Preliminary Study on Somatic Cell Nuclear Transfer in Rabbits in Thailand

MONGKOL TECHAKUMPHU, DVM, Doctorat de 3 e cycle*, PRANEE NUMCHAISRIKA, BSc**,
SOMCHAI SUWAJANAKORN, MD**, KAMTORN PRUKSANANONDA, MD**,
WISUT BOONKASEMSANTI, MD**, VICHUDA AHNONKITPANIT, MSc**,
PRAMUAN VIRUTAMASEN, MD**

Abstract

The objective of the study was to develop the somatic nuclear transfer technique by using rabbits as the model. The oocyte recipients aged 16 h post coitus were collected surgically from 20 superovulated rabbit doe with 28 and 40 mg Follicle Stimulating Hormone (FSH) after mating with a vasectomized male. The metaphase II plate and 1st polar body of oocyte was later aspirated by enucleated micropipette under an inverted microscope. A single donor cell; cumulus cell or cultured or frozen fibroblast cell from passage 1 to 9 were transferred to enucleated oocyte and fused with triple DC pulses, 3.2 kv, 20 μs. The fused embryos were cultivated in TCM 199 $NaHCO_3$ + 10 per cent fetal calf serum (FCS) for 4 days. The cleavage rate (2-cell stage) was 37.2 per cent (32/86) from eight experiments, and 18.8 per cent (6/32) developed to the early morula stage. This study also indicated that the enucleation pipette and the somatic cell type influenced the success.

Key word : Rabbit, Fibroblast Cells, Cumulus Cells, Nuclear Transfer

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NUMCHAISRIKA P, SUWAJANAKORN S, et al
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The cloning by somatic nuclear transfer is an update technique for multiple numbers of identical genetic animals for breeding purposes and presently it was recommended for human medical purposes. The production of human therapeutic proteins and xenotransplantation especially from pig organs to

* Department of Obstetrics and Gynaecology an Reproduction, Faculty of Veterinary Science,
** Reproductive Medicine Unit, Department of Obstetrics and Gynaecology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.

humans were the objectives for cloning application in human being[1]. In the present study, the rabbit was selected to be a model of somatic cell cloning due to its advantages such as a high number of oocytes produced per superovulation, a short gestation period, a well-developed embryo in culture medium and a predicted time of ovulation by mating induction. (induced ovulator)[2]. Futhermore, the size of oocyte the is quite similar to humans and other kinds of farm animals. The objective of this study was to develop the technique of somatic cell cloning.

MATERIAL AND METHOD

Somatic cell donor preparation

Skin biopsies of the ear were obtained from a mature New Zealand White doe. The fibroblast cell population was prepared by the technique described by Vignon et al[3] and were produced by subculture of primary cells in DMEM + 10 per cent fetal calf serum. After 14 days of culture, the fibroblast cells were trypsinized and later subcultivated. The cultivated fibroblast cells at passage 1 to 9, frozen fibroblast cells, provided by Dr. Xavier Vignon, INRA in France during the Workshop of Somatic Cell Cloning in year 2000[1] and the fresh cumulus cells recovered after decoronizaton from matured oocytes were used as donor cells. The cells were washed twice by centrifugation in DMEM +10 per cent FCS (Gibco BRL, USA) at 1,000 g 10 min. The donor cells were kept in DMEM + 10 per cent FCS until transferring to an enucleated oocyte.

Oocyte recipient preparation

According to Techakumphu et al[4,5] the ovulated matured oocytes recovered from 20 mature New Zealand White rabbit does after superovulation with 28 or 40 mg Follicle Stimulating Hormone (FSH, Vetrepharm®, NSW, Australia) were used as oocyte recipient. The oocytes were recovered after ovariohysterectomy at 16 h post coitum and kept in 80 iu of hyaluronidase (Sigma, USA) for 30 min in a CO_2 incubator at 37°C. After the end of incubation, the cumulus were then removed by pipetting from the oocyte.

Oocyte enucleation

The metaphase II and 1st polar body were removed in a microdrop of TCM 199 $NaHCO_3$ + 7 $\mu g.ml^{-1}$ cytochalasin-B (Sigma, USA) under an inverted microscope as described previously[6,7]. The success of metaphase II removal was identified under fluorescence after 5 µm Hoechst #33342 (Sigma, USA).

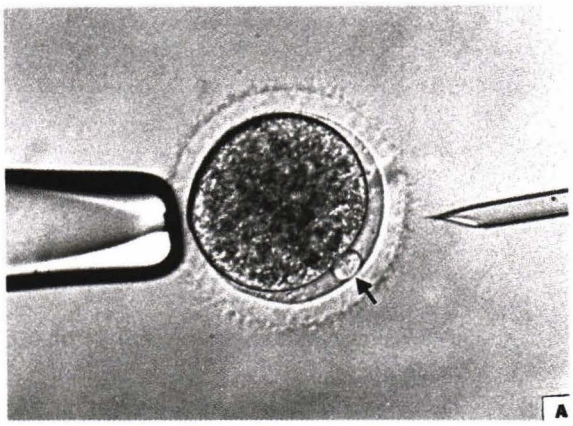


Fig. 1A. A matured oocyte with 1st polar body on the surface of cytoplasm (arrow). The oocyte was maintained by holding a pipette with an internal diameter (ID) of 20 um and the polar body was placed between 4 to 5 O'clock. facing the beveled enucleated pipette (ENP) of 20 µm ID.

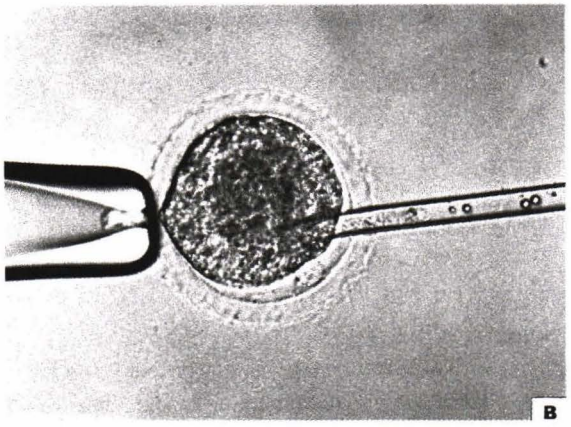


Fig. 1B. The ENP was penetrated through the zona pellucida into the cytoplasm of the oocyte above the polar body. The surrounding cytoplasm around 15-20 per cent was aspirated by delicate, slow aspiration.

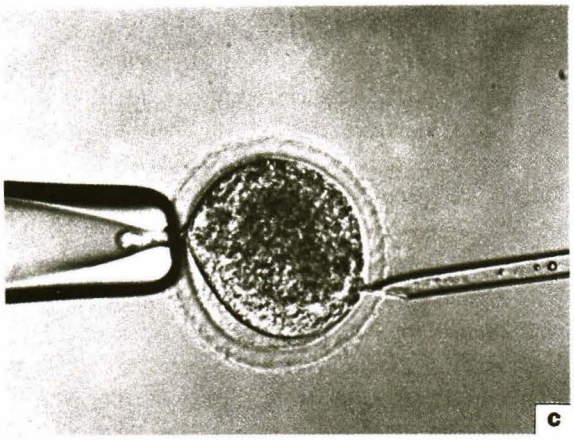


Fig. 1C. The ENP was later pulled back after enucleation.

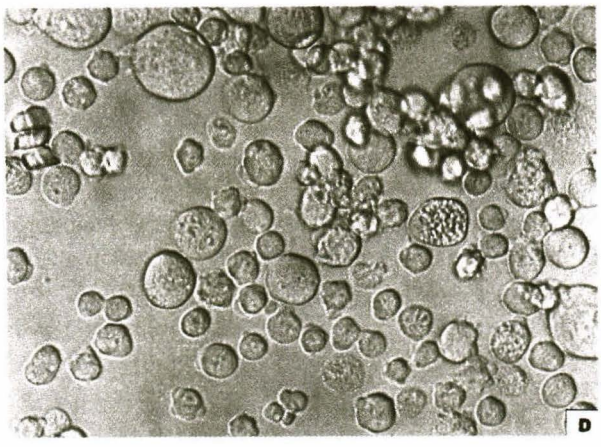


Fig. 1D. A group of fibroblast cells as donor cells.

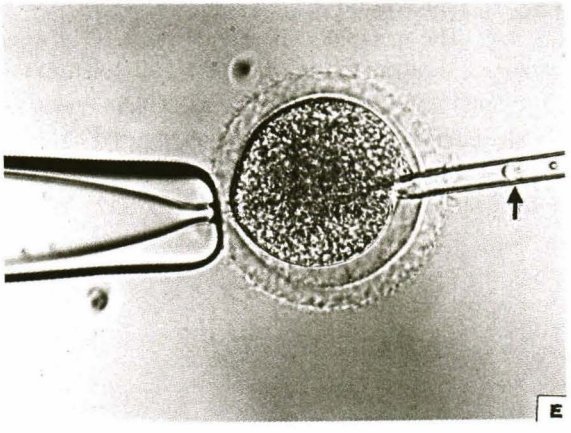


Fig. 1E. The single fibroblast cell (arrow) was aspirated in ENP and its tip was penetrated in the same opening of enucleation.

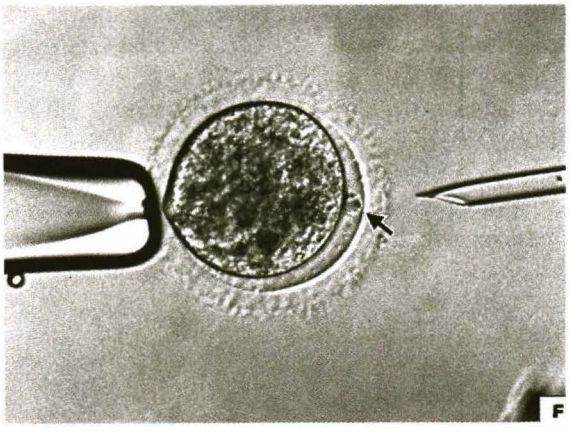


Fig. 1F The donor cell (arrow) was then expulsed into the oocyte under the zona pellucida and attached to the cytoplasm.

Somatic cell transfer

Nuclear transfer was performed by the technique described by Heyman et al[6] and Chesne et al [8]. Each isolated cell (cumulus or fibroblast cell) was inserted under zona pellucida of the oocyte recipient and later fused by electrostimulation (Grass stimulator, Astro-Med, INC. USA), 3.2 kv/cm 20 μ sec 3 pulses in 0.3 M mannitol solution containing 0.1 mm Ca^{++} and Mg^{++}. Each fused embryo was placed in a small drop of TCM 199 $NaHCO_3$ +10 per cent FCS under mineral oil for 4 days at 37°C under 5 per cent CO_2 in air. Cleavage was assessed every 24 h *via* a binocular microscope.

The process of somatic cell nuclear transfer is described in Fig. 1.

RESULT

The results in Table 1 show that 32 from 86 (37.2%) reconstructed embryos cleaved with 43.8 per cent (14/32) at 2-cell stage, 37.5 per cent (12/32) at 4-cell stage and 18.8 per cent (6/32) at 8-16 cell stage (Fig. 2). Cumulus cells can be used as donor cells

providing 69.2 per cent (9/13) of cleavage while frozen and cultivated fibroblast cells can provide the cleavage ranging from 9.1 per cent to 90 per cent.

DISCUSSION

The present paper describes the technique of somatic cell nuclear transfer by using a rabbit oocyte and its fibroblast as a model. Every process influenced the success of embryo reconstruction and *in vitro* development. The essential steps and factors for the somatic cell nuclear transfer are discussed. Firstly, the microtools preparation; the sharpness, cleanliness, angle and diameter (about 20 μm ID) of the enucleated pipette including its spike at the tip of the pipette facilitated the enucleation. Delicate control during somatic cell aspiration and transfer into the oocyte was also required. The possibility of donor cell aspiration in the pipette should be done before enucleation. The holding pipette was also important especially its diameter which should not be wider than 20 μm. For the stimulation protocol, twenty milligrams of FSH in five divided injections are suitable to superovulate the rabbit in his condition(5). A higher milligram of FSH did not increase the ovulation, on the contrary, it reduced the number oocytes per donor. Secondly, the quality of the cytoplasm of the oocyte recipient, according to the superovulation program, can be produced around 20 good matured oocytes per

Table 1. ***In vitro*** **development of reconstructed rabbit embryos after fusion with cumulus and fibroblast cells.**

EXP	Donor cell type	No. oocytes	Cleaved embryos	%	Stage of embryo		
					2°C	4-8°C	≥ 16°C
1	Frozen fibroblast	5	4	80		4	-
2	Frozen fibroblast	19	3	15.8	1	2	-
3	Cumulus cell	13	9	69.2	5	4	-
4	Fibroblast cell (P1)	6	1	16.7	1	-	-
5	Fibroblast cell (P3)	11	3	27.2	2	1	-
6	Fibroblast cell (P4)	10	9	90	4	1	4
7	Fibroblast cell (P6)	11	1	9.1			1
8	Fibroblast cell (P9)	11	2	18.2	1		1
Total		86	32		14	12	6
%			37.2		43.8	37.5	18.8

(P) = passage

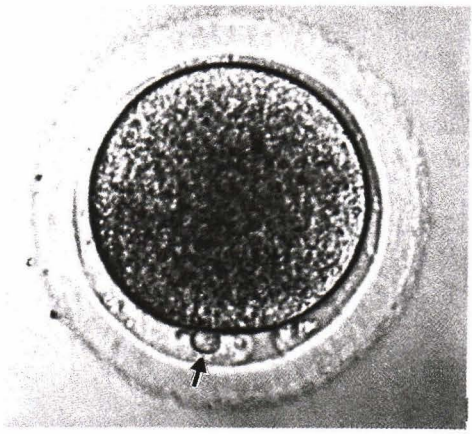


Fig. 2A. Reconstructed embryo with fibroblastic cell on the surface of embryo (arrow) (X400).

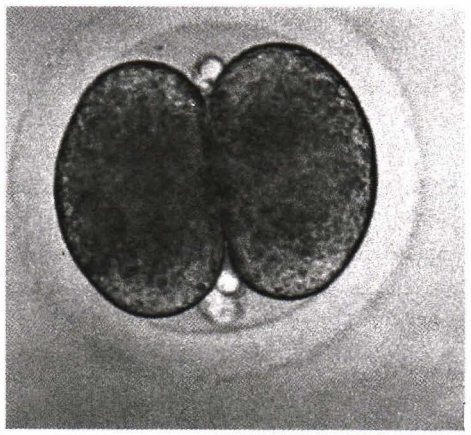
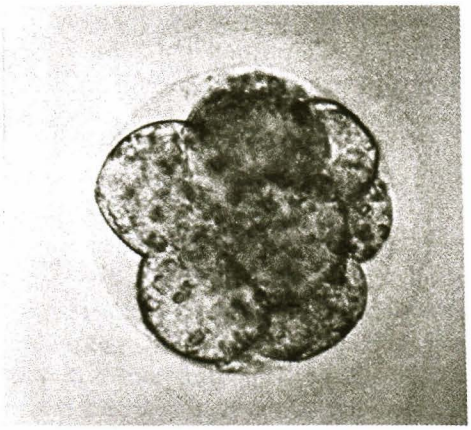
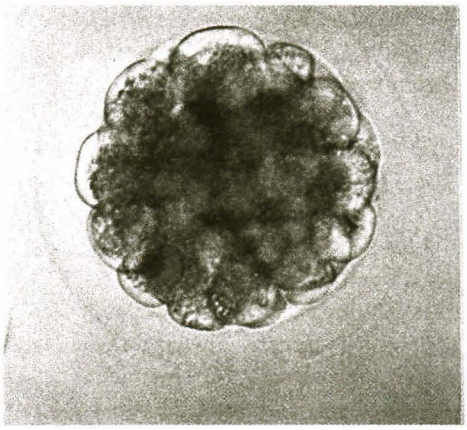


Fig. 2B. Two-cell stage embryo (X400).



C

Fig. 2C. Eight-cell stage embryo (X400).



D

Fig. 2D. Early morula stage (about > 16 cells) (X400).

donor. In the present study more than 90 per cent of ovulated oocytes were used as oocyte recipients in nuclear transfer and the 1st polar body where the chromatin situated nearby could be easily located. Thirdly, the location of the 1st polar body between 4 and 5 o'clock helped to simplify enucleation. It was suggested that the tip of the bevel pipette should be pinched through the zona pellucida at the same level to the polar body before enucleation. The penetration through the zona pellucida should be at the 3 o'clock position and after removing the surrounding cytoplasm (Fig. 1B), the pipette should be pressed downward to polar body for aspiration. In some oocytes, a loosened polar body from the surface of the oocyte can happen and cause failure of polar body removal. The amount of enucleated cytoplasm should not be exceeded in order to have a better contact between oocyte and donor cell. Fourthly, the selection of donor cells was a prerequisite to success, the medium sized with a clear, shiny cytoplasm should be selected. In the present study, the fibroblast in the frozen and cultivated form can be used as a donor cell as well as a cumulus cell. It was demonstrated that fibroblast cells in the cultivated or frozen form and cumulus cells can be used as nuclear donor according to Chesne et al[8].

Furthermore, during the incubation in cytochalasin-B, the depolymerizing product was beneficial to enucleation by preventing the cytoplast lysis. The incubation time in cytochalasin-B should be 5 to 30 mins before enucleation. The success of the study was determined by the cleavage to 2 cells to 8-16 cells by *in vitro* culture. However, due to a high variation of success, further investigation is required for an appropriate morula and blastocyst result. In conclusion, the present study demonstrated that the fibroblast cell in the culture or frozen form can be used as somatic cell cloning which is the first report in a rabbit in Thailand.

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การศึกษาเบื้องต้นของการย้ายฝากนิวเคลียสโดยเซลล์โซมาติกในกระต่ายในประเทศไทย

มงคล เตชะกำพุ, สพบ, ปราณี นำชัยศรีค้า, วทบ**,*
*สมชาย สุวจนกรณ์, พบ**, กำธร พฤกษานานนท์, พบ**,*
*วิสุทธิ์ บุญเกษมสันติ, พบ**, วิชชุดา อานนท์กิจพานิช, วทม**, ประมวล วีรุตมเสน, พบ***

จุดประสงค์ของการศึกษานี้เพื่อพัฒนาเทคนิคการย้ายฝากนิวเคลียสด้วยเซลล์โซมาติกโดยใช้กระต่ายเป็นต้นแบบ โดยเก็บโอโอไซต์ตัวรับจากกระต่ายหลังกระตุ้นการตกไข่เพิ่มด้วยฟอลลิเคิลสติมูเลติง ฮอร์โมน ขนาด 28 และ 40 มิลลิกรัม จำนวน 20 ตัว ทำการผ่าตัด 16 ชั่วโมงหลังผสมด้วยพ่อกระต่ายตัดท่อน้ำเชื้อ และนำท่อนำไข่มาชะล้างเก็บโอโอไซต์ในระยะเมทาเฟส ทู นำโอโอไซต์ที่ได้มาแยกเอาสารพันธุกรรมออกด้วยการดูดด้วยไปเปตขนาดเล็กภายใต้กล้องจุลทรรศน์ หลังจากนั้นนำเอาเซลล์โซมาติกชนิดคิวมูลัสและเซลล์ไฟโบรบลาสต์ที่ได้จากการเลี้ยงในหลอดทดลอง นำมาย้ายฝากแทนเป็นนิวเคลียสของโอโอไซต์ เชื่อมไซโทพลาสซึมของโอโอไซต์กับนิวเคลียสของเซลล์ด้วยกระแสไฟฟ้าอย่างอ่อน ขนาด 3.2 kv 20 µs จำนวน 3 ครั้งติดต่อกัน จากนั้นนำไปเลี้ยงน้ำยาเลี้ยงตัวอ่อนชนิด TCM 199 $NaHCO_3$ + 10% fetal calf serum จากการทดลองทั้งหมด 8 ครั้ง พบว่ามีการแบ่งเซลล์ของตัวอ่อนที่เกิดจากการย้ายฝากนิวเคลียส คิดเป็น 32/86 (37.2%) โดยสามารถพัฒนาเป็นมอรูล่าระยะแรกได้ 6/32 (18.8%) จากการศึกษานี้ยังชี้ให้เห็นว่าผลสำเร็จขึ้นกับไปเปตที่ดูดสารพันธุกรรมและชนิดโซมาติกด้วย

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มงคล เตชะกำพุ, ปราณี นำชัยศรีค้า, สมชาย สุวจนกรณ์, และคณะ
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* ภาควิชาสูติศาสตร์–เธนุเวชวิทยาและวิทยาการสืบพันธุ์สัตว์, คณะสัตวแพทยศาสตร์,
** หน่วยชีววิทยาการเจริญพันธุ์, ภาควิชาสูติศาสตร์–นรีเวชวิทยา, คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย, กรุงเทพ ฯ 10330