CHAPTER V

REPEATED OOCYTE COLLECTION AND ITS INFLUENCE ON PREGNANCY IN DONOR GOATS

Introduction

Surgical collection has been widely used to obtain either *in vivo* matured or immature oocytes for *in vitro* fertilization or nuclear transfer procedures in goats and sheep (Earl et al., 1995; Ptak et al., 1999; Baguisi et al., 1999; Graff et al., 2000; Reggio et al., 2001; Zou et al., 2001; Chesné et al., 2002). It has been shown that high recovery rates (> 80%) can be achieved from the surgical method (Earl et al., 1995; Baldassarre et al., 1996). Nevertheless, to obtain a high number of oocytes from genetically valuable animals, it is necessary to repeat ovum pick-up (OPU) several times (Stangl et al., 1999). However, the oocyte collection or OPU by laparotomy can be repeatedly performed with a limited frequency, due to the occurrence of adhesions of the internal organs to oviduct or uterus. This will make the technology expensive and the animal have to go to slaughter afterwards. All oocytes used for NT study in this dissertation (see CHAPTER IV) were obtained by means of surgical collection. Therefore, this part was done to investigate the post-effect of surgical collection of oocytes in the donor goats. The pregnancy rates were determined after finishing all experiments in CHAPTER IV. Retrospective data, obtained during 2002-2004, were analyzed. The results would provide the information on whether the repeated surgical collection had a detrimental effect on the pregnancy of the oocyte donors, after natural mating with the fertile males.

Materials and methods

Chemicals and media

See Materials and methods in CHAPTER IV for details.

Animals

As from the retrospective data, a total of 53 does were used as oocyte donors for NT experimental program. The donors were of various breeds including Saanen, Thai Native and Mixed Thai Native breeds, 9 months to 5 years of age, which weighed ranging from 20 to 50 kg. All animals were housed indoors throughout the experiment and fed good quality hey.

Treatment of animals

In order to obtain high numbers of oocytes per collection, the estrus of donor goats were synchronized and stimulated with gonadotropins (see Materials and methods in CHAPTER IV). Estrous 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 8 (Day 1 = Day of insertion). Ovarian superstimulation was obtained with FSH (Folltropin®-V, Vetrepharm, Ontario, Canada) given in 6 decreasing doses (50-50, 30-30 and 20-20 mg NIH-FSH-P1, i.m.), every 12 h, starting on Day 5 of CIDR-G insertion (total dose equivalent to 200 mg NIH-FSH-P1) (Menchaca at al., 2002). Estrus was observed 24-48 h after CIDR-G removal. All animals were deprived of food and water for 24 h prior to oocyte collection. The donor goats were anesthetized using a standard protocol (APPENDIX D) before oocyte collection.

Oocyte collection

To obtain the immature oocytes, oocyte collection was performed 24 h after the last injection of FSH by aspiration of follicles of $\geq$ 2 mm diameter with a 21-gauge needle that was attached to silicone tubing connected to a 50 ml conical tube that was maintained in a heating block (V-FTH-2012, Cook, Australia) at 37° C (CHAPTER IV). Negative pressure (-60 mmHg) was applied, during aspiration, using a vacuum pump (V-MAR-5100, Cook, Australia). Aspiration medium was TCM199-Hepes supplemented with 2% FCS, 50 IU/ml penicillin-streptomycin and 50 IU/ml heparin (Pharmacia & Upjohn, USA). The recovered follicular fluid was dispensed directly into 60-mm sterilized plastic dishes. Oocytes were identified under a stereomicroscope. The number of COCs having at least 2 layers of cumulus cells was recorded. To obtain the matured oocytes, oocyte recovery was performed 40-56 h after standing heat. The oviducts of donors were flushed *in situ* retrograde with 20 ml of flushing medium. The flushing medium was DPBS supplemented with 1% calf serum, 100 IU/ml penicillin, 0.1mg/ml streptomycin and 50 IU/ml heparin. The recovered oocytes were evaluated under a stereomicroscope. The procedure was repeated one to three times in the same donors when they were in a good condition. At the end of the experiment (three months after the last collection), donors were kept with the fertile bucks for 8 weeks, in order to examine their fertility.

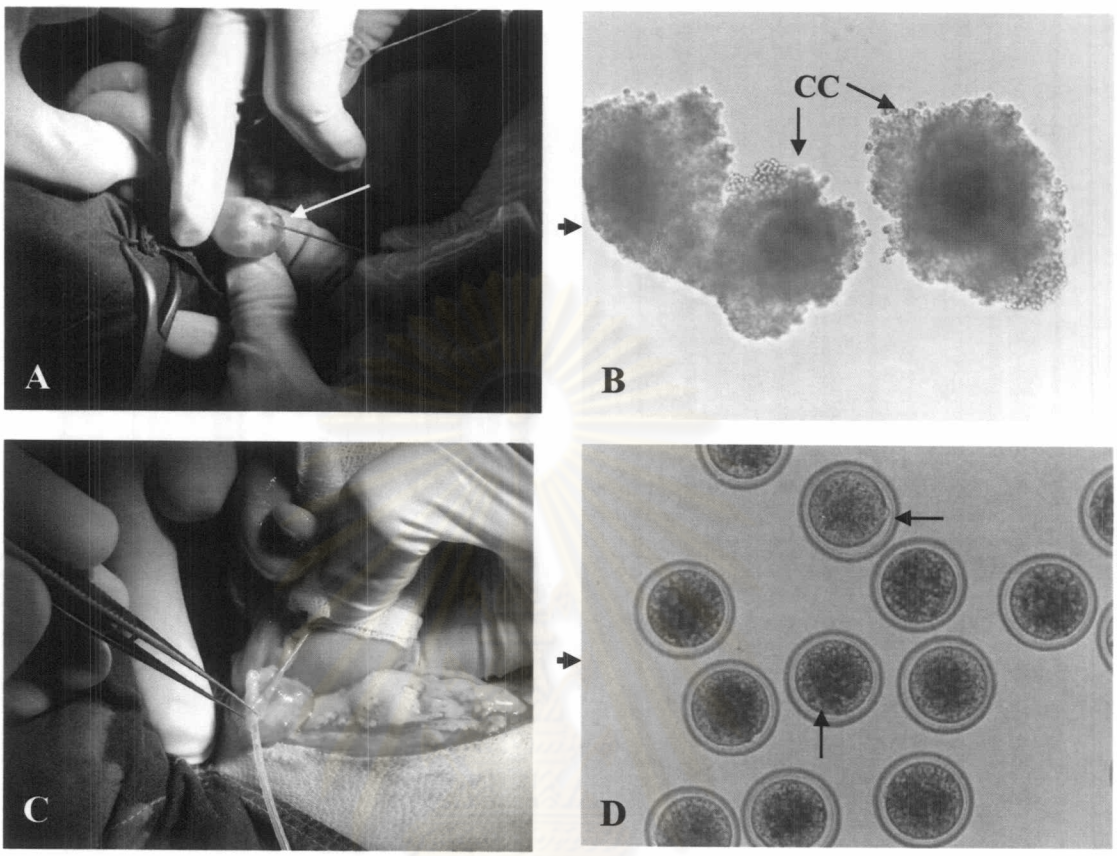

Figure 12. Oocyte collection by aspiration and flushing

A. Oocyte collection by aspiration of follicles exhibiting on the surface of the ovary. Arrow indicates aspiration needle.
B. Cumulus-oocyte complexes recovered from aspirant (immature oocytes). CC: cumulus cells
C. Oocyte collection by oviduct flushing
D. Matured oocytes recovered from flushing medium after collection. Arrows indicate the location of metaphase plate.

Statistical analysis

In order to determine if the repetition of oocyte collection had a detrimental effect on follicular response or oocyte recovery, the results obtained from the first collection were compared to that obtained in the later collection using Student's *t*-test. Numbers of aspirated follicles and recovered oocytes are presented as mean ± SEM. Pregnancy rates were compared by Chi-square test. Differences were considered significant at $P < 0.05$.

Results

Oocyte collection

A total of 89 OPU sessions were performed on 53 does (Table 9). For the immature oocyte collection group, a total of 1,476 follicles were aspirated from 50 does in 69 sessions (21.4 ± 1.5 follicles aspirated per donor/session) and 1,242 oocytes were recovered (18.0 ± 1.4 oocytes per donor/session) resulting in an average recovery rate of 84.1%. For the matured oocytes collection group, a total of 398 corpora lutea (CL) were observed in 19 donors with 20 sessions (19.9 ± 2.4 CL observed per donor/session) and 333 oocytes were recovered (16.7 ± 2.1 oocytes per donor) resulting in an average recovery rate of 83.7%. Large variations in follicle/CL numbers were observed among the donors subjected to the OPU procedure, which may be partially attributed to the age, breed and number of parturitions of the animals used. The animals of the Saanen and some of Mixed Native breeds were adult (3-5 years of age) that used to give birth while the goats of Native and some of Mixed breeds were young (9 months - 2 years old of age) and they never delivered. This study, the effect of age and breed, as well as number of deliveries on follicles

aspirated/CL could not be ruled out and, therefore, statistical analysis was not conducted.

The effect of repeated aspiration on the number of oocytes recovered

The effect of repetition of the OPU procedure on follicles aspirated and oocytes recovered as well as COCs obtained was analyzed in 8-Mixed Native goats that were subjected to OPU two consecutive times. The intervals during the first and second aspirations in these animals were 2-4 months, depending on the conditions of the former incision and collection program. No significant differences in the number of follicles aspirated and oocytes as well as COCs obtained were found between the collections. The number of follicles aspirated, oocytes recovered and COCs obtained from these animals are shown in Table 10. The recovery rates were also similar between two collections (Table 10).

The effect of repeated collection on pregnancy

Thirty-two out of 53 does were naturally mated with fertile males. After 8 weeks with fertile males, 27 does became pregnant, resulting in overall pregnancy rate of 84.4%, 14 from once operation, 8 from twice and 8 from triplet operations, regardless of the breeds and the collection procedures. There were no significant differences on pregnancy rates between three groups (Table 11).

Table 9. Immature and matured oocyte recoveries from multiple follicle stimulated goats

Type of oocytes	No. of sessions (*n*)	Follicles aspirated/ CL (mean ± SEM per goat/session)	Oocytes recovered (mean ± SEM per goat/session)	Recovery rate (%)
Immature oocytes	69 (50)	1,476 (21.4 ± 1.5) range 5-64	1,242 (18.0 ± 1.4) range 5-64	84.1
Matured oocytes	20 (19)	398 (19.9 ± 2.4) range 5-43	333 (16.7 ± 2.1) range 5-34	83.7

n: number of goats

SEM: standard error of the mean

CL: corpora lutea

Table 10. Follicles aspirated and oocytes recovered from a group of 8-Mixed Native goats subjected to the two consecutive collection sessions at 2-4 month-intervals

Collection session	Total follicles aspirated (mean ± SEM per goat)	Total oocytes recovered (mean ± SEM per goat)	Total COCs recovered (mean ± SEM per goat)	Recovery rate (%)
1	168 (21.0 ± 5.0)	146 (18.3 ± 4.4)	125 (15.6 ± 3.4)	86.9
2	203 (25.4 ± 6.9)	176 (22.0 ± 6.7)	149 (18.6 ± 6.4)	86.7

COCs: Cumulus-oocyte complexes

SEM: standard error of the mean

Table 11. Pregnancy rates of donor goats subjected to natural mate with fertile males, regardless of the breeds and collection procedures

No. of operations	No. of does mated	No. of does pregnant (%)
1	16	14 (87.5)
2	8	7 (87.5)
3	8	6 (75.0)
Total	32	27 (84.4)

Pregnancy was determined by ultrasound scanning during 30-40 d after the does were taken out of the males.

Discussion

In this study, the immature and matured oocytes were recovered from FSH-stimulated goats by laparotomy. High recovery rates (> 83%) were obtained from the two collection methods. Repeated OPU in the two consecutive sessions, in the same donors, had no significant influence on the numbers of follicles, oocytes with good quality (COCs) as well as the collection efficiencies (Table 10). Regardless the OPU methods, the donor goats can be repeatedly operated up to 3 times without a detrimental effect on their pregnancy rates.

Overall, follicles aspirated and oocytes recovered by aspiration were slightly higher than those reported previously (Graff et al., 1999; Keefer et al., 2001; Baldassarre et al., 2003; Koeman et al., 2003) when using laparoscopic OPU to collect the oocytes. This may be due to the laparotomic procedure, which allows a direct visualization of the ovaries, associated with of the follicles, during aspiration. Earl et al. (1995) could obtain a high number of collected oocytes, in sheep, by laparotomy. However, the number of follicles aspirated and oocytes recovered by aspiration were slightly fewer than that reported by Reggio et al. (2001) when used the same procedure, laparotomy, as done in this study. *In vivo* matured oocytes have been widely used as recipient cytoplasts in goat NT procedure, at the beginning of NT work, in several laboratories (Baguisi et al., 1999; Zou et al., 2001; Chesne et al., 2002) but there has been no report on the number of oocytes collected by flushing the oviducts. Comparison could be made presumably, therefore, with the pronuclear stage recovery, by flushing, of previous reports (Baldassarre et al., 1995; Kühholzer et al., 1998). In the present study, the numbers of CL observed (ovulations) and oocytes recovered were higher than that previously reported by Baldassarre et al. (1995), 19.6 versus 16.7 (Table 9) and 11.8 versus 9.3 for the numbers of CL and

oocytes recovered, respectively. The numbers of CL and oocytes recovered, in this study, were also higher than those reported by Kuhholzer et al. (1998). Recovery rates, in this study, were similar to those previously reported in goats (Graff et al., 1999; Keefer et al., 2001; Baldassarre et al., 2003; Koeman et al., 2003).

In the present study, the repeated collection by the two aspirations at laparotomy had no detrimental effects on the numbers of follicles through the quality of oocytes recovered. Similar findings were also observed in Nigerian Dwarf goats (Baldassarre et al., 2003), sheep (Graff et al., 1999; Ptak et al., 1999), cattle and buffalo calves (Reis et al., 2002; Techakumphu et al., 2004). Those authors used laparoscopic ovum pick-up (LOPU) or OPU, but not laparotomy to obtain the oocytes. This finding suggests that the laparotomy could be performed twice in each doe, without any negative effects on the number and quality of oocytes.

Repeated OPU, at laparotomy, has no negative influence on the fertility of donor goats, even when repeated up to 3 times, regardless the OPU methods. Although some of goats, in which oviducts and follicles had been manipulated 2-3 times, developed slight to severe adhesions between the ovary and infundibulum, they became pregnant after natural mating. Some donors developed adhesions after receiving only once operation, failed to become pregnant, therefore, no relationship between these adhesions and infertility could be inferred. No information on the effects of repeated OPU by surgery on infertility in goats is available. Therefore it could not be compared to other works on the goat. Similar observations were found in sheep (Stangl et al., 1999). They found that sheep, in which follicles had been punctured 20 times (LOPU method) and developed adhesions, could be pregnant after natural mating. In cattle and buffalo, no effects of repeated OPU on subsequence cycle and reproductive potential were found (Pieterse et al., 1991; Boni et al. 1996;

Broadbent et al., 1996). However, it appeared to slightly alter endocrine profiles and cause minor morphological changes in the ovaries, such as the presence of luteal structures and follicles of various sizes and thicker connective tissue than normal (Petyim et al., 2000).

In conclusion, repeated surgical collection in the two to three consecutive sessions combined with FSH-treatment in donor goats is a practical and efficient regimen for the recovery of oocytes. It provides high numbers of good quality oocytes and it has no effect on pregnancy of the oocyte donors. Further studies are needed to develop other procedures such as laparoscopic ovum-pick up in order to overcome the limitations of repeated recovery in each donor.

SUMMARY AND CONCLUSIONS

This study was divided into two main parts: cloning in cattle and goats. In cattle, the kinetics of the first DNA replication in the 1-cell both of the NT and parthenogenetic embryos was intensively determined. Its effect on the development of the NT embryos was also evaluated after transferring of the donor cells at presumptive G0 stage into the recipient oocytes arrested at MII (non-activated) or interphase (activated) cytoplasts. It was found that the first DNA synthesis period was longer and started earlier in somatic nuclei transferred into activated cytoplasts than in those transferred into non-activated ones. Interestingly, the DNA replication was complete in the nuclei transferred into non-activated cytoplasts whereas it was not so in the activated cytoplasts, which correlated to the developmental potential of NT embryos. A very significant higher development rates to the blastocyst stage was obtained from the non-activated derived NT embryos, compared to that obtained from activated derived group (51.1% versus 22.8%, respectively, $P<0.001$). The developmental potential *in vivo* of NT embryos derived from both groups should be further investigated.

In goats, the effect of activation protocols on the development of NT embryos both *in vitro* and *in vivo* was determined. The similar results, in the fusion, cleavage and development rates to the blastocyst stage, as well as in the pregnancy rates were obtained when used either ionomycin or ethanol, in combination with 6-DMAP plus CB, as activation protocols. However, only one recipient doe, received NT embryos derived from ionomycin treatment group, could maintain pregnancy by midterm. Furthermore, the retrospective study was conducted to investigate whether the

repeated surgical oocyte collection has a detrimental effect on the efficiency of oocyte collection, the numbers of follicles and oocytes recovered as well as pregnancy in oocyte donors after natural mating. It was found that the repeated surgery, in this study, had no effects on all parameters described above. Other procedure for oocyte collection, which is invasive and repeatable such as laparoscopic ovum pick up should be established.

Taken together, the results obtained from this study, both in cattle and goats, provide:

1. More information inside of nuclear reprogramming events occurring in the early stage that affect the development of NT embryos.
2. A new approach for improving the efficiency and success of NT procedure.
3. New information on somatic nuclear transfer in goats.
4. Basic information on oocyte collection and somatic nuclear transfer in goats in Thailand.

In conclusion, a molecular characteristics related to nuclear reprogramming events occurring on NT embryos during the 1-cell stage has been explored in bovine in this study. The completion of DNA synthesis during the first cell cycle affects the developmental ability of NT embryos. It can be said that now we know how the cell states of donor cells and the recipient cytoplasts influence the *in vitro* development of NT embryos when transferred the nuclei at quiescent stage into recipient cytoplast at different stages. For cloning in goats, NT procedure for this species has been developed in Thailand, evidently by the production of NT embryos and the occurrence of implantation. However, the efficiency is needed to improve.