Production and lambing rate of blastocysts derived from in vitro matured oocytes after gonadotropin treatment of prepubertal ewes

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J Anim Sci 1999. 77:2234-2239.

Production and Lambing Rate of Blastocysts Derived from In Vitro Matured Oocytes After Gonadotropin Treatment of Prepubertal Ewes[1]

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ABSTRACT: The aim of this study was to evaluate the effect of gonadotropin treatment on the in vitro maturation, blastocyst production, and developmental potential to term of oocytes collected from Sardinian neonatal and prepubertal ewes at 4 to 6 wk of age. Cumulus-oocyte complexes were recovered at 24 h after withdrawal of a 1/6th size progestagenated pessary from the donors, of which each received 120 IU FSH/LH and 400 IU PMSG in a single dose 36 h before sponge removal. Treated donors produced a greater ($P < .01$) number of oocytes per animal (86.2 ± 7.9) compared with slaughterhouse (untreated) prepubertal ewes (55.5 ± 6.1) of the same age or with treated neonatal ewes (6.1 ± 0.7) 10 d old. During oocyte maturation, there were no differences in the percentage of germinal vesicle break-down (78.08 vs 74.24), metaphase I (89.13 vs 87.18), and metaphase II (77.91 vs 76.38) when evaluated after 8, 14, and 24 h of maturation, respectively, between oocytes from treated and slaughterhouse (untreated) prepubertal ewes. The embryo cleavage (71.1 vs 73.7) and blastocyst rates (22.2 vs 19.8) were similar in the treated and the untreated prepubertal ewes after transfer of in vitro matured oocytes into ligated oviducts of temporary recipients. The in vitro viability rates of vitrified blastocysts (81.2 vs 76.9) and the in vivo survival rates (46.1 vs 50.0) of embryos derived from in vitro matured and in vivo fertilized oocytes showed no difference. The data suggest that gonadotropin treatment increases oocyte production per animal but has no effect on oocyte quality because embryo production and lambing rates of blastocysts derived from in vitro matured oocytes were not markedly different from those derived from untreated prepubertal ewes of the same age.

Key Words: Gonadotropins, Oocytes, Blastocyst, Lambing, Sheep

 J. Anim. Sci. 1999. 77:2234–2239

Introduction

Oocytes derived from the antral follicles of prepubertal animals, including cows (Armstrong et al., 1992), sheep (Armstrong et al., 1995), goats (Martino et al., 1995), and pigs (Pinkert et al., 1989), can be matured and fertilized in vitro with good results. However, the developmental ability of oocytes obtained from prepubertal animals was less than that from adults, and they resulted in a smaller number of viable embryos than those from adults (Palma et al., 1993).

Many factors may reduce the developmental competence of oocytes collected from prepubertal animals. Using calf oocytes, Duby et al. (1996) suggested that defective cytoplasmatic maturation can occur with a reduced release of calcium ion content. Levesque and Sirard (1994) observed some differences in protein patterns and content compared with oocytes derived from adult ewes. Oocytes naturally acquire their capacity for maturation, fertilization, and development at puberty when the hypophysial-gonadal axis and local factors start to operate in the ovarian follicle of prepubertal animals (Foster, 1988). Several studies indicate that ovaries from prepubertal animals can be stimulated to grow with exogenous hormones (Earl et al., 1994; O'Brien et al., 1996).

The treatment of juvenile animals with exogenous hormones for oocyte collection and subsequent in vitro embryo production combined with other breeding technologies, such as embryo transfer, may reduce the generation interval and improve the genetic programs for livestock (Nicholas, 1996). Thus, this study was conducted to determine whether treatment with exogenous hormones improves 1) the number of oocytes recovered from neonatal and prepubertal ewes and oocyte developmental potential, 2) in vitro survival of in vitro-matured (**IVM**)/in vivo-derived blastocysts following vitrification, and 3) viability of IVM/in vivo-derived blastocysts after embryo transfer compared with slaughterhouse (untreated) material from prepubertal ewes.

[1]This study was supported by Ministero dell'Università e della Ricerca Scientifica e Technologica (MURST, 40%).

Received July 23, 1998.

Accepted January 18, 1999.

Materials and Methods

Animals and Collection of the Oocytes

Seven neonatal ewes 4 to 8 d old and 11 prepubertal ewes 30 to 40 d old were treated with 1/6 of a pregestagenated pessary containing fluorogestone acetate (**FGA**; 40 mg; Chronogest, Intervet, The Netherlands) for 6 d. Each animal received at 36 h before pessary removal a single i.m. injection of 120 IU FSH/LH (Pluset, Serono, Roma, Italy) and 400 IU of PMSG. The cumulus oocyte complexes (**COC**) were collected by follicular aspiration from ovaries of treated noenatal and prepubertal ewes via midventral laparotomy and exposure of the genital tract under general anaesthesia (pentothal sodium 15 mg/kg body weight; Farmaceutici Gellini, Aprilia, Italy) at 24 h after spong removal in TCM 199 medium (Sigma Chemical Co., St. Louis, MO) containing 50 U of penicillin/mL and 50 μg of streptomycin/mL (Sigma Chemical Co.) and .1% (wt/vol) polyvinyl alcohol (**PVA**; Sigma Chemical Co.). The COC were obtained from ovaries of slaughterhouse (untreated) prepubertal ewes collected in a local abattoir and transported in Dulbecco's PBS at 20 to 25°C (Sigma Chemical Co.) supplemented with antibiotics, arriving at the laboratory within 1 h. Ovaries were washed three times in fresh PBS and sliced using a microblade, and the contents released into the dissection medium.

The COC were selected according to morphological criteria: intact and several dense cumulus cell layers and homogenous distribution of lipid droplets in the cytoplasm. The COC were washed three times in the same medium before in vitro maturation. Oocytes obtained from the treated and the slaughterhouse (untreated) prepubertal ovaries were selected according to their dimensions using an inverted microscope equipped with a calibrated eyepiece, and small oocytes (< 135 μm) and those with signs of atresia were discarded. For each ovary, the number of oocytes recovered was recorded.

Four experiments were performed to evaluate the competence of slaughterhouse (untreated) prepubertal ovine oocytes and those from the treated ewes.

Experiment 1: In Vitro Maturation

The COC obtained from neonatal and prepubertal ewes were divided and allocated in the following maturation system. Treated and slaughterhouse (untreated) oocytes were cultured in parallel for the first 24 h in TCM 199 supplemented with 10% (vol/vol) heat-treated fetal calf serum (**FCS**; Sigma Chemical Co.) 10 μg of FSH/mL, and 10 μg of LH/mL (Pergonal, Serona Italy). The oocytes (25 to 30) from the two sources were put into separate Petri dishes (35 mm) containing 2 mL of the same culture medium and maintained on a rotary shaker at low speed during the entire period in the same incubator. The culture conditions were maintained at 39°C in a humidified atmosphere with 5% CO_2 in air. At 0, 8, 14, and 24 h of the maturation period, oocytes from treated and slaughterhouse (untreated) prepubertal ewes were fixed for 24 h in acetic acid ethanol solution (1:3; vol/vol), stained with 1% (wt/vol) of Lacmoid (Sigma Chemical Co.), and examined under a phase contrast microscope at 200 to 400 ×. Stages of nuclear maturation of oocytes were evaluated as germinal vesicle break-down (GVBD), metaphase I (M I), or metaphase II (MII).

Experiment 2: Embryo Production from Oocytes In Vitro Matured After Transfer into Temporary Recipient Ewes

In this experiment, we examined the ability of treated and slaughterhouse (untreated) in vitro-matured oocytes to develop after transfer to the ligated oviducts of recipient ewes for in vivo fertilization and culture for 5.5 d.

After 24 h of in vitro maturation, under the same conditions as described in Exp. 1, oocytes were washed with fresh TCM 199 + 10% FCS and surgically transferred to recipient ewes. For this purpose, estrus was synchronized in temporary recipient Sardinian ewes with progestagenated pessaries containing 40 mg of FGA, inserted for 14 d. All recipients were anesthetized with 10 mg/kg body weight of sodium pentothal in saline solution. The abdominal cavity was opened, and the genital tract was exposed. Only oviducts adjacent to a non-ovulatory ovary were used for the fertilization and temporary in vivo culture. Frozen semen was thawed in a 35°C water bath, and the content of the straw was centrifuged with TCM 199 supplemented with PVA (.1%) to remove the freezing medium. Sperm (50 μL; 100×10^6/mL) of the same fresh medium was injected via the ampulla into the oviduct of each recipient using a tom-cat catheter connected to a syringe. Twenty to 30 oocytes were injected via the ampulla into the oviduct of each recipient in about 15 to 20 μL of medium, and the oviducts were ligated just anterior to the uterotubal junction. The oviducts of recipients were flushed 5.5 d later, and ova/embryos were recovered from the flushing medium and observed under an inverted microscope to assess the developmental stage. Embryos that had developed beyond the 16-cell stage were immediately stained in Hoechst 33342 (Sigma Chemical Co.) to count the number of nuclei under a microscope equipped with epifluorescence (Diaphot 300, Nikon, Tokyo, Japan). Embryos at the blastocyst stage, evaluated using morphological criteria such as the formation of a blastocoel, were used for viability assessment in vivo and in vitro.

Experiment 3: In Vitro Survival of Vitrified Blastocysts Derived from Ooocytes from Treated and Slaughterhouse (Untreated) Prepubertal Ewes

Blastocysts collected from temporary recipients, at random, were vitrified using our procedures (Naitana et al., 1997). The basic solution for vitrification and

Table 1. Effects of PMSG/FSH treatment on oocyte recovery from ovaries of neonatal and prepubertal ewes

Age of donors	Source of oocytes	Number of ovaries	Recovered oocytes	Oocytes/animal (mean ± SEM)
5–10 d	Treated	14	42	6.1 ± 0.7[a]
32–40 d	Treated	22	979	86.2 ± 7.9[b]
32–40 d	Untreated	28	770	55.6 ± 6.1[c]

[a,b,c]Values within columns with different superscripts are different ($P < .01$).

warming procedures was PBS with .3 mM sodium pyruvate, 3.3 mM glucose supplemented with .1% (wt/vol) PVA (30 to 70 × 10^3 molecular weight, Sigma Chemical Co.), and antibiotics. Briefly, embryos were moved, after exposure at room temperature (15 to 20°C) to the basic solution, into 200 μL drops of 1.4 M glycerol for 5 min, then into 200 μL drops of 1.4 M glycerol and 3.6 M ethylene glycol for 5 min before being transferred into a 20-μL column of 3.4 M glycerol and 4.6 M ethylene glycol, and loading into the center of .25 mL straws using a fine glass capillary pipette. In the straws, the embryos and vitrification solution were separated by four air bubbles (60 μL) from two columns (160 μL) of .5 M sucrose solution.

After sealing with gelatine powder, about one-half of the straw, including the vitrification solution, was immediately plunged into LN_2 within 25 to 30 s; the rest of the straw was then immersed slowly to prevent bursting. The straws were maintained into LN_2 until they were used. For warming to the biological temperature, the straws were transferred from LN_2 into a water bath at 35°C for 10 s. The content of each straw was expelled into Petri dishes (Falcon, Becton Dickison, Franklin Lakes, NJ) and stirred gently to facilitate the mixture of the two solutions. The embryos were retrieved and transferred into 200 μL drops of .25 M sucrose for 3 min to allow for removal of intracellular cryoprotectants.

After washing, vitrified-warmed embryos were maintained for 10 min at room temperature in the basic solution for a rehydration and equilibration period. Embryos were cocultured in TCM 199 with 10% FCS and sheep oviductal epithelial cells (**SOEC**) prepared according to the method of Gandolfi and Moor (1987) in a humidified atmosphere of 5% CO_2 in air at 39°C. They were then examined at 12-h intervals for 6 d. The embryos that completed the escape from the zona pellucida or reformed their blastocoelic cavity were considered viable.

Experiment 4: In Vivo Viability of Blastocysts Derived from In Vitro-Matured Oocytes from Treated and Slaughterhouse (Untreated) Prepubertal Ewes

To assess the viability of the blastocysts developed in Exp. 2 in vivo, blastocysts were transferred to mature Sardinian ewes, which were synchronized with the same protocol used in Exp. 3, that received 500 IU of PMSG after pessary removal. Estrus was detected using a vasectomized ram. Permanent recipients were anesthetized as described. The uterine wall of one horn was perforated with a needle and the catheter was inserted about 3 cm into the lumen where the blastocyst was deposted. The ewes carried pregnancies to term, and, after lambing, the duration of pregnancy and the weight of each newborn were recorded.

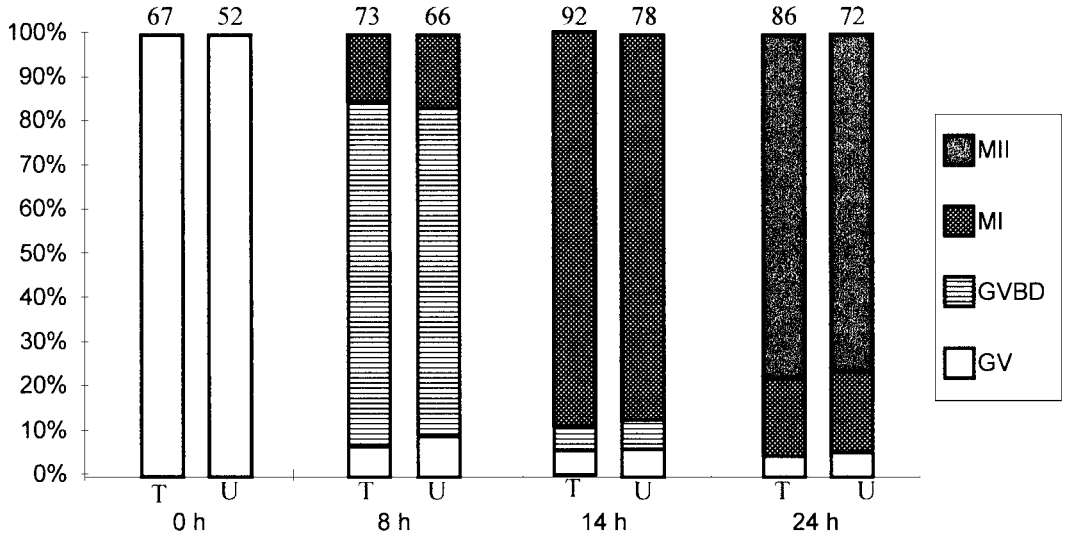

Figure 1. Germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII) during meiotic progression of in vitro culture of oocytes collected from treated (T) and slaughterhouse (untreated) (U) prepubertal ewes.

Table 2. Embryo production from prepubertal (32- to 40-d-old) treated and slaughterhouse (untreated) ewes after in vitro maturation and in vivo fertilization oocytes in temporary recipients[a]

Prepubertal ewes	Number of oocytes			Stage of development	
	Transferred	Recovered	Cleaved	<16 Cells	Blastocyst
Treated	253	177 (69.9)[b]	126 (71.1)	98	28 (22.2)
Untreated	271	198 (73.0)	146 (73.7)	117	29 (19.8)

[a]No differences between treatments $P > .05$.
[b]Values in parentheses are the percentages.

Statistical Analysis

Differences in meiotic progression and in developmental competence of neonatal and prepubertal oocytes were subjected to chi-squared analysis and the Fisher exact test. The means of recovered oocytes were tested for significant differences between treatments by one way analysis of variance using Tukey multiple range test. Differences were considered statistically significant at $P < .05$.

Results

The effect of gonadotropin stimulation on oocyte production of prepubertal ewes is shown in Table 1. Four- to 6-wk-old treated prepubertal ewes produced significantly more oocytes (86.2 ± 7.9) than did slaughterhouse (untreated) ewes (55.5 ± 6.1) of the same age, or did treated noenatal ewes (6.1 ± 0.7) 5 to 10 d old.

In four replicate experiments, the gonadotropin treatment did not modify the percentage of oocytes reaching germinal vesicle break-down (78.08 vs 74.24%) at 8 h, metaphase I (89.13 vs 87.18%) at 14 h, and metaphase II (77.91 vs 76.38%) at 24 h during meiotic progression in in vitro culture in parallel conditions for the treated and the slaughterhouse (untreated) prepubertal ewes (Figure 1). No significant difference was observed in the cleavage (71.1 vs 73.7%) and blastocyst rates (22.2 vs 19.8%) between treated and slaughterhouse (untreated) prepubertal ewes after in vitro culture and subsequent transfer into ligated oviducts of temporary recipients (Table 2). Similar in vitro viability rates were obtained in blastocysts derived from in vitro-matured and in vivo-fertilized oocytes from treated (81.2%) and slaughterhouse (untreated) (76.9%) prepubertal ewes after cryopreservation by vitrification and thawing (Table 3). No marked differences were found between treated (50.0%) and slaughterhouse (untreated) (46.1%) animals in the lambing rates of these blastocysts transferred to synchronized recipients (Table 4).

Discussion

Our data demonstrated that, as observed in adult animals, prepubertal ewes showed a high variability of ovarian responses after superovulatory stimulation. Gonadotropin treatment increased the number of follicles of 30- to 40-d-old prepubertal lambs compared with unstimulated lambs of the same age and with 5- to 10-d-old lambs, which exhibited poor response to exogenous hormones. These findings are particularly intriguing because the hormonal profile of prepubertal lambs of different ages was not significantly different, and the plasma level of gonadotropin hormones in lambs was similar near birth and at 10 wk of age (Tassell et al., 1978). This effect may be due to the immature reproductive tract, which needs to develop progressively the full capacity to respond to the hormonal stimulation as the lambs reach puberty. In fact, previous evidence demonstrated that antral

Table 3. In vitro survival rates of vitrified blastocysts obtained from prepubertal (32- to 40-d-old) oocytes of treated and slaughterhouse (untreated) ewes after 24 h of in vitro maturation[a]

Prepubertal Ewes	Vitrified blastocysts	Reexpansion after 24 h of in vitro culture	Hatched blastocysts
Treated	16	14 (87.5)[b]	13 (81.2)
Untreated	13	11 (84.6)	10 (76.9)

[a]No differences between treatments $P > .05$.
[b]Values in parentheses are the percentages.

Table 4. Viable blastocysts obtained from prepubertal treated and slaughterhouse (untreated) ewes in vitro matured and in vivo fertilized oocytes[a]

Prepubertal ewes	Transferred blastocysts	Recipient ewes	Pregnancy	Lambing
Treated	10	5	4	5 (50.0)[b]
Untreated	13	8	5	6 (46.1)

[a]No differences between treatments $P > .05$.
[b]Values in parentheses are the percentages.

follicles do not respond to exogenous gonadotropins before 4 wk of age (Mansour, 1959; Mauleon, 1969) when the development of granulosal layers and thecal cells appear to reach maturity (Kennedy et al., 1974). These authors also observed that the ovarian weight and the presence of growing and vesicular follicles increased about 11 times between birth and 8 wk.

The present report also demonstrated that the quality of the oocytes, assessed by meiotic progression and by embryo production, was similar between treated and slaughterhouse (untreated) animals, confirming another report (O'Brien et al., 1997). At present, the effects of gonadotropin treatment on oocyte quality are not clear; some studies showed a positive influence (Armstrong et al., 1994), and others showed a negative influence on oocyte quality (Moor et al., 1985). From previous studies, full oocyte maturation is not necessarily indicated by the metaphase II stage but rather by the culmination of several processes including cytoplasmic organelle distribution (Thibault et al., 1987) and protein synthesis (Moor and Crosby, 1986). In fact, O'Brien et al. (1996) observed some cytoplasmic differences in the dimension of mitochondria and cortical granules after gonadotropin stimulation of 4- to 6-mo-old prepubertal lambs. Our previous experiences showed other differences, such as reduced oocyte size (Ledda et al., 1996a), defective coupling between granulosa cumulus cells and oocytes, and a decrease in amino acid uptake (Ledda et al., 1996b) and level of maturation promoting factor (Bogliolo et al., 1997).

Similar numbers of embryos were produced from oocytes collected from treated and untreated prepubertal animals. This means that in prepubertal lambs, contrary to what is observed in adult animals, gonadotropin treatment can prevent or delay atretic changes in follicular growth without influencing full cytoplasmic maturation and oocyte quality. This was confirmed by assessing the in vitro developmental potential of these embryos, after vitrification, with similar results in untreated and treated animals, indicated by a good resistance to exposure to low temperatures and to a high concentration of cryoprotectants. In the same way, no differences in lambing rates were found after transfer into synchronizated recipients between embryos derived from stimulated and unstimulated lamb oocytes.

Previous studies (Ledda et al., 1996, O'Brien et al., 1997) indicate that oocytes obtained from untreated prepubertal sheep showed lower developmental efficiency than those from adults. However, Earl et al. (1994) showed a similar developmental potential of oocytes derived from prepubertal sheep treated with exogenous hormones and those from adults. However, there are important differences between these studies, in particular, age of donors, breeding, management, and hormone treatment. Also, the oocytes used in the study by Earl et al. (1994) were obtained from in vivo maturation, which may have improved their developmental competence. At present, even though considerable progress has been obtained in reproductive technology, the in vitro maturation system remains inadequate to complete oocyte growth, a deficiency that may particularly affect the maturation of oocytes derived from prepubertal animals.

Apart from the central role of the gonadotropins, other molecules such as growth factors may play an important role in intraovarian regulation (Wathes et al., 1995). The presence of these factors in the follicles and the identification of specific receptors suggest an active involvement of these molecules in follicular growth and the full maturation of oocytes by autocrine and paracrine mechanisms (Monniaux and Pisselet, 1992; Teissier et al., 1994). An in vitro study confirmed the potential use of these growth factors to improve the efficiency of oocyte maturation in mammalian species (Hsu and Hammond, 1990; Lorenzo et al., 1994). Even though gonadotropin treatment does not influence the improvement of full oocyte maturation, our results confirm other studies in prepubertal calves (Revel et al., 1995) that gonadotropin injection produces more oocytes that have the same rate of in vitro development and survival after embryo transfer into adult recipients as oocytes from untreated animals.

Implications

Gonadotropin treatment increases the number of oocytes from prepubertal lambs and, therefore, the potential production of blastocysts, but no differences are found in the quality of these oocytes. Further efforts are needed to understand the basic mechanisms of oocyte maturation during puberty, to improve gonadotropin treatment of prepubertal animals, and to perfect in vitro culture conditions in order to increase the developmental potential of these oocytes.

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