CHAPTER IV

THE EFFECT OF ACTIVATION PROTOCOLS ON THE DEVELOPMENT OF CLONED GOAT EMBRYOS

Introduction

Activation is an important step in the NT procedure and ethanol and ionomycin have been widely used as activating agents in several species, such as cattle (Presicce and Yang, 1994a, 1994b; Lui et al., 1998), mice (Hardy and Handyside, 1996), sheep (Loi et al., 1998), and goats (Baguisi et al., 1999; Chen et al., 2001; Keefer et al., 2002; Zou et al., 2002; Chesné et al., 2002). There are a few reports on the development of goat oocytes after parthenogenetic activation by treatment with ionomycin and ethanol, both followed by exposure to 6-diethylaminopurine (6-DMAP) (Ongeri et al., 2001; Ongeri and Krisher, 2001). Nevertheless, the comparison of the development of NT goat embryos with either ionomycin or ethanol, as an activating agent, has not been reported. The objective of this study was to compare the developmental competence *in vitro* and *in vivo* of NT goat embryos, after activation, by using either ionomycin or ethanol, both followed by incubation in 6-DMAP-CB. In a preliminary study, the effect of activation with CHX or ethanol on the *in vitro* development of NT goat embryos derived from *in vivo* and *in vitro* matured oocytes was tested (Publication 6 in APPENDIX E) to examine whether CHX or ethanol could activate goat cytoplasts as was in bovine species (CHAPTER III). I was found that the NT embryos did develop to the blastocyst stage after activation with ethanol whereas they did not do so with CHX (see Publication 6

in APPENDIX E). Consequently, ethanol was further used as activating agent, in this study, to determine the development of NT embryos *in vitro* and *in vivo*, compared with ionomycin.

Materials and methods

Chemicals and media

Unless otherwise indicated, all chemicals used in this study were obtained from Sigma-Aldrich Company (St. Louis, MO) and the media from Gibco Invitrogen Corporation (Grand Island, NY).

Preparation of donor cells

Donor cells were prepared from an ear skin fibroblast cell line taken from a 3-year-old Native female goat by using the protocol previously described for bovine (see Materials and methods in CHAPTER III). The culture medium was Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS and 1% penicillin-streptomycin. Primary cells were trypsinized with 0.05% trypsin-EDTA, frozen in 10% dimethyl sulfoxide in FCS and stored in liquid nitrogen. For the experiments, the cells were thawed and cultured for 3-4 d. When the cells had grown to 80-90% confluence, they were starved, by culturing in a medium supplemented with 0.5% FCS for 4-10 d. Before NT, the cells were harvested by trypsinization, resuspended in DMEM, supplemented with 10% FCS and immediately transferred into the recipient oocytes. Cells cultured between passages 3-8 were used for NT.

Preparation of recipient oocytes

The oocytes were recovered from superovulated goats, which had been raising at the Embryo Transfer Research Center, Paak Chong, Nakhonratchasima province. The oocyte donors were of various breeds including Saanen, Thai Native and Mixed Thai Native breeds, aged 9 months to 5 years, and weighed 20 to 50 kg. The gonadotropin treatment (Figure 7) was performed with a modified program of Reggio et al. (2001). The estrus cycle was synchronized with the insertion of a controlled internal drug release device (CIDR-G, 0.3 g progesterone, Eazi Breed, InterAg, New Zealand), which was removed on Day 7 (Day 1 = day of insertion). Ovarian superstimulation was obtained with FSH (Folltropin®-V, Vetrepharm Canada Inc., Ontario, Canada) by giving 6 decreasing doses (50-50, 30-30 and 20-20 mg NIH-FSH-P1, i.m.), one injection every 12 h, starting on Day 5 of CIDR-G insertion (total dose equivalent to 200 mg NIH-FSH-P1). Oocyte collection was performed 24 h after the final FSH injection (Reggio et al., 2001). Ovaries were exteriorized and the oocytes were aspirated from follicles with a diameter ≥ 2 mm, by using a 21-gauge needle attached to silicone tubing that was connected to an electric vacuum pump (Cook Veterinary Products, Eight Mile Plains, Australia) (Figure 8B). COCs having at least 2 layers of cumulus cells (Figure 9C) were selected and matured in maturation medium for 22-23 h, at 38.5 °C, in a humidified air atmosphere with 5% CO_2. The maturation medium consisted of M199 supplemented with 10 % FCS, 10 μg/ml FSH, 10 μg/ml pLH, 1 μg/ml 17-β estradiol, 10 mM β-mercaptoethanol (Songsasen and Apimeteetumrong, 2002), 0.55 mg/ml pyruvate and 50 IU/ml penicillin-streptomycin. After maturation, the cumulus cells were removed by culturing the COCs for 3 min in M199-Hepes containing 0.5 mg/ml hyaluronidase and pipetting for 1 min. Cumulus cell-free oocytes were then cultured in M199,

supplemented with 10 % FCS and 0.5 μg/ml Hoechst 33342, for 20-30 min at 38.5°C in a humidified air atmosphere with 5 % CO_2 before enucleation. Oocyte enucleation was performed in M199-Hepes supplemented 10 % FCS, 5 μg/ml CB and 0.5 μg/ml Hoechst 33342 overlaid with mineral oil on the stage of an inverted microscope equipped with Narishige micromanipulators and epifluorescent illumination. The metaphase plate and the first polar body were removed with a glass enucleation pipette (20 μm outside diameter). Confirmation of successful enucleation was achieved by visualizing the metaphase plate under ultraviolet light (Figure 9F).

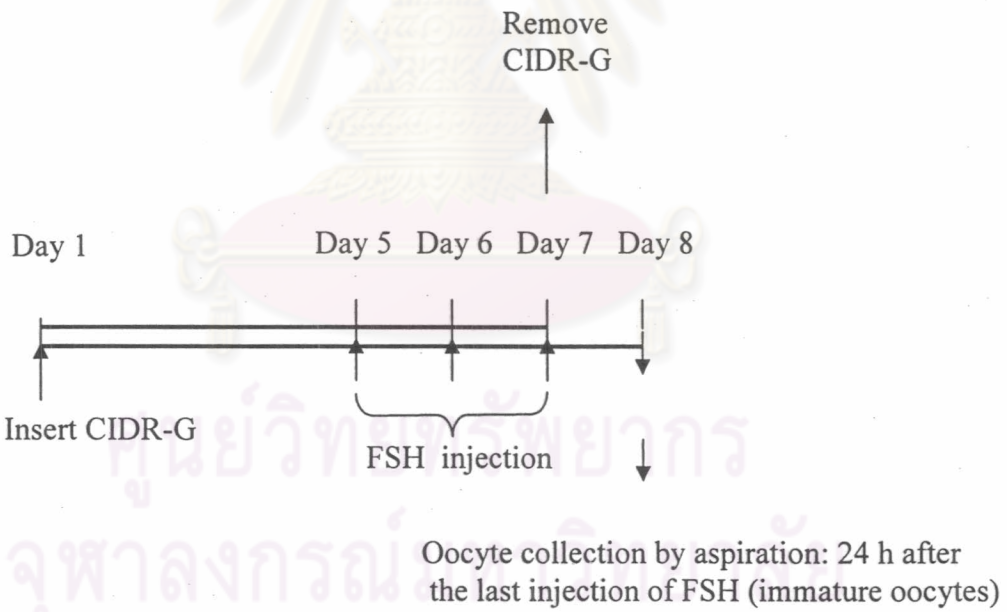

Figure 7. Protocol of gonadotropin treatment for ovarian superstimulation in donor goat

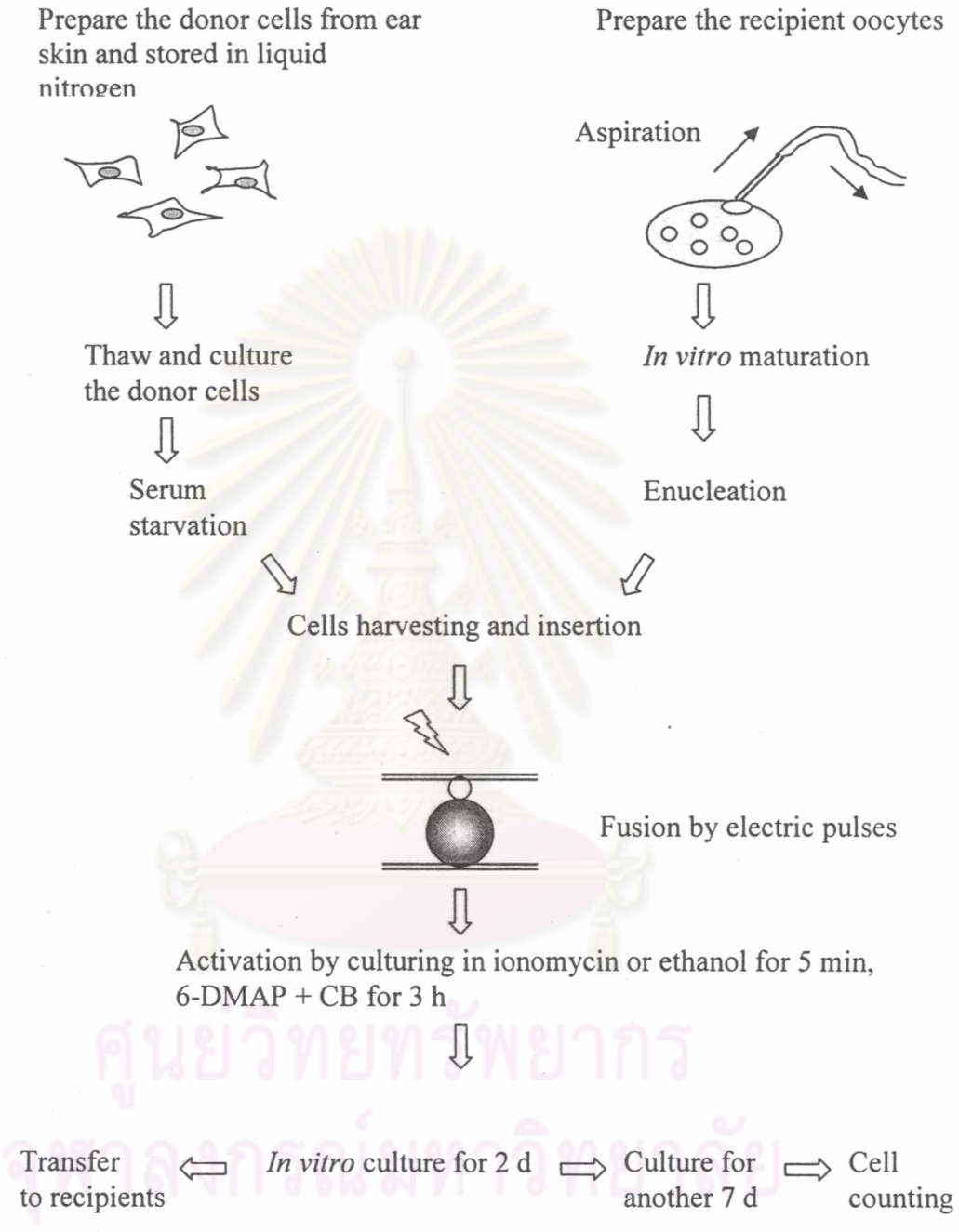

Figure 8. Nuclear transfer procedure used to produce cloned goat embryos

Somatic cell nuclear transfer and activation

The enucleated oocytes were fused with donor cells by applying 2 DC pulses of 2.0 kV/cm for 50 μsec (Chesne et al., 2002). Two hours after fusion, the reconstructed embryos were activated, either by exposure to 5μM ionomycin for 5 min at 38.5 °C, in M 199 supplemented with 10% FCS or to 7% ethanol for 5 min at 38.5 °C in M 199, supplemented with 10% FCS and followed by culturing in 2 mM 6-DMAP and 5 μg/ml CB for 3 h (Susko-Parris et al., 1994; Chesne et al., 2002). Fusion was observed at the end of the activation period. The fused embryos were then cultured in B_2 medium (CCD, Paris, France), supplemented with 7% FCS and Vero cells, for 9 d at 38.5°C in a humidified air atmosphere with 5% CO_2. The cleavage and development of NT embryos were observed on Day 2 and Days 7-9 (Day 0 = day of fusion). At the end of the culture period, the NT embryos were fixed and stained with Hoechst 33342 by the method of Begin et al. (2003). The numbers of nuclei were counted under ultraviolet light. The embryo, on Day 9 of culture, which had greater than 16 nuclei but less than 32 nuclei, was classified as morula (Koeman et al., 2003). The embryo, on Day 9 of culture, which had greater than 32 nuclei, was classified as blastocyst (Koeman et al., 2003). NT procedure used to produce goat embryos is shown in Figure 8.

Parthenogenetic activation of oocytes

Parthenogenetic activation of oocytes was served as a control group, for the development of NT embryos in the same way as was in the NT group. Denuded matured oocytes were exposed to 0.5 μg/ml Hoechst 33342, for 20-30 min at 38.5°C in a humidified air atmosphere with 5 % CO_2 and then observed for signs of MII and the first polar body, under ultraviolet light. Electric stimulation was done with the

same technique as described above for NT embryos. Stimulated oocytes were then cultured for 2 h in M199, supplemented with 10 % FCS. After culture, they were activated, with either ionomycin or ethanol, followed by culturing in 6-DMAP plus CB, as described above and then further cultured under the same conditions as the NT embryos. Activated oocytes were observed for cleavage and development into the morula and blastocyst stages. At the end of the culture period, the numbers of nuclei in the parthenogenetic embryos were counted under ultraviolet light.

Preparation of recipient animals and embryo transfer

The recipient goats were of Thai Native and Mixed Thai Native breeds, aged 1 to 3 years, and weighed 20 to 40 kg, which had been raising at the Embryo Transfer Research Center, Paak Chong, Nakhonratchasima province. The recipient goats were synchronized by the same protocol as used for the oocyte donors, but not given FSH. An injection of 175 μg of $PGF_{2\alpha}$ (Estrumate®, Schering-Plough Animal Health, NSW, Australia) was given 36 h before CIDR-G removal. Estrous was detected by using vasectomized male goats 24-48 h after CIDR-G removal. On Day 2 (Day 0 = day of fusion), NT embryos at the 2-4 cell stage from each activation treatment were transferred into 10 recipients (6-12 embryos/recipient) on Day 2 of their cycle (Day 0 = estrus). Ultrasonography scanning was performed on all recipients on Days 30, 45 and 60 of gestation for pregnancy examination and fetal development. The genomic DNA was compared between cultured skin fibroblasts, blood samples collected from the donor cells and recipient animal and fetus tissue (taken out at the end of the gestation period) using DNA microsatllite analysis (DNA Technology Laboratory, BIOTEC).

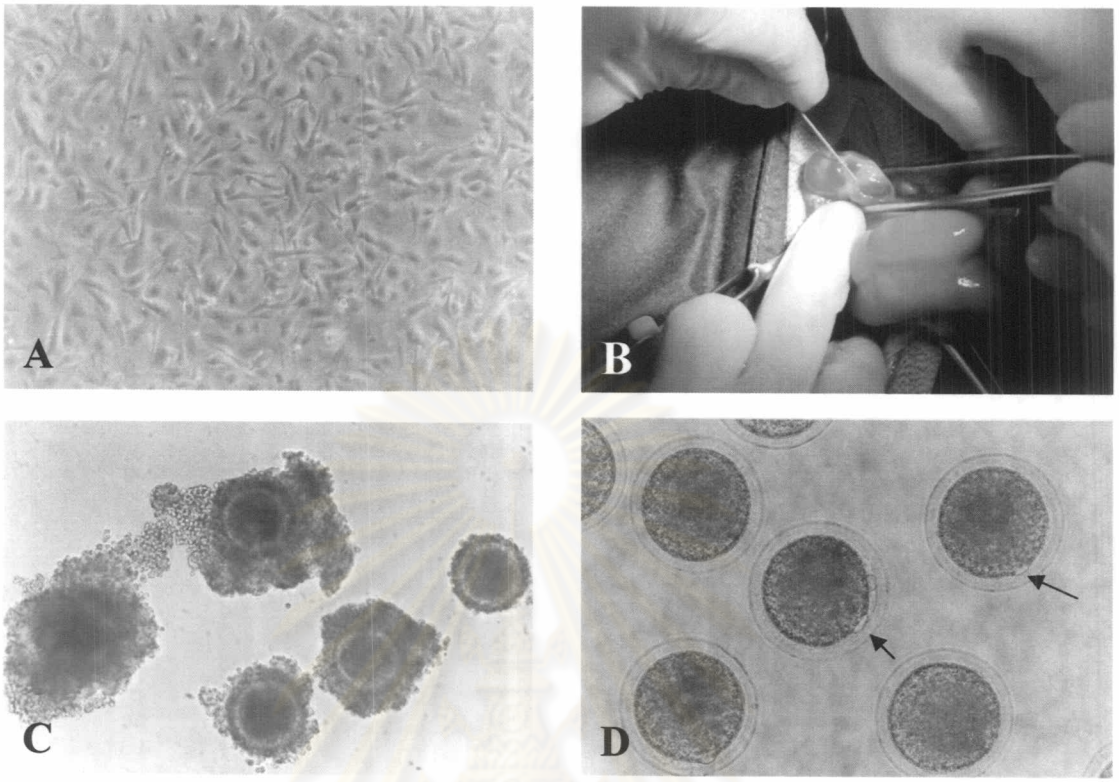

Figure 9. Representations of the somatic NT procedure with ear skin fibroblasts in goats

A. Fibroblast cells at passage 5 cultured in 60-min dish for 4 d (X100).

B. Oocyte collection by direct aspiration from stimulated ovary

C. Immature oocytes (cumulus-oocyte complexes) collected from a donor goat (X80)

D. A higher magnification, *in vitro* matured oocytes after removal of cumulus cells. Arrows indicate the location of metaphase plate (X150).

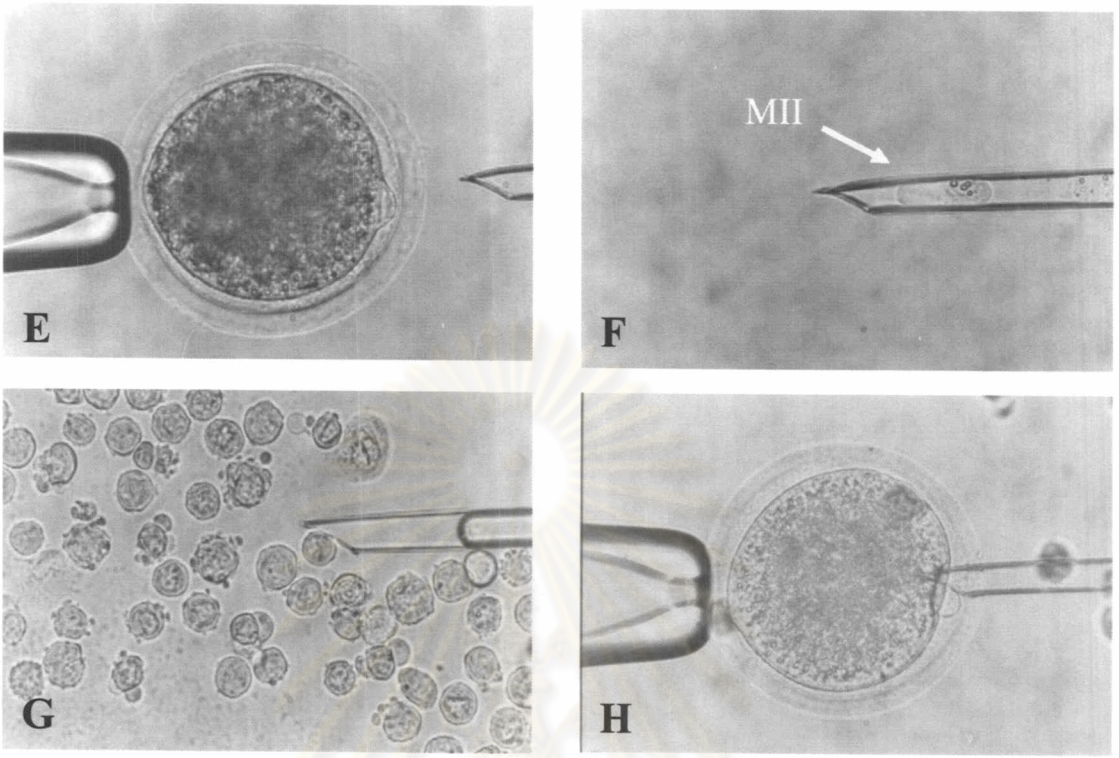

Figure 9. Representations of the somatic NT procedure with ear skin fibroblasts in goats (Continued)

E. Position of oocyte during enucleation. Note a matured oocyte is being held with a holding pipette and the clear zone is located near an enucleation pipette (X200).

F. Metaphase plate (MII) was aspirated with an enucleation pipette. The MII exhibiting blue fluorescence of Hoechst 33342 is visualized under ultraviolet light (X200). Arrow indicates the metaphase plate.

G. Suspension of donor cells after trypsinization (X200)

H. A donor cell is being inserted into perivitelline space of an enucleated oocyte (X200).

Statistical analysis

Fusion, cleavage rates and development into morula and blastocyst stages in both groups of NT embryos, as well as the parthenogenetic embryos were compared by means of Fisher's exact test and SAS software. Pregnancy rates were also compared with Fisher's exact test and SAS software. Differences were considered significant at $P < 0.05$.

Results

The effect of activation protocols on developmental potential of NT embryos and parthenogenetic embryos *in vitro*

The rate of fusion of NT embryos was not significantly different (Table 6) in the ionomycin and ethanol treatment groups (86.3% and 82.9%, respectively). The rate of development to the morula and blastocyst stages of NT embryos (Figure 10B) showed no significant differences between the ionomycin and ethanol treatment groups (9.5% and 5.9%, respectively). There were no significant differences in the cleavage rates of NT embryos and parthenogenetic embryos derived from both activation treatments but the development rate to the morula or blastocyst stages of NT embryos, derived from both treatments, was significantly lower ($P < 0.01$) than that of the parthenogenetic embryos (Table 6, Figure 10D). Cell numbers in blastocysts produced from both groups fixed at Day 9 after NT or activation were lower than 100 (Table 7).

The effect of activation protocols on developmental potential of NT embryos *in vivo*

A total of 46 NT embryos at the 2-4 cell stage derived from the ionomycin treatment group (Figure 10A) were transferred into five recipients (Table 8) with each

recipient receiving, on average, 9.0 ± 1.0 (mean ± SEM) embryos. A total of 37 NT embryos at the 2-4 cell stage, derived from the ethanol group, were transferred into five recipients (Table 8), with each recipient receiving, on average, 7.4 ± 0.4 (mean ± SEM) embryos. Because of the small number of recipients receiving NT embryos derived from the ionomycin and ethanol treatment groups, there were no significant differences in the rates of pregnancy at Day 30 (60%, 3/5 and 20%, 1/5, respectively). Pregnancy status was observed in all three recipients from the ionomycin group through to Day 60 (Figure 11B) but it was not observed after Day 45 in the recipient from the ethanol group. One mummified fetus (Figure 11C; Crown-Rump Length, CRL = 5 inches) connected with cotyledons (number of cotyledons = 54) was taken out of a recipient in the ionomycin group on Day 145 of gestation. Analysis of DNA of this fetus showed it to be genetically identical to the cultured skin fibroblasts and blood sample from the donor cell goat (Figure 11).

Table 6. Effect of activation protocols on the developmental potential of NT embryos and parthenotes *in vitro*

Activation protocols	Groups	No. of activation /reconstructed	No. of fused (%)	No. of [c] cleaved (%)	No. of [c] (%) Morula/Blastocyst
Ionomycin[a]	NT	73	63 (86.3)	57 (90.5)	6 (9.5)[d]
	Parthenotes	26	-	22 (84.6)	11 (42.3)[e]
Ethanol[b]	NT	82	68 (82.9)	56 (82.4)	4 (5.9)[d]
	Parthenotes	12	-	11 (91.2)	4 (33.3)[e]

NT experiments were replicated 6 times in both activation protocols.

Parthenogenetic activations were replicated twice.

[a] Five micromole ionomycin followed by incubation in 2 mM 6-dimethylaminopurine + 5 μg/ml cytochalasin B for 3 h

[b] Seven percent (v/v) ethanol followed by incubation in 2 mM 6-dimethylaminopurine + 5 μg/ml cytochalasin B for 3 h

[c] Percentage based on number of embryos fused/cultured

[d, e] Values with different superscripts in the same column differ significantly ($P < 0.01$)

Table 7. Cell numbers of morulae/blastocysts at Day 9 after nuclear transfer or parthenogenetic activation

Activation protocols	Groups	No. of embryos examined	Cell number	*
			Morula	Blastocyst
Ionomycin	NT	6	22, 32, 30, 16	89, 61
	Parthenotes	11	32, 25	64, 66, 52, 74, 70, 56, 121, 69, 90
Ethanol	NT	4	32, 22	40, 35
	Parthenotes	4	20, 32	70, 50

* Due to a limited numbers of embryos were obtained, statistical analysis could not be performed.

Table 8. Effect of activation protocols on the developmental potential of NT embryos *in vivo*

Activation protocols	No.of cultured	(%) No.of (%) [c] 2-4 cell stage	No.of transferred	No.of recipients	No.of pregnant (%) Day 30	Day 45	Day 60	No.of kids
Ionomycin [a]	68	56 (82.3)	45	5	3 (60)	3 (60)	3 (60)	1[d]
Ethanol [b]	47	37 (80.9)	37	5	1 (20)	1 (20)	0	0

[a] Five micromole ionomycin followed by incubation in 2 mM 6-dimethylaminopurine + 5 µg/ ml cytochasin B for 3 h

[b] Seven percent (v/v) ethanol followed by incubation in2 mM 6-dimethylaminopurine + 5 µg/ ml cytochasin B for 3 h

[c] Percentage based on number of embryos cultured

[d] Mummified fetus

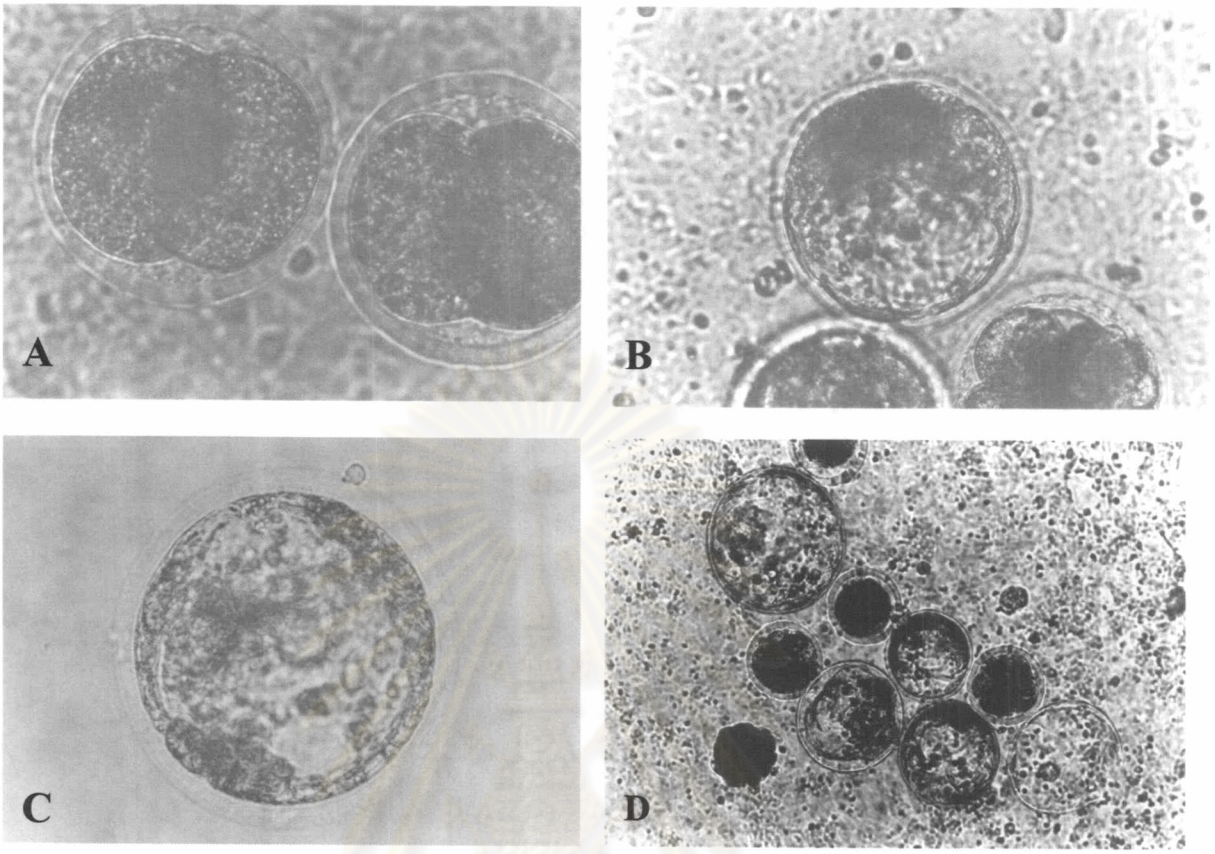

Figure 10. Goat embryos produced by NT or by parthenogenetic activation of oocytes

A. Two-cell NT embryos 2 d post fusion produced by NT, using *in vitro* matured oocytes as recipients, activated with ionomycin (X300)
B. Blastocyst 8 d post fusion produced by NT using *in vitro* matured oocytes as recipients, activated with ionomycin (X200)
C. Blastocysts 8 d post fusion produced by NT, using *in vitro* matured oocytes activated with ethanol (X300)
D. Blastocysts 8 d post fusion produced by parthenogenetic activation of *in vitro* matured oocytes activated with ionomycin (X80)

Vero cells used for co-culture are seen at the bottom of the photographs.

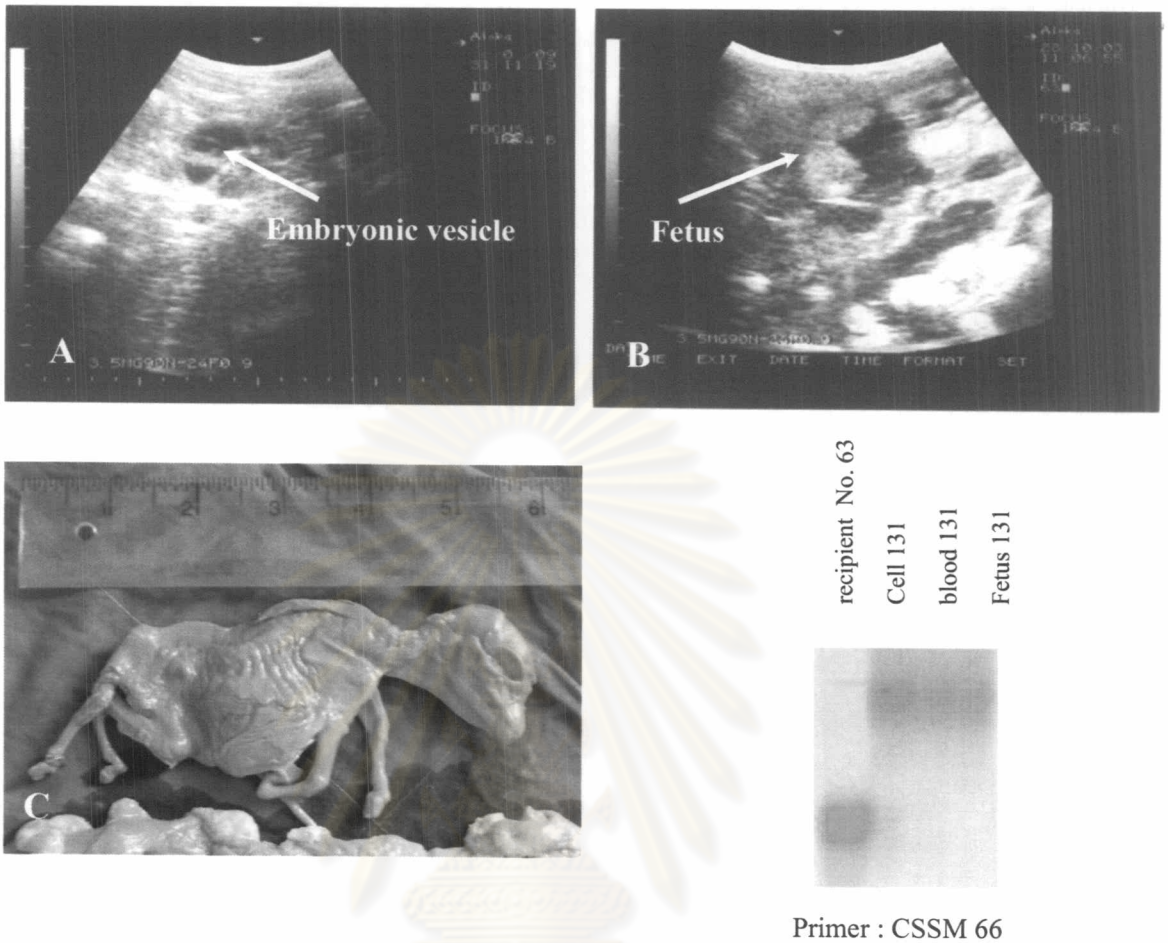

Figure 11. Development of cloned goat embryos after transfer into the recipient

A, B. Pregnancy was detected by ultrasound scanning at Days 27 (A) and 60 (B) of gestation. Note that eight NT embryos at 2-to 4-cell stage derived from ionomycin treatment were transferred into a recipient.

C. Mummified fetus, with 5 inch-crown-rump length and 54 cotyledons, was surgically recovered at Day 145 of gestation. DNA analysis confirms that the fetus is cloned.

Discussion

In this study, the NT embryos derived from the two different activation protocols appear to have a similar developmental competence *in vitro*. Fusion rates achieved from both activation protocols (86.3% and 82.9% in the ionomycin and ethanol groups, respectively) were similar to that previously reported in sheep (Wilmut et al., 1997) but higher than that reported by Reggio et al. (2001) in goats (85% versus 63%, respectively), with ionomycin as the activating agent. There were no significant differences in the fusion and cleavage rates between the two different protocols in our study. This suggests that the activation protocols had no effect on the fusion and cleavage rates of NT goat embryos.

The morula/blastocyst rate was low in both treatment groups (in 9.5% ionomycin and 5.9% ethanol groups) under our conditions, and the rate was much lower than that (34.4%) previously reported by Zou et al. (2001), when used in in-vivo matured cytoplasts as recipients and NT embryos cultured *in vivo* for 6 d. In the case of the ionomycin group, the rate was also much lower than that previously reported by Chesné et al. (2002), who used *in vivo* matured oocytes as recipient cytoplasts. This may be due to the *in vivo* conditions offering a better environment than the *in vitro*. In our study, there was no significant difference in the development rate to the morula/blastocyst stages of parthenogenetic embryos, derived from both activation treatments, which corresponds to a recent report (Ongeri et al., 2001). Our results were different from that reported by Loi et al. (1998) for parthenogenetic sheep embryos. Those authors showed a highly significant difference in the development of the blastocysts derived from ionomycin treatment, after culturing in uteri for 7 d, when compared with ethanol treatment (58.4% versus 19.1%, respectively).

Higher development rates were achieved in parthenogenetic embryos when compared to NT embryos in the present study. This may be due to matured oocytes being treated by combined activation, electric stimuli and ionomycin or ethanol as was done in these experiments. It has been demonstrated that the combined treatment with electric and chemical stimulation yields the highest rate of blastocyst formation (Hyun et al., 2003). In our results, it should be noted that mean cell numbers per blastocyst in both NT and parthenogenetic embryos was lower than 100 (Table 7), which may indicate delayed and/or abnormal development (Booth et al., 2003).

Ethanol has been used in cattle to effectively induce oocyte activation (Presicce and Yang, 1994a, 1994b; Zakhartchenko et al., 1999) as well as in rabbits (Liu et al., 2002). Presicce and Yang (1994) demonstrated that a combined ethanol and cycloheximide (a protein synthesis inhibitor) treatment would induce activation of both young (20 h) and aging (40 h) IVM bovine oocytes at very high frequencies (90-100%). In addition, ethanol can induce a single Ca^{2+} rise, which results both from extracellular entry and from mobilization of intracellular stores (Loi et al., 1998). On the other hand, ionomycin exclusively mobilizes intracellular calcium stores when used as an activating agent (Loi et al., 1998). In the present study, we exposed the reconstructed embryos from each group to each activating agent for the same period, followed by 6-DMAP treatment. Similar development rates *in vitro* were achieved by both activation treatments. Therefore, from our results, either ionomycin or ethanol can activate goat oocytes.

The culture system may also contribute to the low development of embryos. The efficiency of the culture system may be expressed in terms of the percentage of embryos that cleaved and reached more than the 8-cell stage (Yadav et al., 1998).

In addition, the culture of preimplantation mouse embryos in the presence of serum, influences the regulation of growth-related imprinted genes, leading to aberrant growth and behavior (Fernandez-Gonzalez et al., 2003). It has been demonstrated that *in vitro* development to the morula-blastocyst stage was higher when NT goat embryos were cultured at low oxygen tension, with a defined medium (36.3%), than in co-culture with Vero cells (27.5%) (Chesné et al., 2002). The optimal culture system for developing goat embryos needs to be further investigated. In the present study, NT embryos were transferred at early cleavage stages to avoid long-term culture *in vitro*. The avoidance of long-term culture may have alleviated some of the detrimental effects of *in vitro* culture (Keefer et al., 2001). In sheep and cattle, it was observed that there was an increased incidence of large offspring, after the transfer of embryos cultured in the presence of serum, indicating the possibility of detrimental effects of *in vitro* culture on fetal development (Young et al., 1998).

A 60% (3/5) pregnancy rate at Day 30 was achieved after embryo transfer of NT embryos derived from the ionomycin treatment. This pregnancy rate, based on the number of pregnant recipients, per total number of recipients, was slightly higher than that previously reported by others (Keefer et al., 2001; Reggio et al., 2001). Similar to the findings of earlier studies (Keefer et al., 2001; 2002), prenatal loss after Day 30 ultrasound scan was not observed in the ionomycin treatment group in the present study. A 20% (1/5) pregnancy rate at Day 30 was achieved after embryo transfer of NT embryos, derived from the ethanol treatment, which was lower than that reported (55.5%) by Baguisi et al. (1999) but those authors lost all thereafter. This is similar to the results of our study, in which recipient received embryos derived from the ethanol group exhibited vesicle formation and were resorbed after Day 45 of gestation.

As for the ionomycin group, three of them were found pregnant at term. From the final diagnosis, it was found that two recipients showed signs of pseudopregnancy, with multiple vesicles in their uteri, while the other one carried a mummified fetus with a crown rump length of 5 inches and 54 cotyledons (Figure 11C). The cause of these abnormalities may be due to incomplete reprogrammation of the donor nuclei and consequent low quality cloned embryos, as resulted in a low number of nuclei, under our conditions. Koo et al. (2002) suggested that early fetal losses or abnormalities might be due to aberrant allocations of NT embryos to the ICM and TE cells during early development. The total cell number, as well as the number of TE cells, of NT embryos, was significantly lower than that of either in vivo-derived or IVF embryos in cattle. They also suggested that the NT embryos having a reduced number of TE cells at the preimplantation stage, or incomplete blastocyst formation, might form an abnormal placenta, leading to developmental failures and later fetal loss. In the present study, we transferred NT embryos into recipients as early as possible to avoid undesired factors affecting the developmental potential from *in vitro* culture. The pregnancy could not be maintained to term when transferring NT embryos derived from ethanol treatment. This result strongly confirms the report of Baguisi et al. (1999).

In conclusion, our results demonstrate that somatic nuclei, obtained from locally bred goats, could be activated by ionomycin or ethanol treatment in combination with 6-DMAP plus CB treatment. Although no live offspring could be produced, embryonic implantation occurred in the recipients in our study. More experiments need to be performed to confirm the results. Further studies need to investigate the relative contributions of the type of somatic nuclei and the

remodeling/reprogramming capabilities of recipient oocytes, as well as appropriate culture systems for the development of NT goat embryos.