

Prepubertal Propagation of Transgenic Cloned Goats by Laparoscopic Ovum Pick-Up and *In Vitro* Embryo Production

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ABSTRACT

The use of laparoscopic ovum pick-up (LOPU) followed by *in vitro* embryo production was evaluated in the early propagation of cloned goats. Ten kinder goats produced by somatic cell nuclear transfer technology were used as oocyte donors. Half of the donor animals were subjected to LOPU at 2–3 months of age (prior to induction of lactation), whereas the other five goats were subjected to LOPU at 6–7 months of age (following induction to lactation). They were stimulated with 80 mg NIH-FSH-P1 (Folltropin[®], Vetrepharm, Canada) together with 300 IU eCG (Novormon[®], Vetrepharm, Canada) administered intramuscularly 36 h prior to LOPU. The number of follicles aspirated and oocytes recovered was higher in the younger group of donors (57 ± 7 and 41 ± 4 vs. 28 ± 2 and 25.8 ± 2 , $p < 0.05$), however, oocytes from animals in the late prepubertal age showed higher developmental capacity resulting in higher transferable embryo yield (81.4% vs. 67.8%, $p < 0.01$), pregnancy rate (80% vs. 40%, $p < 0.05$) and total kids born (27 vs. 15, $p < 0.01$). In conclusion, LOPU in combination with *in vitro* embryo production techniques is an efficient method for the early propagation of valuable goats produced by somatic cell nuclear transfer.

INTRODUCTION

THE PRODUCTION OF RECOMBINANT PROTEINS of pharmaceutical and biomedical interest in the milk of transgenic animals provides an economical advantage over more traditional production systems based on microorganisms and animal cells (Wall, 1996; Echelard et al., 2000; Niemann et al., 2000). Transgenic animals can be produced by a number of different techniques, including pronuclear microinjection (Ebert, et al., 1991; Baldassarre et al., 1999 and 2003), sperm-mediated transgenesis (Celebi et al., 2003), and somatic cell nuclear transfer (Schnieke et al., 1997; Keefer et al., 2001, 2002).

Somatic cell nuclear transfer (SCNT) has recently improved the efficiencies to generate transgenic animals (Coleman, 1999). In this system, female cell lines are transfected *in vitro* and selected based on transgene integration before they are used in a nuclear transfer program. As a result, the progeny born are virtually guaranteed to be transgenic, and, therefore, fewer animals are needed to produce the desired number of transgenic founder animals.

Shortly after they are born, the founder animals can be checked for expression of the transgene by means of hormonal induction of lactation (Camuso et al., 2000). If the expression levels are adequate (usually in the gram per litter levels), it is

desirable to propagate those animals as fast as possible in order to generate the transgenic production herd. For such process, usually referred to as scale-up, it is ideal to generate male lines from the founder female animals that have shown adequate expression levels, as males are very cost efficient to propagate using standard artificial insemination technology (Baldassarre et al., 2000).

In the present study, we have evaluated the use of laparoscopic ovum pick-up (LOPU) in conjunction with *in vitro* embryo production, as a method to propagate SCNT-derived transgenic founder goats at young (prepubertal) ages. Furthermore, the effect of age at LOPU as a consequence of practicing the procedure before or after induction of lactation was evaluated.

MATERIALS AND METHODS

Animals

Ten SCNT-derived goats were used as oocyte donors. These somatic cell clones were produced from two transfected fetal fibroblast cell lines using techniques previously described (Keefer et al., 2001, 2002). One set of five goats was derived from designated line 503, while the other set of five goats was derived from line 601. Both lines were derived from the same 28-day-old fetus. Half of the animals were submitted to LOPU at 2–3 months of age, while the other half of the animals was submitted to LOPU at 6–7 months of age. Each age group was composed of three founders from one cell line and two founders of the other line. Thirty-eight goats of standard breeds (Nubian, Saanen, Boer) were used as recipients.

Induction of lactation

The SCNT-derived donors were induced to lactate at 2–4 months of age following established procedures (Cammuso et al., 2000). Briefly, goats were treated with estradiol and progesterone for 2 weeks to stimulate mammary gland development followed by a 3-day lactogenic treatment with dexamethasone. Goats were milked for a period of 2–3 months before they were dried-off. Half of the animals were hormonally induced to lactate prior to oocyte recovery, while the other half of the animals was submitted to LOPU before they were induced to lactate.

Hormonal treatment of donor and recipient goats

Donor goats were stimulated with 80 mg NIH-FSH-P1 (Folltropin[®], Vetrepharm, Canada) together with 300 IU eCG (Novormon[®], Vetrepharm, Canada) administered intramuscularly 36 h prior to LOPU.

Recipient goats were heat synchronized using intravaginal sponges containing 60 mg medroxyprogesterone acetate (Veramix[®], Upjohn, Canada) for 10 days combined with a luteolytic treatment of 125 μ g cloprostenol (Estrumate[®], Schering, Canada) and 300 IU eCG (Novormon[®], Vetrepharm, Canada) administered intramuscularly 24 h prior to sponge removal, which took place approximately 15 h prior to LOPU in donors.

Anesthesia

Donors and recipients were deprived of food and water for 24 h prior to laparoscopy. Anaesthesia was induced with intravenous administration of 0.35 mg/kg body weight of diazepam (Diazepam[®], Sabex, Canada) and 5 mg/kg body weight of ketamine (Ketalean[®], Bimeda-MTC, Canada), and maintained with Isoflurane (Isoflo[®], Abbot, Canada) via endotracheal intubation.

Chemicals and reagents

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Laparoscopic ovum pick-up (LOPU)

Oocytes were recovered by aspiration of follicular contents under laparoscopic observation as previously described (Baldassarre et al., 2002b). The collection medium was TCM 199 supplemented with 0.05 mg/mL Heparin (Hepalean[®], Organon Teknica, Canada) and 1% (v:v) fetal bovine serum.

Washing and grading of oocytes

Follicular contents were poured into a Petri dish and observed under a stereomicroscope. The cumulus–oocyte complexes (COCs) were located and washed in M199 supplemented with penicillin (100 IU/mL), streptomycin (0.1 mg/mL), kanamycin (0.05 mg/mL), and 0.5% bovine serum albumin. They were then transferred into the IVM drops and selected for subsequent use based on their cellular vestments and cytoplasm uniformity.

Maturation of oocytes

In vitro maturation was performed in 50- μ L drops of maturation medium under mineral oil. Maturation medium consisted of M199 supplemented with bLH (0.02 U/mL; Sioux Biochemicals, USA), bFSH (0.02U/mL; Sioux Biochemicals, USA), estradiol β -17 (1 μ g/mL), sodium pyruvate (0.2 mM), kanamycin (50 μ g/mL) and 10% heat-inactivated estrus goat serum. *In vitro* maturation was performed at 39°C in humidified atmosphere with 5% CO₂ in air for 24–27 h.

In vitro fertilization

The expanded cumulus cells were partially removed from matured COCs by repeated pipetting or dissection using a needle. Oocytes were washed in fertilization medium and transferred to 40- μ L drops of fertilization medium under mineral oil. Fertilization medium consisted of TALP medium (Parrish et al., 1986) supplemented with 20% heat-inactivated estrus goat serum.

Fresh semen was collected on the day of IVF from one male of the Saanen breed using an artificial vagina and was kept in the dark at 20°C for 2–3 h. Two mL of warm (37–38°C). Defined Medium (Younis et al., 1991) supplemented with 6 mg/mL fatty acid free BSA (mDM) were added to the tube and mixed with the semen. A 100- μ L aliquot of the semen mix was overlaid on top of a 45:90% Percoll gradient, which was then centrifuged at room temperature for 30 min at 500 \times g. The pellet was resuspended in 4 mL of mDM and centrifuged for 10 min at 70 \times g. The washed pellet was resuspended in capacitation medium containing 0.5mM 8-bromo-cAMP, 100 nM ionomycin, and 10 μ g/mL heparin in 1 mL of mDM. The sperm-capacitation mixture was incubated at 39°C for 15 min. The sperm concentration was adjusted to 10 \times 10⁶ sperm per mL, and 5 μ L were added to the fertilization drops containing the oocytes (final sperm concentration; 1 \times 10⁶ sperm per mL). The IVF drops were then incubated at 39°C in a humidified atmosphere incubator with 5% CO₂ in air.

Embryo transfer

At 15–20 h after insemination, the presumptive zygotes were transferred into the oviducts of recipient goats, which were first examined laparoscopically to confirm at least one recent ovula-

tion. A midventral laparotomy was established and the reproductive tract was exteriorized for the transfer of embryos contained in a Tomcat catheter (Sovereign[®], Kendall Company, Mansfield, MA). The catheter was introduced into the oviduct through the fimbria. The number of embryos transferred per recipient varied between 5 and 8.

Pregnancy detection

Pregnancy was detected by ultrasound using a SonoVet[®] 600 scanner (Medison Corp., Korea) with a transrectal 7.5-MHz linear array probe at 28 and 56 days following embryo transfer.

Identification of transgenic animals

Genomic DNA was extracted from the blood or skin of 2-week-old putative transgenic offspring using standard molecular biology techniques (Sambrook et al., 1989). The DNA was screened for the presence of the transgene by PCR using two different sets of primers specific to the transgene. Confirmation of all PCR positive animals was performed using Southern blotting analysis and the Roche Molecular Biochemicals (Laval, QC, Canada) DIG system for detection.

Statistical analysis

Results were statistically analyzed using Prism[™] (GraphPad Software Inc., San Diego, CA). The data collected from follicles aspirated, oocytes recovered and embryos transferred per donor were analyzed using the unpaired Student's *t*-test with Welch's correction for unequal variances. The data for transferable embryo yield (embryos transferred/oocytes recovered), pregnancy rate and kids born were analyzed using Pearson's Chi-Square.

Animal ethics

All protocols used in this study have been approved by Animal Care Committee of Nexia, in compliance with the guidelines from the Canadian Council of Animal Care.

RESULTS

Overall, 425 follicles were aspirated (42.5 \pm 19 per goat) and 334 oocytes recovered (33.4 \pm 10 per goat) resulting in a 79% recovery rate. *In vitro*

maturation and fertilization resulted in 244 transferable zygotes (24.4 ± 11 per goat) that were transferred into 38 recipients (6.4 embryos per recipient). A total of 21 recipients were detected pregnant by ultrasound at 28 days, of which 20 went to term and delivered 42 kids. Five kids (12%) were born dead or died shortly after birth due to weakness at birth. From the total of 42 kids born, 33 were characterized to be transgenic (78%) by PCR and Southern Blotting.

No differences were found between the two sets of SCNT-derived goats (lines 503 and 601) in terms of the follicles aspirated (42.8 ± 22 vs. 42.2 ± 19), oocytes recovered (33.2 ± 12 vs. 33.6 ± 10), pregnancy rate ($11/21 = 52\%$ vs. $10/17 = 59\%$), kids born (23 vs. 19).

The overall efficiency of IVM/IVF was high, with 73% of the total number of oocytes recovered resulting in transferable zygotes.

The age of the animals at the time of LOPU had a significant impact on most variables (Table 1). While the number of follicles available for aspiration and the oocytes recovered was significantly higher in the animals of <100 days of age (57 ± 7 and 41 ± 4 vs. 28 ± 2 and 25.8 ± 2 , $p < 0.05$), older animals resulted in a higher yield of transferable embryos after IVM/IVF (81.4% vs. 67.8%, $p < 0.01$), higher pregnancy rate (80% vs. 40%, $p < 0.05$), and higher number of kids born (27 vs. 15, $p < 0.01$). Only one of the 10 donors failed to produce at least one transgenic offspring during this experiment (<100 days of age group).

A high transgenesis rate (78.6%) was found in the progeny as a result of all donors having multiple integration sites.

DISCUSSION

Overall, the propagation program was very successful in that 33 transgenic kids were obtained from 10 transgenic founder animals that were less than 8 months of age at the time of LOPU. As only one donor failed to produce any transgenic progeny, the program resulted in the successful propagation of 90% of the donors.

It is noteworthy that for those animals that were submitted for LOPU at <100 days of age the program resulted in offspring from them being born before they reached age and development to be bred for the first time. As a result, a significant shortening of the generation interval was possible.

Not surprisingly, the oocytes collected from older animals showed increased developmental ability resulting in higher fertilization, pregnancy and development to term. Since these animals were used as donors following hormonal induction of lactation, the results indicate that such treatment did not have a detrimental effect on subsequent LOPU-IVF. Furthermore, O'Brien et al. (1997) reported higher developmental capacity in oocytes from prepubertal sheep that were primed with estradiol and progesterone prior to gonadotropin treatment and oocyte collection. To the best of our knowledge, this is the first report describing the *in vitro* production of embryos using oocytes collected from goats shortly after hormonal induction of lactation. Hence, further research is required to establish if the hormonal treatment used for induction of lactation prior to oocyte collection may have enhanced the quality

TABLE 1. EFFECT OF AGE AT THE TIME OF LOPU ON THE MOST IMPORTANT PARAMETERS TO EVALUATE THE EFFICIENCY OF LOPU/IVF FOR THE EARLY PROPAGATION OF VALUABLE GOATS

Parameter/age at LOPU	<100 days of age	>180 days of age	p value
Number of LOPU donors	5	5	n/a
Average follicles aspirated	57.0 ± 16	28.0 ± 5	<0.05
Average oocytes recovered	41.0 ± 9	25.8 ± 6	<0.05
Embryos transferred (ET)	139	105	n/a
ET/oocytes recovered (%)	67.8%	81.4%	<0.01
Recipients transferred	23	15	n/a
Initial pregnancy (%)	9 (40%)	12 (80%)	<0.05
Pregnancy losses	1 (11%)	0	ns
Kids born (avg./recipient)	15 (1.9)	27 (2.2)	
Perinatal deaths	3	1	n/a
Kids transgenic (%)	13 (87%)	20 (74%)	n/a

of the oocytes recovered from the animals subjected to LOPU at this later age.

Results from the early prepubertal age group (<100 days of age) were quite acceptable, significantly higher than those reported in calves (Revel et al., 1995) and comparable to those reported in lambs (Earl et al., 1994; Ledda et al., 1999) and standard prepubertal goats (Baldassarre et al., 2002a).

In conclusion, LOPU in combination with *in vitro* embryo production techniques have proven to be a powerful tool for the early propagation of valuable transgenic female founders produced by SCNT. Our results suggest it is more efficient to perform the procedure at 6–7 months of age, after completing the induced lactation program. In addition, the study demonstrates good reproductive results can be obtained from prepubertal goats produced by somatic cell nuclear transfer.

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