Zygote **12** (August), pp. 257–261. © 2004 Cambridge University Press
DOI:10.1017/S0967199404002849 Printed in the United Kingdom

Effect of GnRH injection timing in the production of pronuclear-stage zygotes used for DNA microinjection

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Date submitted: 08.04.04. Date accepted: 09.07.04

Summary

This study was aimed at developing a hormonal treatment protocol in order to optimize the proportion of pronuclear-stage embryos to be used for DNA microinjection in a goat transgenic founder production programme. A total of 46 adult BELE® and 47 adult standard goats (1–5 years old) were used as donors and recipients, respectively. They were heat-synchronized using intravaginal sponges containing 60 mg medroxyprogesterone acetate for 10 days with an injection of 125 μg cloprostenol on the morning of the eighth day. Recipients were injected with 400 IU eCG at the time of sponge removal while donors received a total of 133 mg NIH-FSH-P1 (Folltropin-V) given twice daily in decreasing doses over 3 days starting 48 h before sponge removal. Ovulation was induced in donors by injecting 100 μg of GnRH at 24 h (GnRH24) or 36 h (GnRH36) after sponge removal. Embryo recovery was performed by oviduct flushing following a standard mid-ventral laparotomy procedure. The proportion of embryos in the pronuclear stage of development was higher in the GnRH36 group (90% vs 34%, $p < 0.01$). Embryos were microinjected with a DNA expression cassette followed by transfer to the oviduct of synchronized recipients. A higher, yet not statistically significant, pregnancy rate was found in the recipients transferred with pronuclear-stage embryos compared with those transferred with 2-cell-stage embryos (64% vs 37%, chi-square $p = 0.06$). One transgenic female founder was produced from the group of recipients transferred with pronuclear-stage microinjected embryos.

Keywords: GnRH, Goat, Microinjection, Ovulation, Transgenics, Zygote

Introduction

The production of large transgenic animals has been extensively reviewed (Wall *et al*., 1992; Ebert & Schindler, 1993; Pursel & Rexroad, 1993; Wall, 1996; Ziomek, 1998; Neiman & Kues, 2000). The use of expression vectors that target the production of rc-proteins in the milk of transgenic animals is an alternative to microorganisms or animal cell production systems. The use of the BELE (Breed Early Lactate Early) goat system has been proposed as an alternative system for producing recombinant proteins in the milk of transgenic ruminants (Karatzas & Turner, 1997). This system utilizes Nigerian Dwarf goats (early sexual maturity, less seasonal and small in size) as donors and standard breeds of goats (Saanen, Toggenburg, Alpine, Nubians) as recipients. Although less efficient compared with somatic cell nuclear transfer (Schnieke *et al*., 1997; Cibelli *et al*., 1998; Baguisi *et al*., 1999; Keefer *et al*., 2001, 2002), DNA microinjection of zygotes remains a reliable and predictable method for the production of transgenic founder animals (reviewed by Neiman & Kues, 2000). In addition, pronuclear (PN) microinjection may be the preferred procedure for the production of transgenic animals with respect to commercial applications where intellectual property restrictions may apply for the use of NT technology.

Hormonal regimes to induce multiple ovulations in goats are established (reviewed by Cognie, 1999 and Cognie *et al*., 2003). However, using standard superovulation protocols the follicles often ovulate within a wide range of hours resulting in variable

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stages of development of the embryos collected (Ebert *et al*., 1991; Gootwine *et al*., 1997; Baldassarre *et al*., 1999; Echelard *et al*., 2000). It is also accepted that transgenesis rates are more favourable if microinjection is performed at the early pronuclear stage of development, i.e. 15–20 h after fertilization (reviewed by Wang & Yang 2002). Consequently, the ability to recover an evenly staged group of zygotes is very important for the overall success of a DNA microinjection programme. The purpose of this study was to improve the homogeneity in development of the embryos collected, by synchronizing ovulation using GnRH injections at two times following sponge removal.

Materials and methods

Animals

A total of 46 adult BELE goats (1–5 years old) were used as embryo donors in this study, while 47 goats of standard breeds (Nubian, Alpine, Saanen, Toggenberg) served as recipients for the microinjected embryos. The study was carried out between mid-February and mid-March (late breeding season for goats in Canada).

Heat synchronization and superovulation

The timing of oestrus was synchronized in donors and recipients with the placement of intravaginal sponges (Veramix, Upjohn, Orangeville, Ontario, Canada) containing 60 mg medroxyprogesterone acetate for 10 days and an injection of 125 μg cloprostenol (Estrumate, Shering-Canada, Pointe Claire, Quebec) on the morning of the eighth day. Recipients were injected with 400 IU eCG (Equinex, Ayerst, St Laurent, Quebec, Canada) at sponge removal while donors received a total equivalent to 133 mg NIH-FSH-P1 (Folltropin-V, Vetrepharm, London, Ontario, Canada) given twice daily in decreasing doses over 3 days starting 48 h prior to sponge removal.

GnRH treatment groups

Donor BELE goats were randomly assigned to one of the two GnRH treatment groups. They received 100 μg of GnRH (Factrel, Ayerst, Canada) in a single intramuscular injection given either 24 h (GnRH24) or 36 h (GnRH36) following sponge removal.

Heat detection and breeding

Heat was detected in donors and recipients using vasectomized bucks wearing marking harnesses and the time of the onset of oestrus was recorded. Donors were hand bred at 36 and 48 h after sponge removal using bucks of known fertility.

Anesthesia

Donors and recipients were deprived of food and water for 24 h prior to embryo recovery and transfer. Anesthesia was induced with intravenous administration of 0.35 mg/kg body weight of Valium (diazepam, Sabex, Quebec, Canada) and 5 mg/kg body weight of ketamine (Ketalean, Bimeda-MTC, Ontario, Canada), and maintained with isoflurane (Isoflo, Abbott, Montreal, Canada) via endotracheal intubation.

Chemicals and reagents

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

Embryo recovery

Embryo recovery was performed at 72 ± 2 h following sponge removal. Donor goats were explored by laparoscopy prior to recovery and were not subjected to surgery if they responded with fewer than three ovulations or if enough embryos had already been recovered for the number of recipients scheduled for that day. For embryo recovery a mid-ventral laparotomy was established and the reproductive tract was exteriorized. A 16 G gavage needle was threaded into the oviduct by way of the infundibulum and a 22 G Angiocath catheter (Becton Dickinson, Utah) was inserted in the ipsilateral uterine horn close to the uterotubal junction. The oviduct was then flushed retrogradely with 10 ml of medium (EmCare, ICP, New Zealand) supplemented with 1% fetal calf serum (FCS). The flushing medium was collected into a 50 ml sterile tube connected to the 16 G gavage needle at the oviduct end.

Embryo manipulation and microinjection

Following flushing, the medium containing the recovered embryos was observed under a stereomicroscope equipped with a 35 °C transparent warm plate, for the purpose of counting and separation of fertilized and non-fertilized eggs. Embryos harvested from all donors were pooled together and placed in 100 μl droplets of B_2 medium (Meditech, Canada) supplemented with 10% FCS and covered with mineral oil, and briefly cultured at 38.5 °C with 5% CO_2 and 95% air.

When flushing of all donors was completed, the embryos were placed in 1.5 ml centrifuge tubes filled with 400 μl EmCare containing 10% FCS, microcentrifuged at 15 600 *g* for 3–4 min and then placed into B_2 + 10% FCS droplets for culture prior to microinjection.

Microinjections were performed under Olympus Nomarski optics. Centrifuged zygotes and 2-cell (2C) stage embryos were manipulated in a microinjection chamber containing TCM199 with 25 mM HEPES + 10% FCS using a pair of Narishige micromanipulators. They were injected with a DNA construct consisting of the goat beta-casein promoter ligated to the sequence encoding human tissue plasminogen activator (htPA), in a 1–2 ng/ml concentration. Noticeable swelling of the nuclei was the criterion for successful microinjection.

Microinjected embryos were cultured for an additional 1–2 h in B_2 containing 10% FCS. Intact zygotes and 2-cell stage embryos were loaded into a Tomcat catheter (Sovereign, Kendall Company, Mansfield, MA) along with 10 μl EmCare + 1% FCS for embryo transfer.

Embryo transfer

Successfully microinjected embryos were transferred into the oviduct of oestrus-synchronized recipient goats following laparoscopic exploration in order to confirm the presence of at least one recent ovulation. For embryo transfer, a mid-ventral laparotomy was established and the reproductive tract was exteriorized. A Tomcat catheter (Sovereign, Canada) containing the embryo was introduced through the fimbria 2–3 cm into the oviduct and the embryos were injected into the lumen. Three to five embryos at the same stage of development were transferred per recipient depending on the embryo to recipient ratio of each day.

When possible, embryos at the same stage of development were transferred into the same recipient. Five recipients transferred with a mix of both PN and 2C staged embryos were not included for the purpose of statistical analysis.

Pregnancy detection

Initial pregnancy was detected by ultrasound at 28–35 days following transfer, using a SonoVet 600 scanner (Medison, Seoul, Korea) equipped with a transrectal 5 MHz linear array.

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 presence of the transgene in the isolated DNA was tested by polymerase chain reaction (PCR) using two 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, PQ, Canada) DIG system for detection.

Table 1 Effect of timing of GnRH injection following sponge removal on total number of ovulations (TOTOVL) and total number of ova recovered (TOTOVA)

Treatment	Superovulated	Flushed	TOTOVL (per donor)	TOTOVA (per donor)
GnRH24	22	17	220 (12.9)	191 (11.2)
GnRH36	24	16	198 (12.4)	159 (9.9)
Total	46	33	418 (12.7)	350 (10.6)

Values within the same column do not differ significantly ($p > 0.05$, *t*-test).

Table 2 Effect of the timing of GnRH injection on the stage of development of embryos recovered

Treatment	PN stage	2C stage	UFO	Total
GnRH24	47^a (24.6%)	90^c (47.1%)	54^e (28.3%)	191
GnRH36	113^b (71.1%)	12^d (7.5%)	34^e (21.4%)	159
Total	160	102	88	350

PN, pronuclear stage; 2C, 2-cell stage; UFO, unfertilized ova. Values within the same column with different superscripts differ significantly ($p < 0.01$, chi-square).

Statistical analysis

The program used for statistical analysis was GraphPad Prism (GraphPad Software, San Diego, CA). The data collected for total ovulations and total ova recovered were analysed using the unpaired Student's *t*-test with Welch's correction for unequal variances. The data for the developmental stage of embryos recovered and pregnancy rates were analysed using Pearson's chi-square test.

Animal welfare

Studies were conducted in conformance with guidelines of the Canadian Council Animal Care. All protocols were previously approved by the Institutional Committee of Animal Care (ACCN).

Results

No significant difference was found in the timing from sponge removal to the onset of oestrus between the two GnRH treatment groups (42 ± 8 vs 42 ± 7 h for GnRH24 and GnRH36, respectively). Of the 46 donors that were superovulated only 33 (17 of the GnRH24 and 16 of the GnRH36) were flushed. Of those, 4 goats did not ovulate (2 from each treatment group) and 9 animals were not flushed for logistic reasons.

The embryo recovery results are summarized in Tables 1 and 2. No difference was observed in the

Table 3 Pregnancy rates and number of kids born after embryo transfer of pronuclear (PN)-stage or 2-cell (2C)-stage embryos

Embryo stage	TET	Recipients transferred	Pregnant at 30 days (%)	Kids born	Transgenic
PN	103	28	18 (64%)	23	1
2C	66	19	7 (37%)	12	0
Total	169	47	25 (53%)	35	1

TET, total embryos transferred.
Values within the same column do not differ significantly ($p > 0.05$, chi-square).

average number of ovulations per donor (12.9 vs 12.4) and the average number of ova recovered per donor (11.2 vs 9.9) between the GnRH24 and GnRH36 treatment groups, respectively ($p > 0.05$) (Table 1).

No difference was observed in the percentage of fertilized ova (72% vs 79%) between the GnRH24 and GnRH36 groups, respectively ($p > 0.05$). However, timing of GnRH injection had a statistically significant effect on the stage of development of embryos recovered. The majority of PN-stage embryos were collected in the GnRH36 treatment group, whereas the GnRH24 treatment resulted in higher percentage of 2C embryos ($p > 0.01$) (Table 2).

The data from embryo transfer, initial pregnancy and kids born are summarized in Table 3. Recipients receiving PN-stage microinjected embryos had a higher pregnancy rate (64%) than those carrying 2C-stage embryos (37%); however, this difference was not statistically significant ($p = 0.06$).

Discussion

In the present study, we explored the use of GnRH for better controlling the timing of ovulation in superovulated goats. The results obtained in terms of total number of ovulations, ova recovered and fertilized embryos were comparable to those reported by others (Ebert *et al.*, 1991; Gootwine *et al.*, 1997; Echelard *et al.*, 2000).

The use of GnRH and timing of injection following sponge removal influenced the stage of development of embryos recovered, a key parameter for the success of a transgenic founder generation programme. Furthermore, working with BELE goats and the synchronization and superovulation regime described, the GnRH36 protocol provided almost 80% fertilized ova of which 90% were at the pronuclear stage of development. It has been previously suggested that successful integration of foreign DNA is more likely following microinjection of PN-stage embryos than when performed in 2C-stage embryos (reviewed by Wang & Yang, 2002).

In the dataset presented, the only transgenic animal produced resulted from the group microinjected at the PN stage, but the small number of embryos microinjected does not allow any firm conclusions to be drawn. The transgenic animal produced represents 4% of the kids born and 1% of the embryos microinjected and transferred within the PN-stage group.

The data indicate that PN-stage embryos have higher developmental competence following microinjection and transfer to recipients, but perhaps a larger number of transfers would have been necessary in order to show statistical significance. If confirmed, this difference in the viability following microinjection could be reflecting a difference in the fragility and/or the ability to heal of the nuclear envelope between the two different embryo stages. Additionally, since the onset of oestrus was approximately 42 ± 7 h following sponge removal in both treatment groups, the GnRH24 protocol may have promoted a premature ovulation of follicles resulting in lower developmental capacity of the microinjected embryos. The GnRH24 protocol may result in similar results of embryo development at flushing if embryo collection is advanced to 60 h after sponge removal, but this protocol may be more difficult to schedule.

Improvements in the efficiency in the production of transgenic goats by pronuclear microinjection have recently been proposed by our group (Baldassarre *et al.*, 2002, 2003). This improvement is based on DNA microinjection of zygotes produced after *in vitro* maturation and fertilization of immature oocytes recovered by laparoscopy from gonadotropin-treated donor goats. The method offers the possibility of recovering a rather large number of oocytes per donor (av. approximately 13 oocytes/donor), a precise control of stage of development at DNA microinjection, and utilizes a less traumatic surgical method (laparoscopy) which allows repeating the procedure at least twice as many times in the same donor compared with laparotomy-based zygote collection. However, given the high cost of laparoscopic equipment and the skill required for the procedure, it is foreseeable that microinjection of *in vivo* produced zygotes will remain a common method for transgenic goat production.

Acknowledgements

Authors are grateful to Janice Pierson for her contributions to the preparation of this manuscript. Special thanks go to the Caprine Production Farm team for taking great care of the animals and to the Molecular Biology team for DNA preparation and animal testing.

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