Effects of epidermal growth factor (EGF) on oocyte maturation, in vitro fertilization (IVF) and blastocyst formation in ewes treated with follicle stimulating hormone (FSH)

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INTRODUCTION

The world population is increasing at dramatic rates; therefore, the demand for meat and milk products will continue to grow (CAST, 1999). Consumers also are demanding a more uniform, higher quality, nutritious product at a low price, requiring producers to raise animals that meet these specific demands. Altering herd genetics is a slow and expensive process because of the limitation of having a set number of offspring produced by each animal. However, through the use of assisted reproductive technology (ART) each animal has the ability to produce an increased number of offspring, thereby allowing the producer to more easily alter herd genetics to meet the consumers demands. Using ART, such as induction of superovulation, IVF or embryo transfer, farmers will be able to increase the number of offspring produced by genetically superior parents.

New introductions and improvements to ART have facilitated the creation, advancement and preservation of animal genetics as well as continued improvement of animal reproductive efficiency (Gordon, 1997). In sheep reproduction, ART has been introduced to obtain multiple embryos, in vivo or in vitro, and to obtain transgenic or cloned animals (Schnieke et al., 1997; Wilmut et al., 1997; Cognie, 1999). To obtain large numbers of oocytes (eggs) or embryos from ewes, the method of inducing follicular development with follicle stimulating hormone (FSH) is widely used (Gordon, 1997; Cognie, 1999; Stenbak et al., 1999, 2000). Optimal culture conditions are critical to obtain the high rates of fertilization and blastocyst formation for embryo transfer or cryopreservation (Jablonka-Shariff et al., 1994, 1996; Gordon, 1997). Media containing gonadotropins, estradiol, growth hormone, and growth factors including EGF and/or other supplements have been shown to exert positive effects on oocyte maturation, IVF and blastocyst formation in several species including sheep (Longergan et al., 1996; Izadyar et al., 1998; Lim and Hansel, 1999; Abeydeera et al., 2000; Guler et al., 2000; Watson et al., 2000). The aim of this study was to determine the effects of EGF on the rate of fertilization and blastocyst formation in vitro in sheep in which multiple follicles development was induced with FSH.

MATERIALS AND METHODS

Ewes of mixed breeds (n=15) that exhibited an estrous cycle of normal duration (15-17 days) immediately proceeding the treatment were used for these experiments during the reproductive season of fall and winter 2000. Day 0 of the estrous cycle (standing estrus) was determined by using vasectomized rams. Beginning on day 13 of the estrous cycle, all ewes received twice daily i.m. injections of FSH for two days (on day 13, 5 units/injection, and on day 14, 4 units/injection) as described before (Stenbak et al., 1999, 2000). On day 15 ewes were slaughtered and ovaries collected.

The number of follicles was counted on each ovary and oocytes were then collected 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; Stenbak et al., 1999, 2000).

By using a stereoscope, dish with oocytes was searched and the recovered oocytes were transferred to a petri dish with fresh collection media without heparine. 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 (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). For each ewe, half of the oocytes was incubated in stabilized (incubated overnight under oil at 39^0 C, 5% CO_2, and 95% air) maturation media without addition of EGF, and other half was incubated in the same media containing 10 ng of EGF (Sigma). Dose of EGF was chosen on the basis of previously published experiments (Longergan et al., 1996; Abeydeera et al., 2000; Guler et al., 2000).

The oocytes were matured for 21-24 hours at 39^0 C, 5% CO_2, and 95% air followed by cumulus cells were removal by using a 1% hyaluronidase (Type I-S; Sigma) treatment. Then, the oocytes were again evaluated for health based on morphology (Thompson et al., 1995). Oocytes classified as healthy were used for in vitro fertilization (IVF). The 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 0-1 of the estrous cycle (O'Brian et al, 1997; Brown and Radziewic, 1998; Wang et al., 1998).

Frozen semen, which was pooled from 4 Hampshire 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 sperm was then centrifuged, counted and used for in vitro fertilization. 0.5-1.0 x 10^6 sperm/ml was added to the oocytes (up to 20 oocytes/500 µl/well). The oocytes were incubated with the sperm for 17-20 hours at 39^0 C, 5% CO_2, 5% O_2 and 90% N_2. Then, 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), and cultured at the same media for 36 h at 39^0 C, 5% CO_2, 5% O_2 and 90% N_2. The dishes were then evaluated to determine the number of cleaved zygotes. The cleaved zygotes were then washed three times in culture media (SOF) containing glucose (Stenbak et al., 1999). After 48 h, developmental stage of zygotes was evaluated and zygotes were transferred to fresh culture media with glucose. On day 8-9 of culture (day 1=day of fertilization) presence of blastocysts was determined.

Statistical analysis

All data are reported as means $\pm$ the standard errors. Data were analyzed by using general linear models (GLM) procedure of the Statistical Analysis System (SAS, 1985) with the main effect of EGF presence in the maturation media. When the F-test was significant ($P<0.05$), differences between specific means were evaluated using Bonferroni's multiple comparison procedure (Kirk, 1982). Correlations between specific parameters were evaluated by using SAS.

RESULTS

For all ewes, number of follicles was 25.0±2.4/ewe, number of recovered oocytes was 22.1±2.1/ewe, oocyte recovery rate was 88.3±2.7/ewe, number of healthy oocytes was 17.7±1.6/ewe, number of atretic oocytes was 4.4±0.7/ewe and percentage of healthy oocytes was 80.9±2.0/ewe.

Total number of oocytes used for IVF was 232, 109 oocytes were incubated without EGF, and 123 oocytes were incubated with EGF.

EGF affected morphology of cumulus oocyte complex (COC). After maturation, cumulus cells were more expanded in cultures with EGF than in cultures without EGF.

Table 1 shows the effects of EGF on the rates of fertilization, and morula and blastocysts formation.

Table 1. Effects of EGF on parameters of IVF procedures. Data are expressed per one ewe.

	No. of oocytes used for IVF	No. of cleaved zygotes	No. of not fertilized oocytes	Fertilization rate (%)	No. of cultured zygotes	No. of morulas on day 6-7	% of morulas on day 6-7	No. of blastocysts on day 8	% of blastocysts on day 8
0 ng EGF/ml	7.8±0.8	5.7±0.7	2.1±0.4	73.8±5.1	4.6±0.4	1.9±0.4	39.1±7.7	0.6±0.2	13.4±4.3
10 ng EGF /ml	8.2±0.9	6.5±0.9	1.7±0.3	78.1±4.1	5.2±0.8	2.2±0.6	40.5±8.5	1.4±0.7	21.0±6.6
P value for 0 vs 10 ng of EGF	0.730	0.487	0.432	0.512	0.496	0.668	0.905	0.245	0.352
Overall (no EGF effects)	15.5±1.7	11.9±1.6	3.5±0.6	75.7±3.9	8.9±1.3	3.9±1.0	40.6±7.1	1.9±0.8	18.4±4.9

All parameters were similar ($P>0.05$) for no treated and EGF treated COC. However, the number of blastocysts on day 8 of culture tended ($P<0.25$) to be greater in the EGF-treated group. The rate of blastocyst formation (% of blastocysts on day 8) was positively correlated (0.490, $P<0.06$) with number of follicles/ewe.

DISCUSSION

Assisted reproductive technologies are powerful tools in animal industry for genetic improvement and also for enhancing reproductive efficiency. For ART methods to be efficient, the number of follicles and the number of oocytes and embryos obtained from animals must be optimized. In this regard, existing protocols for oocyte retrieval, maturation, and in vitro fertilization techniques need improvements especially in terms of consistency of the responses.

For ART, induction of multiple follicles is only effective if multiple oocytes or embryos can be collected (Gordon, 1997). In the present study, we stimulated follicular growth with FSH-treatment for two days which resulted in induction of about 25 follicles per ewe. This length of FSH-treatment has been shown to be beneficial for obtaining high number of oocytes and for high rates of fertilization (Stenbak et al., 1999).

In this experiment, the rate of in vitro fertilization was 76%, and the rate of morula and blastocyst formation was 41% and 18%, respectively. Similar rates of fertilization were reported by others for sheep (68%, Slavik, et al., 1992; 70%, Stenbak et al., 1999; 72%, Ledda, et al., 1997; 74%, Dattena et al., 2000; 80%, O'Brien, et al., 1996, 1997; 72-83%, Watson, et al., 1994). However, in other studies, the rate of morula and blastocyst formation ranged from 15 to 70% (Gardner et al., 1994; Watson et al., 1994; Thompson et al., 1995; O'Brien et al., 1996, 1997; Walker et al., 1996; Wang et al., 1998; Dattena et al., 2000). These differences probably are due to media composition, culture conditions, age of oocyte donor, and breed of ewes.

Numerous supplements of maturation, fertilization and/or culture media have been tested to improve production of embryos in vitro (Gardner et al., 1994; Watson et al., 1994; Thompson et al., 1995; Holm et al., 1996; O'Brien et al., 1996, 1997; Walker et al., 1996; Ledda et al., 1997; Wang et al., 1998). In addition to gonadotropins and estradiol, growth factors including EGF have been demonstrated to have some positive effects on oocyte maturation, IVF, and blastocyst formation in several species (Longergan et al., 1996; Park et al., 1997; Izadyar et al., 1998; Rieger et al., 1998; Lim and Hansel, 1999; Abeydeera et al., 2000; Guler et al., 2000). EGF is present in the ovaries of several species, and affects cellular functions through EGF receptors (Reeka et al., 1998; Yoshida et al., 1998; Qu et al., 2000). In the present experiment, EGF increased formation of blastocysts from 0.6 to 1.4/ewe. This demonstrates that in the presence of EGF, formation of blastocysts doubles, which confirms previously published reports. These results indicate and confirm previous reports that EGF is a very important supplement to maturation media and it should be recommended for use in in vitro embryo production in FSH-treated sheep. The results of this study could ultimately lead to improved and efficient methods for obtaining large numbers of high quality oocytes and embryos for transfer programs. Improvement in these techniques will enhance the overall efficiencies of ART for farm use.

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