Effects of FSH treatment in seasonally anestrous ewes on egg production, retrieval, and quality for use in *in vitro* fertilization procedures.

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INTRODUCTION

Through the use of assisted reproductive technology (ART), we will be able to extend the use of genetically superior animals, and perhaps increase the number of offspring. These technologies include such procedures as estrus synchronization, superovulation, artificial insemination, embryo transfer, *in vitro* fertilization (IVF), and cloning. Because they exhibit seasonal breeding and multiple ovulations, sheep have a tremendous potential for improvement and manipulation of reproduction with the use of ART. Most sheep normally exhibit estrous cycles and mate during late summer, fall, and early winter. During winter, spring, and early summer they exhibit anestrous, and thus, are reproductively inactive. Reproductive performance of sheep, in some cases, can be maximized by utilizing this anestrous period or non-breeding season. Manipulations to maximize reproductive performances during seasonal anestrous include hormonal stimulations and estrous synchronization. However, many improvements are still needed to enhance the reproductive efficiency of ewes during seasonal anestrous.

Very little research has been conducted to study out-of-season effects on oocyte (egg) quality for *in vitro* fertilization. IVF requires a large number of eggs collected from ewes. The method of inducing follicular development with follicle stimulating hormone (FSH) has been widely used (Gordon, 1997, Cognie, 1999). The ewe naturally releases FSH from the anterior pituitary gland in response to gonadotropin releasing hormone (GnRH) to promote follicular development during the breeding season. When injected into ewes for two or more days at regular intervals, FSH usually promotes development of a large number of follicles on each ovary during the breeding season and out-of-season (Jablonka-Shariff, 1994, 1996; Gordon, 1997). Synchro-Mate-B (SMB; a synthetic progestogen) is often used to synchronize estrus among animals. However, it also helps to stimulate the animals to begin their reproductive cycles during the non-breeding season. The aim of this study was to determine the effects of FSH and Synchro-Mate-B on the number of follicles, the recovery and quality of oocytes, and the ability of these oocytes to fertilize *in vitro* (in the laboratory) when oocytes were collected from ewes during seasonal anestrous.

MATERIALS AND METHODS

Seasonally anestrous ewes of mixed breeds were used for this experiment during the winter and spring of 1999. Half of the ewes were implanted with Synchro-Mate-B (½ implant; Merial Limited, Athens, GA) and left in place for 14 days. SMB contains norgestomet, a potent synthetic progestin that can stimulate reproductive hormone cycles upon its removal in anestrous ewes. On day 14 (day 0 = day of SMB implantation), SMB implants were removed through a small incision made in the skin. The other half of the ewes were not implanted with SMB.

Induction of multiple follicular growth and oocyte collection

Ewes (n = 49) were randomly assigned to three groups which were given one of three treatments: no treatment (control, n = 12), FSH injected for two days (2D, n = 21) or 3 days (3D, n = 15). Beginning on the morning of Day 12 (3D) or Day 13 (2D) after SMB implantation, ewes received twice daily (morning and evening) intramuscular injections of FSH (porcine FSH with 10% luteinizing hormone; Sioux Biochemical, Sioux Center, IA). Injections were as follows: Day 1, 5 units (1.0 ml)/injection; Day 2, 4 units (0.8 ml)/injection; Day 3, 3 units (0.6 ml)/injection (total dose: 2 day treatment = 18 units; 3 day treatment = 24 units). SMB was removed on Day 14 and a laparotomy was performed on Day 15 at 15 hours after the removal of the SMB implant, to count follicles and retrieve oocytes.

At laparotomy, the ovaries were exteriorized and the number of follicles were counted on each ovary. In addition, for each follicle, the surface diameter was measured and follicles were classified as <3mm (small), 3-8mm (medium), and >8mm (large) before oocyte collection. An ovariectomy was performed and oocytes were collected in the laboratory. Oocytes were then collected by aspiration using a 22-gauge 1-inch needle and a syringe containing approximately 0.2 ml of collection media that consists of TCM-199 (Sigma, St. Louis, MO), 2% heat inactivated fetal bovine serum (FBS; Gibco, Gaithersburg, MD), heparin (Sigma), and penicillin/streptomycin (Watson et al., 1994). Each collected follicle was washed/flushed three times with the collection media. The media and follicular fluid from each follicle was emptied into petri dishes.

By using a stereoscope, each dish was searched and the recovered oocytes were transferred to a petri dish with fresh collection media at which point all oocytes from individual ewes were combined. 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 in maturation media before being transferred into stabilized maturation media (TCM-199, 10% FBS, ovine FSH [oFSH-RP-1; NIAMDD-NIH, Bethesda, MD], ovine LH [oLH-26; NIADDK-NIH], estradiol [Sigma], glutamine [Sigma],

sodium pyruvate [Sigma], and penicillin/streptomycin; Watson et al., 1994).

In vitro fertilization of collected oocytes

Oocytes collected from ewes were subjected to *in vitro* fertilization and evaluated for fertilization rates. The oocytes were matured for 21-24 hours at 39 C, 5% CO_2, and 95% air. After maturation procedures, the oocytes were again evaluated for health based on morphology. Oocytes classified as healthy were separated and used for in vitro fertilization (IVF). The cumulus cells were removed by a 1% hyaluronidase (Type I-S; Sigma) treatment and the healthy oocytes were transferred to stabilized fertilization media, consisting of synthetic oviductal fluid (SOF; Tervit et al., 1972) and 2% heat inactivated sheep serum collected from sheep on day 3 of the estrous cycle (O'Brien et al., 1997).

Frozen semen, which was pooled from 4 NDSU rams, was thawed and viable sperm were separated using the swim up technique (Yovich, 1995). In the swim up technique, the healthy and viable sperm from a semen fraction swim into the media (Modified Sperm Washing Medium, Irvine Scientific, Santa Ana, CA) which lays on top of the thawed semen pool. This media containing the motile healthy sperm is then centrifuged, counted and used for in vitro fertilization. 0.5-1.0 x 10^6 sperm/ml were added to the oocytes (up to 20/500 µg/well). The oocytes were incubated with the sperm for 17-20 hours at which time the embryos were washed three times with culture media without glucose (SOF supplemented with BSA, glutamine, MEM amino acids, BME amino acids [Sigma], and penicillin/streptomycin; Catt et al., 1997). The dishes were evaluated, 48-60 h after adding sperm to the oocytes, to determine the number of cleaved oocytes (i.e., embryos).

Statistical analysis

All data is reported as means per ewe $\pm$ the standard errors.

Data was analyzed as a 2x3 factorial with SMB and FSH-treatments as the main effects. Numbers of follicles and oocyte numbers and percentages of matured oocytes for non-treated and FSH-treated ewes were analyzed by using the general linear models (GLM) procedure of the Statistical Analysis System (SAS, 1985). When the F-test was significant, differences between specific means were evaluated using t-tests. Relationships between treatments were evaluated using least squares difference (LSD).

RESULTS

Ewes treated with Synchro-Mate-B had similar ($P > 0.05$) numbers of follicles, numbers of oocytes, and oocyte health when compared to ewes that did not receive SMB. Therefore, data were combined among SMB implanted and non-implanted ewes.

The number of small, medium, and large follicles in non-treated and FSH-treated ewes during the non-breeding season are presented in Table 1.

Table 1: Number of small, medium, and large follicles in non-treated and FSH-treated ewes during seasonal anestrous.

		Number of follicles			
Treatment	n	<3mm	3-8mm	> 8mm	Total
None	13	5.5±0.99[a,b]	1.4±0.29 [a]	0.08±0.08	6.92±0.92[a]
2D FSH	21	6.9±1.44[a]	11.8±1.12 [b]	0	18.67±1.93[b]
3D FSH	15	3.1±0.90[b]	14.5±2.41 [b]	0.4±0.4	18.00±2.58[b]

[a, b,] - means ± SEM differ within a column, $P < 0.05$.

n - number of ewes.

FSH treatment increased ($P < 0.01$) the number of medium and total number of follicles, but did not affect the number large follicles present. The non-treated ewes and the ewes treated with FSH for 2 days had a greater number ($P < 0.05$) of follicles that measured less than 3 mm.

Table 2 presents the number of eggs collected from small, medium, and large follicles from non-treated and FSH-treated ewes during seasonal anestrous.

Table 2. Number of oocytes recovered from small, medium, and large follicles for non-treated and FSH-treated ewes during seasonal anestrous.

		Number of oocytes recovered			
Treatment	n	<3mm	3-8mm	>8mm	Total
None	13	4.3 ±0.94	1.2±0.32[a]	0.08±0.08	5.62±0.98[a]
2D FSH	21	4.8±1.07	10.6±1.09[b]	0	15.4±1.73[b]
3D FSH	15	2.2±0.76	12.8±2.13[b]	0.3±0.3	15.3±2.28[b]

[a, b] - means ± SEM differ within a column, P<0.05.

n - number of ewes.

FSH-treatment increased (P < 0.05) the number of eggs recovered from medium sized and total follicles. However, FSH-treatment did not affect the number of eggs recovered from small and large follicles.

Table 3 presents the recovery rate, number and percentage of healthy oocytes recovered from non-treated and FSH-treated ewes during seasonal anestrous.

Table 3. Recovery rate, and percentages of healthy and atretic oocytes for non-treated and FSH-treated ewes during seasonal anestrous.

Treatment	n	Total recovery rate (%)	# of healthy oocytes	% healthy oocytes
None	13	79.5±9.1	4.62±0.76 [a]	84.2±4.8
2 day FSH	21	81.6±3.3	13.2±1.5 [b]	86.7±2.6
3 day FSH	15	84.6±3.3	11.9±1.8 [b]	80.1±4.3

[a, b]- means ± SEM differ within a column , P<0.05.

n - number of ewes.

The recovery rate of oocytes and the percent of healthy oocytes did not differ between non-treated and FSH-treated ewes. However, the number of healthy oocytes was greater (P < 0.05) for the FSH-treated ewes than the non-treated ewes.

Fertilization rate for this study was low. Ewes treated with FSH for 2 and 3 days only had 6%

fertilization and the non-treated ewes had a 14 % fertilization rate.

DISCUSSION

Assisted reproductive technologies are powerful tools in animal industry for genetic improvement and also for enhancing reproductive efficiency. However, in the sheep industry these technologies are less than optimal for use during seasonal anestrous. Improving breeding rates during seasonal anestrous would contribute to increasing reproductive efficiency in ewes, as well as extend the use of these valuable techniques.

In the present study, follicular growth was induced in seasonally anestrous ewes by FSH. This study is similar to an earlier study conducted during the breeding season using the same treatment groups (Stenbak et al., 1999). In the present study SMB did not have any effect on follicular development, oocyte retrieval, and the health of the oocytes. FSH induced follicular development in SMB and non-implanted ewes. The total number of follicles from ewes treated with FSH for 2 days and 3 days was 18. In addition, non-treated ewes had the smallest number of follicles with an average of 7 follicles per ewe. In an earlier study conducted during the breeding season, ewes treated with FSH for 2 days exhibited 16 total follicles, whereas, ewes treated with FSH for 3 days had 18 total follicles per ewe and non-treated ewes exhibited 8 total follicles (Stenbak et al., 1999). These data support other reports that FSH treatments for ART are an effective way to induce follicular development in ewes (Jablonka-Shariff et al., 1994, 1996; Gordon, 1997). From this study, it appears that there is no seasonal effect in the follicular response to FSH in ewes.

Understanding how superovulation techniques affect the quality of oocytes will lead to improved ART. The number of healthy oocytes was higher in FSH-treated ewes than in non-treated ewes. However, the percent of healthy oocytes after maturation was not significantly different among treatment groups. The number of healthy oocytes and the percent of healthy oocytes is similar to the previous experiment that we conducted during the breeding season (Stenbak et al., 1999).

Unfortunately, the fertilization rates in this study were very low (only 7%). Other studies have shown that *in vitro* fertilization is possible out-of-season (Pugh et al., 1991). However, the fertilization rate of oocytes collected out-of-season was only 53%, whereas fertilization rates during the breeding season range from 68-80% (Pugh et al., 1991; Slavik, et al.,1992; Ledda, et al., 1997; O'Brien, et al., 1996,1997; Watson, et al., 1994). The ewes in the Pugh et al. (1991) study received similar FSH treatment as our study. However, contrary to our study, their experiment was performed on Coopworth ewes, a mix of Border Leicester and Romney (New Zealand), where seasonal affects are negligible. Most data from the present study were consistent with our previous study conducted during the breeding season in which we obtained approximately 70% *in vitro* fertilization rates (Stenbak et al., 1999). The

semen for both the present and the previous experiments were from the same pool, so the low fertilization rates found in the present study probably was not due to poor semen quality, but rather due to seasonal effects.

In an effort to determine why IVF rates were so low in the present study, we implanted SMB into 6 ewes for 14 days. After removal of SMB, the sheep were allowed to cycle before they were re-implanted for another 14 days. This was done to mimic a normal estrous cycle prior to oocyte collection. The sheep received 2 days of FSH as described earlier and oocytes were collected and processed as in the above study. We found that the fertilization rate in these ewes was 28%, which is greater ($P < 0.068$) than the 7% found in the ewes injected with FSH for 2 days in the above study. This is still lower than the results in the Pugh et al. (1991) study. It does indicate that prior treatment with SMB to mimic estrous cycles may be necessary for *in vitro* fertilization. However, it also shows that much work is needed to improve IVF rates of seasonally anestrous oocytes. Other techniques, besides SMB implants, are also available to aid in inducing the estrous cycle during seasonal anestrous. Such techniques include light manipulation, melatonin supplementation, progestogen and PMSG treatments (Gordon, 1997).

The results of this study will ultimately lead to improved and efficient methods for obtaining large numbers of high quality oocytes and embryos for embryo transfer programs. Improvement in these techniques will enhance the overall efficiencies in the sheep industry during the breeding season and out-of-season.

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