data indicate that the interval between CIDR treatment and oocyte collection affects IVF rates in FSH-treated ewes during anestrous. Therefore, progestogen treatment protocols used in ovine IVF programs should be carefully designed to minimize adverse effects on fertilization rates. During seasonal anestrous the rates of IVF (about 30-60%) are lower than during the normal reproductive season (about 70-80%; Watson et al., 1994; Ledda et al., 1997; O'Brien et al., 1997; Grazul-Bilska et al., 2002; Stenbak et al., 2001) and further embryonic development *in vitro* is minimal. As a result, 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.

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