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การพัฒนาเทคนิคการย้ายฝากนิวเคลียสของเซลล์โซมาติกเพื่องานวิจัยทางการแพทย์และการเกษตร (ปีที่ 2)

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Report

Development of Somatic Cell Nuclear Transfer Technique for Biomedical and Agricultural Research (year II)

By

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Subject: Development of Somatic Cell Nuclear Transfer Technique for Biomedical and Agricultural Research (year II)
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Year: December 2005

Abstract

There are two objectives of the somatic nuclear transfer studies. The first objective was to evaluate the possibility to produce the inter-species cloned embryos from donor cells of different animals transfer to rabbit, pig or swamp buffalo oocytes. The second objective was to study the donor cell type can significantly affect the *in vitro* development of cloned rabbit embryos. The experiments are described in three chapters:

Chapter 1: The *in vitro* development of inter-species cloned embryos, reconstructed using a rabbit recipient oocyte
Chapter 2: The *in vitro* development of inter-species cloned embryos, reconstructed using pig and swamp buffalo oocytes
Chapter 3: The *in vitro* development of cloned rabbit embryos derived from various primary somatic cell types

From the study, we found that, rabbit oocytes can be served as recipient cytoplasm for donor cells from different kinds of animals. Pig and buffalo oocytes are possibly be used as recipient cytoplasm for producing intra- and inter-species cloned embryos but the developmental rates of the cloned embryos are limited. The result of the *in vitro* development of cloned embryos derived from various primary somatic cell types showed that the donor cell type can significantly affect the *in vitro* development of cloned rabbit embryos. The outcome of research is to obtain basic knowledge on reprogramming of foreign donor nuclei to recipient cytoplasm, establishing the genetic banking and offering an opportunity to genetic rescue of endangered mammal.
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Key words: somatic cell nuclear transfer, rabbit, *in vitro* development, inter-species, primary cell types

ชื่อโครงการวิจัย การพัฒนาเทคนิคการย้ายฝากนิวเคลียสของเซลล์โซมาติกเพื่องานวิจัยทางการแพทย์และการเกษตร (ปีที่ 2)

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บทคัดย่อ

วัตถุประสงค์ของงานวิจัยนี้เพื่อในการศึกษาความเป็นไปได้ในการย้ายฝากนิวเคลียสของเซลล์โซมาติกของสัตว์ต่างชนิดฝากในโอโอไซต์ของกระต่าย สุกรและกระบือปลัก และเพื่อศึกษาการย้ายฝากนิวเคลียสด้วยเซลล์จากร่างกายชนิดต่าง ๆ

รายงานวิจัยนี้แบ่งเป็น 3 บท คือ

บทที่ 1 การศึกษาการพัฒนาในหลอดทดลองของโคลนตัวอ่อนต่างสปีชีส์ที่สร้างจากการใช้โอโอไซต์ของกระต่ายเป็นโอโอไซต์ตัวรับ

บทที่ 2 การศึกษาการพัฒนาในหลอดทดลองของโคลนตัวอ่อนต่างสปีชีส์ที่สร้างจากการใช้โอโอไซต์ของสุกรและกระบือเป็นโอโอไซต์ตัวรับ

บทที่ 3 การศึกษาการพัฒนาในหลอดทดลองของโคลนตัวอ่อนกระต่ายที่เกิดจากการใช้เซลล์ โซมาติกชนิดต่าง ๆ

จากการศึกษานี้แสดงให้เห็นว่าโอโอไซต์กระต่ายสามารถใช้เป็นเซลล์ตัวรับต่อเซลล์โซมาติกตัวให้ของสัตว์ชนิดต่าง ๆ ที่ศึกษา ในขณะที่โอโอไซต์ของสุกรและกระบือปลักสามารถใช้ได้แต่มีโคลนตัวอ่อนมีการพัฒนาที่จำกัด ในส่วนผลการศึกษาการใช้เซลล์ชนิดต่าง ๆ เป็นเซลล์ตัวให้ของการพัฒนาของโคลนตัวอ่อนกระต่าย พบว่าชนิดของเซลล์ร่างกายให้ผลการพัฒนาของโคลนตัวอ่อนกระต่ายแตกต่างกันไป ผลของงานวิจัยนี้ให้ประโยชน์ในด้านความรู้พื้นฐานต่อความสัมพันธ์ระหว่างเซลล์ตัวให้ที่ถูกเริ่มกำหนดการทำงานเป็นนิวเคลียสตัวให้ใหม่ในไซโทพลาสซึมของโอโอไซต์ตัวรับ และเป็นประโยชน์ในการพัฒนาการเก็บรักษาพันธุกรรมของสัตว์ป่าด้วย

คำสำคัญ: การย้ายฝากนิวเคลียสของเซลล์โซมาติกสัตว์ต่างชนิด โอโอไซต์กระต่าย การพัฒนาในหลอดทดลอง เซลล์ร่างกายชนิดต่าง ๆ

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Lists of Symbols

^{o}C = degree celsius
6-DMAP = 6-dimethylaminopurine
Ca^{++}= calcium
cm = centrimetres
DMSO = Dimethyl sulfoxide
FCS = foetal calf serum
h = hour
hpc= hours post coitum
kg = Killogram
kV= kilovolts
M = Molar
min = minute
ml = millilitres
Mg^{++} = Magnisium
mg = milligrams
μm = micrometers
μs = microseconds
n = number
NaCl = sodium chrolide solution
NT = nuclear transfer
SNT = somatic nuclear transfer
PBS = Phosphate Buffer Saline
Rpm = round per minute
SOF = Synthetic Oviductal Fluid

CHAPTER 1

THE IN VITRO DEVELOPMENT OF INTER-SPECIES CLONED EMBRYOS, RECONTRUCTED USING A RABBIT RECIPIENT OOCYTE

Abstract

In the present work, we evaluated the possibility to produce the inter-species cloned embryos from donor cells of cow, swamp buffalo, pig or domestic cat, transferred into rabbit oocyte. Furthermore, donor cells from two endangered mammals, marble cat (*Pardofelis marmorata*) and Asian elephant (*Elephus maximus*) have been introduced into rabbit oocyte as well. A single donor cell in starvation stage were transferred into rabbit oocyte after removal of metaphase II and 1st polar body, enucleated oocytes. Reconstructed inter-species cloned oocytes were later activated by two sets of electrical stimulation, 1h apart, (3DC pulses, 1.2Kv.cm^{-1}, 20 μs each in mannitol 0.3 M. After the second set of electrostimulation, they were incubated in cyclohexamide 5mg.ml^{-1} and 6-DMAP 2mM in TCM199 for 1 h then washed and cultivated in TCM 199$NaHCO_3$ or SOF+10% fetal calf serum for 7 days. Two types of fusion-activation protocol, 3.2 kV/cm, 3 pulses, 20 μs and 2.0 kV/cm, 2 pulses, 20 μs were studied in elephant-rabbit cloning. The *in vitro* development was observed daily, the cleavage, and morula-blastocyst rates were recorded. The result showed that bovine-rabbit, buffalo-rabbit, pig-rabbit, cat-rabbit, marble cat-rabbit and elephant-rabbit can develop to morula and blastocyst with the rates comparable to control, rabbit-rabbit. The fusion-activation protocol influences the success of elephant-rabbit development. In conclusion, rabbit oocytes can be served as recipient cytoplasm for donor cells from different kinds of animals. The outcome of research is the basic knowledges on reprogramming of foreign nuclei to cytoplasm, establishing the genetic banking and offering an opportunity to genetic rescue of endangered mammal.

Key words: interspecies cloning, rabbit oocyte, *in vitro* development

Introduction

Cloning technology has several applications include bio-medical applications, such as the production of pharmaceuticals in the blood or milk of transgenic cattle and also is useful in the agriculture production (Faber et al., 2004). The somatic cell nuclear transfer (SCNT) was widely studied since the birth of Dolly, the first sheep cloning by transferring mammary gland cell into recipient oocyte cytoplasm (Wilmut et al., 1997). This breakthrough technique open the window of research area in cloning for the pursposes of animal breeding, human cell therapy and endangered species conservation. In general, oocyte source for recipient cytoplast was easily obtained from slaughtered materials or alive by ovum pick up of domestic animals, however in wildlife, the limit of oocyte source is one of major obstacle for cloning. Inter-species cloning using an enucleated oocyte of another species such as rabbit or cattle for receiving a a donor cell nucleus of one species are widely studied. Several reports showed that by using inter-species cloning, the cloned embryos can be produced successfully. Interspecies cloning was reported by using oocytes from bovine (Dominko et al., 1999; Hwang et al., 2001; Kitiyanant et al., 2001; Li et al., 2002), ovine (White et al., 1999), cat (Hwang et al., 2001; Gomez et al., 2004) and rabbit (Chen et al., 2002). This showed that the ooplasm of domestic species oocyte can be used as the recipient cytoplasm of a somatic cell nucleus of an endangered species. The objectives of the study were firstly to produce the inter-species cloned embryos from donor cells of cows, swamp buffalo, pig, domestic cat transferred into rabbit oocytes served as recipient oocytes. Secondly, it was for studying the *in vitro* development of cloned embryos after introducing donor cells from two wild animals, marble cat (*Pardofelis marmorata*) and Asian elephant (*Elephus maximus*) into rabbit oocytes.

Materials and Methods

Chemicals

Chemicals were purchased Sigma Chemical Co. (St Louis, MO), unless otherwise stated.

Establishment of donor cell line

Fibroblast from ear stripping of six species, adult and foetal rabbit, domestic cat, marble cat, pig, cattle, swamp buffalo and elephant were used as donor cells. Adult and foetal rabbit cells come from frozen stock (-196^{o}C) since the 1st year project in 2004. The marble cat cells were prepared from the sample collected from Khao Kheow Open Zoo, Zoological Park of Thailand as described by Suwimonteerabutr et al. (2004). For the elephant fibroblast, the ear skin fibroblast were obtained from a stillborn Asian elephant donated to the faculty of Veterinary Science for anatomical study. Tissues were cut into pieces and digested with 0.25% trypsin (Gibco, USA) for 30 min at 37^{o}C. The digested cells and tissue were cultured in DMEM supplemented with 20% fetal calf serum, FCS (Gibco, USA) in a 5% CO_2 incubator at 37^{o}C. Cells were passaged at 70-80% confluence and cells at passage 5 were used as donor cells in starvation status. The cell starvation was performed by

FCS for 5 days before usage. Cell culture of fibroblast and assessing procedures were described previously (Vignon et al., 1998).

Preparation of recipient oocytes

The ovulated mature oocytes with homogenous color and good contour (Fig. 1.1) recovered from mature New Zealand White does rabbit after superovulation were used as recipient oocytes as described previously (Techakumphu et al., 2004). The oocytes were incubated in TCM-199 supplemented with 0.1% hyaluronidase for 15 min in 5% CO_2 at 38.5^{o}C. The cumulus cells were removed from the oocytes by gentle pipetting. The oocytes were later incubated in TCM-199 + 2.5M Hepes supplemented with 7μg/ml cytochalasin-B for 30 min.

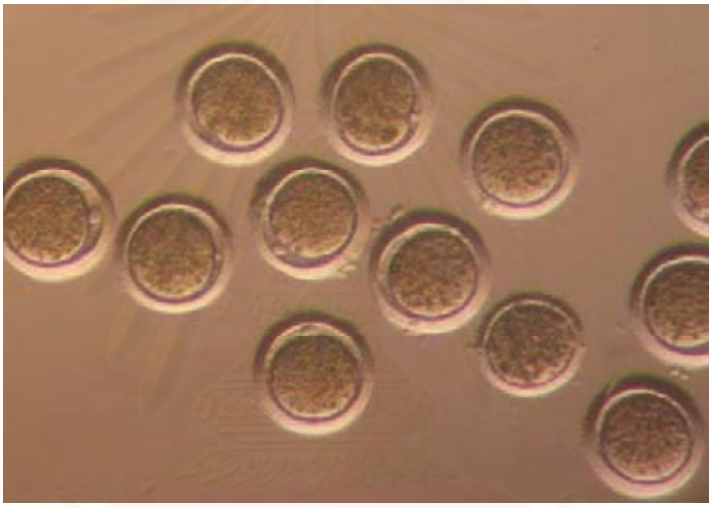

Figure 1.1: Recipient rabbit oocytes, collected from a superovulated doe (x150)

Enucleation and nuclear transfer

The procedure of enucleation and nuclear transfer has been described previously (Techakumphu, et al., 2004). Briefly, the metaphase II plate and first polar body together with small amount of cytoplasm by micromanipulation technique were removed. Enucleation were performed under an inverted microscope and the successful enucleation was confirmed after staining with 5 μg ml^{-1} Hoechst 33342 and investigated under fluorescence microscope.

For nuclear transfer, a single donor cell was introduced in the perivitilline space in close contact with the plasma membrane of an enucleated oocyte as shown in Fig. 1.2.

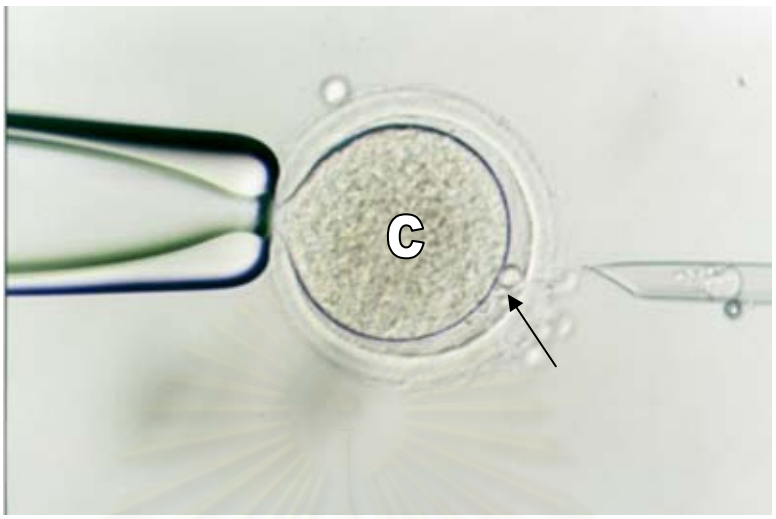

Figure 1.2: A single donor nuclear cell (arrow) from any species was introduced into vittelline space contacting to the surface of rabbit enucleated cytoplasm ,C (x 300)

Fusion and activation

The reconstructed oocytes were transferred to a fusion chamber containing 100 µg of fusion medium (0.3 M mannitol with 0.1 mM Ca^{2+} and Mg^{2+}). Fusion was induced by 3.2 kV/cm, 3 pulses, 20 µs (E1) (Chesne et al., 2002). In elephant-rabbit, a second fusion program, 2.0 kV/cm, 2 pulses, 20 µs (E2) (Chen et al., 2002) was used. The reconstructed oocytes were then washed in M199 (Gibco, USA) supplemented with 10% FCS (Gibco, USA) three times and incubated in the same medium for 1 h at 38.5°C in a humidified air containing 5% CO_2. The reconstructed oocytes were checked for fusion with the stereomicroscope and the fused oocytes were activated by the same electrical pulses and incubated in 5 µg ml^{-1} of cyclohexamide and 2mM of 6-DMAP for 1 h.

The activated oocytes were washed in TCM-199+10% FCS for at least three time before transferring them into a small culture drop of synthetic oviductal fluid (SOF) or B2 culture medium (Menezo, France) covered with mineral oil, at 38.5°C, in 5% CO_2 in air.

Embryo culture and assessment of development

The embryo development was observed and recorded for cleavage up to blastocyst stage every 24 h under a microscope.

Statistical analysis

Differences in the percentages of oocytes developing to a particular stage between electrical pulses and type of donor cell will be determined by chi-square analysis.

Result

1. *In vitro development of cloned embryos using cattle ear fibroblast as donor cells*

From Table 1.1, it was shown that the percentages of cloned embryos for culture, cleavage rate, developmental rate to 2-8, >8-16, compact morula and blastocyst was comparable to cloned rabbit embryos using adult fibroblast as donor cell. The percentage of development of cloned embryos from bovine cell line 2 was higher than bovine line 1 (Table 1.1).

Table 1.1: *In vitro* development of cloned embryos using cattle as donor cell compared to adult rabbit fibroblast

Donor cell	n	2-8 cell	>8-16 cell	morula	blastocyst
		n(%)			
bovine (cell line 1)	133	27(20)	0	0	0
bovine (cell line 2)	90	34(38)	10(11)	4(4)	3(3)
rabbit	74	22(30)	6(8)	5(7)	2(3)

2. *In vitro development of cloned embryos, using swamp buffalo, pig ear fibroblast as donor cell compared to foetal rabbit fibroblast cells*

For swamp buffalo and pig, a higher rate of development was found when using pig fibroblast as donor cell compared to swamp buffalo. The pig-rabbit cloned embryos can develop to with 2% of blastocyst which similar to rabbit-rabbit cloning when using adult fibroblast (Table 1.1). However, this figure was lower when using foetal fibroblast as donor cell for rabbit-rabbit cloning. (Table 1.2)

Table 1.2: *In vitro* development of cloned embryos using swamp buffalo, pig ear fibroblast as donor cell compared to fetal rabbit fibroblast

Donor cell	n	2-8 cell	>8-16 cell	morula	blastocyst
		n(%)			
buffalo	84	30(36)	7(8)	2(2)	0
pig	87	40(46)	7(8)	4(5)	2(2)
foetal rabbit	60	39(65)	22(37)	16(27)	8(13)

3. *In vitro development of cloned embryos using elephant ear fibroblast as donor cells*

The elephant-rabbit cloned embryos can develop to morula and blastocyst stages which was equivalent to rabbit-rabbit cloned embryos (Table 1.3).

Table 1.3. *In vitro* development of cloned embryos using elephant fibroblast as donor cell compared to foetal rabbit fibroblast

Donor cell	n	2-8 cell	>8-16 cell	morula	blastocyst
		n(%)			
elephant	74	44(59)	17(23)	6(8)	4(5)
foetal rabbit	48	26(54)	13(27)	5(10)	2(4)

4. In vitro development of cloned embryos using domestic cat or marble cat ear fibroblast as donor cells

The cleavage rates of domestic cat-rabbit and marbled cat-rabbit were 48% and 53%. The blastocyst rates were 4 and 6% respectively. These percentages were lower than those of control (rabbit-rabbit) (Table 1.4)

Table 1.4: *In vitro* development of cloned embryos using domestic cat, marbled cat fibroblast as donor cell compared to fetal rabbit fibroblast

Donor cell	n	2-8 cell	>8-16 cell	morula	blastocyst
		n(%)			
domestic cat	46	22(48)	8(17)	6(13)	2(4)
marble cat	47	25(53)	7(15)	5(11)	3(6)
rabbit	20	14(70)	7(35)	5(25)	3(15)

4. *In vitro development of inter-species cloned embryos using rabbit oocyte as recipient*

The overall result was demonstrated in Table 1.5, showed that donor cell from different species in this study, bovine, buffalo, pig, elephant can be used for inter-species cloning, the inter-species cloned embryos can cleave with the percentage above 50% in elephant-rabbit group. Inter-species cloned embryos can develop beyond 16 cell stage to morula and blastocyst except in buffalo-rabbit group. The fusion, cleavage and 8-cell stage formation rates were not significantly different when in bovine-rabbit, buffalo-rabbit, pig-rabbit, marble cat-rabbit and cat-rabbit compared to adult rabbit-rabbit ($P>0.05$). The cleavage rate of bovine, buffalo and pig-rabbit was significant lower when compared to foetal rabbit-rabbit ($P=0.02$, 0.07, 0.01 respectively). The low rate of morula formation makes it impossible to compare among the groups, however, it tended to provide the highest rate in foetal rabbit-rabbit group. Cell line can influence on development as shown in bovine-rabbit group and also the source of rabbit fibroblast from adult fibroblast or foetal had an effect on

embryo survival. The figure of inter-species cloned embryos was showed in Fig. 1.3 to 1.9.

Table 1.5. *In vitro* development of inter-species cloned embryos using rabbit oocyte as recipient

Donor cell	n	2-8 cell	>8-16 cell	morula	blastocyst
		n(%)			
cattle 1	133	27(20)	0	0	0
cattle 2	90	34(38)	10(11)[a]	4(4)	3(3)
buffalo	84	30(36)	7(8)[b]	2(2)	0
pig	87	40(46)	7(8)[c]	4(5)	2(2)
elephant	74	44(59)	17(23)	6(8)	4(5)
domestic cat	46	22(48)	8(17)	6(13)	2(4)
marbled cat	47	25(53)	7(15)	5(11)	3(6)
rabbit*	74	22(30)	6(8)	5(7)	2(3)
rabbit**	60	39(65)	22(37)[d]	6(27)	8(13)

* adult fibroblast, ** fetal fibroblast
a,d, P=0.02; b,d, P=00.7; c,d, P=0.01

5. *In vitro development of elephant-rabbit cloned embryos with different program of activatio*n

Table 1.6 showed the result of development of elephant-rabbit cloned embryos by using two different activation protocol. It was found that a higher rate of fusion, cleavage and blastocyst rates were found when using E1 than E2 protocol (P<0.05).

Table 1.6: Summary of *in vitro* developmental of elephant-rabbit cloned embryos, being fused by different electrical pulses

Electrical pulses	Reconstructed n	Fused/Culture n (%)	Cleavage n (%)	Blastocyst n (%)
E1	61	43 (70.5)[a]	28 (65)[a]	3 (7)[a]
E2	69	36 (52.2)[b]	17 (47)[b]	1 (3)[b]

[a, b] Values with different superscripts within the same column differ significantly (P<0.05)

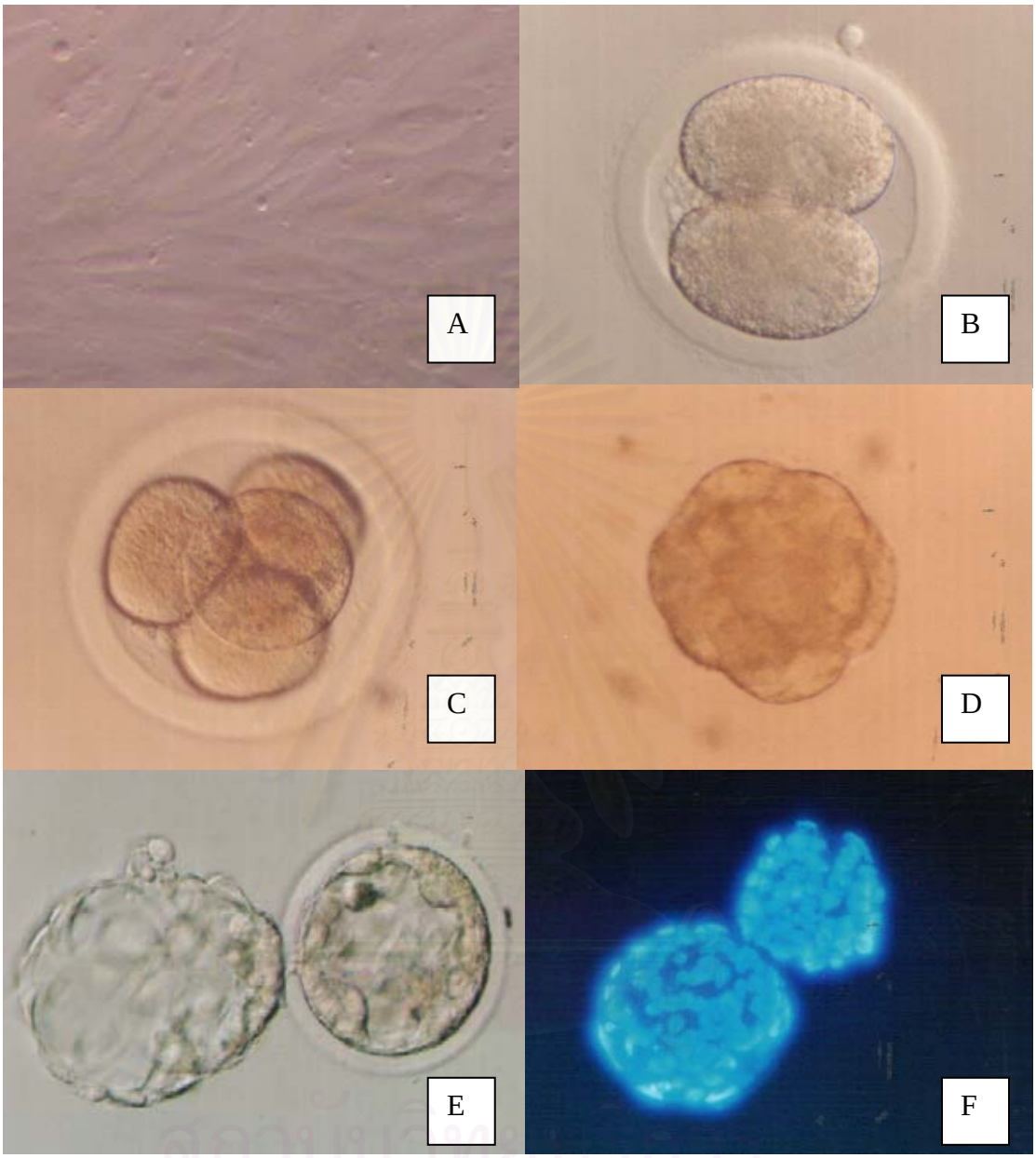

Figure 1.3: Rabbit-Rabbit cloned embryos at different stages, served as control: A, rabbit fibroblast cells for donor cells (x200), B; 2-cell stage (x300), C; 4-cell stages (x300), D; compact morula stage (x200), E; hatching blastocyst (x300), F; hatching blastocyst, investigated under fluorescence (x200)

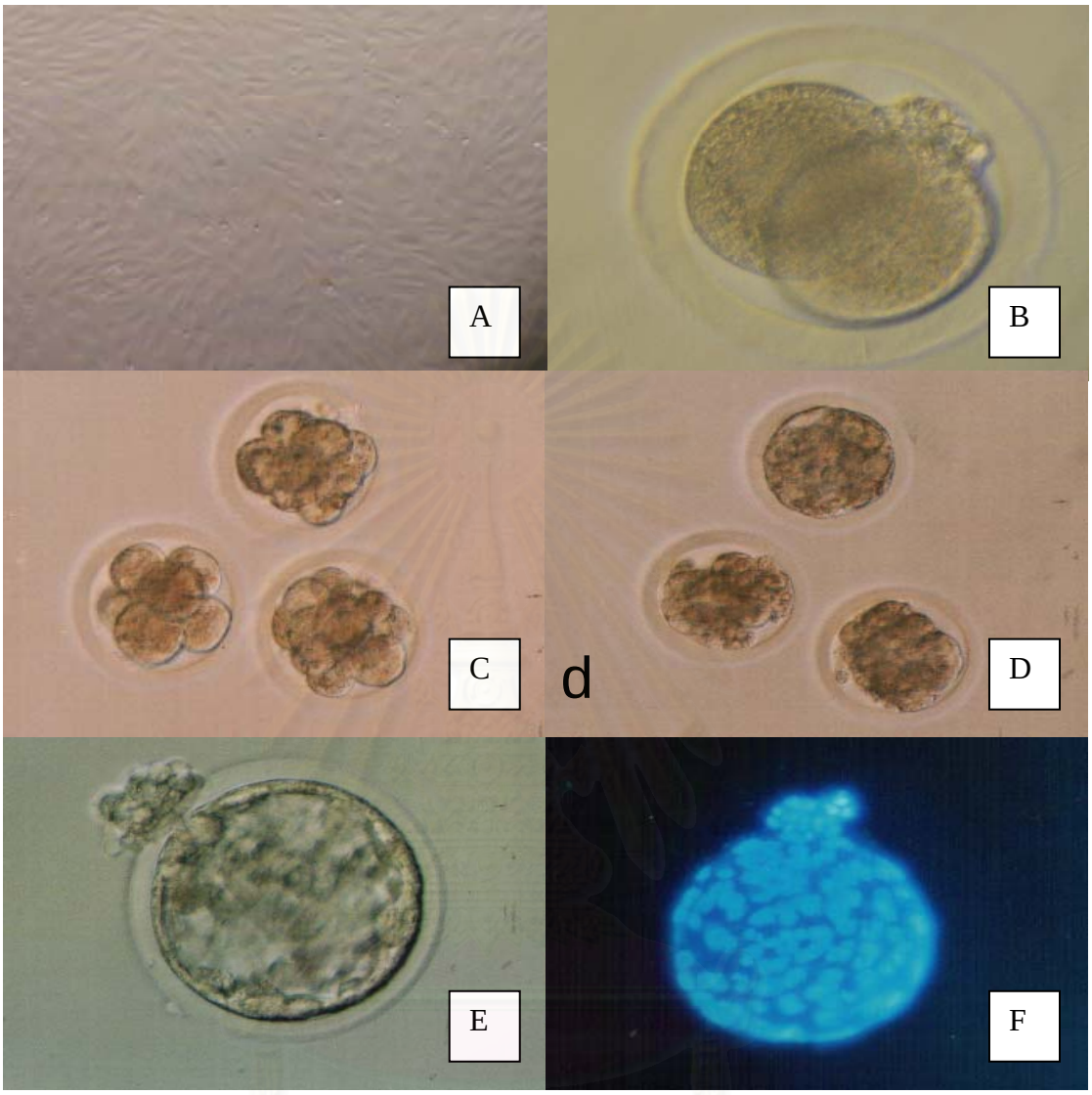

Figure 1.4: Cattle-Rabbit cloned embryos at different stages: A, cattle fibroblast cells for door cells (x200), B; 2-cell stage (x300), C; 8-cell stages (x150), D; morula and early blastocyst stages (x150), E; hatching blastocyst (x300), F; hatching blastocyst, investigated under fluorescence (x300)

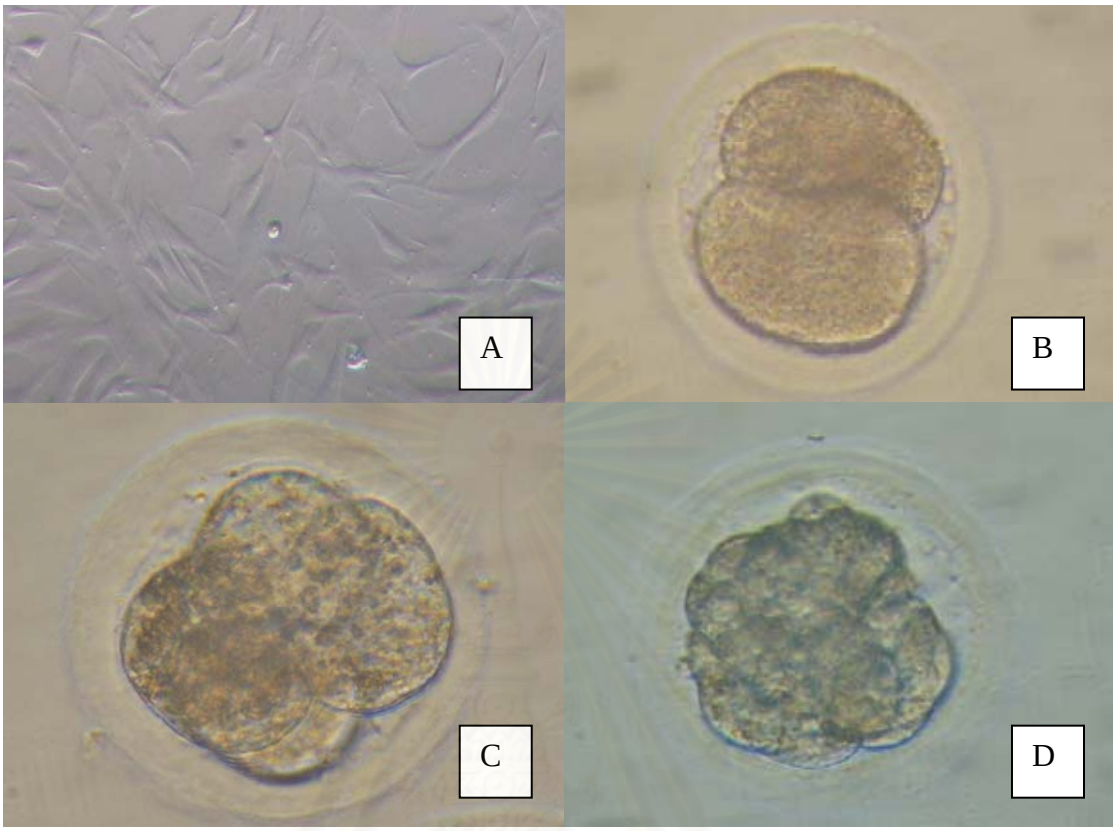

Figure 1.5: Buffalo-Rabbit cloned embryos at different stages: A, buffalo fibroblast cells for donor cells (x200), B; 2-cell stage (x300), C; 4-cell stages (x300), D; morula stage (x300)

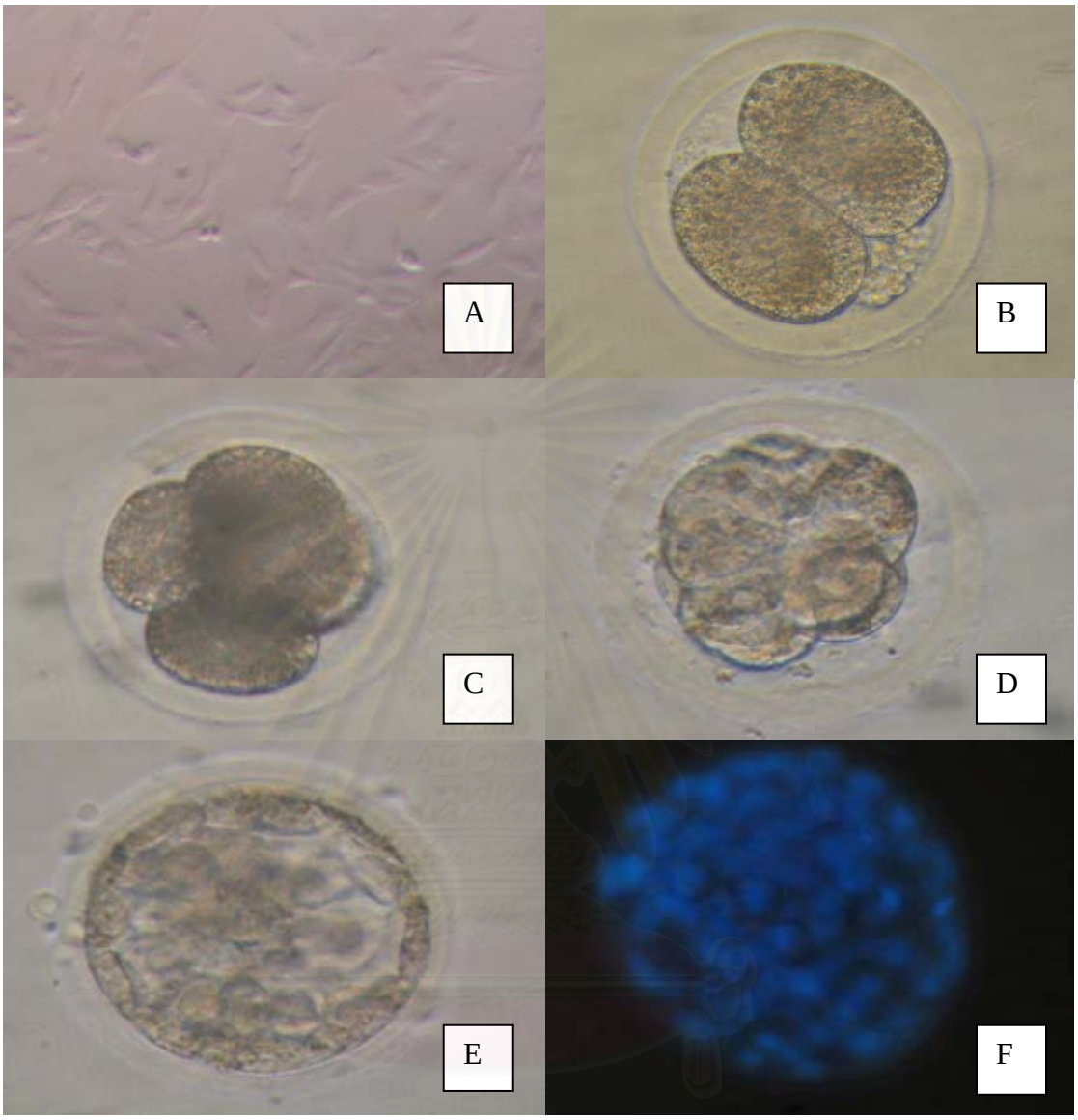

Figure 1.6: Pig-Rabbit cloned embryos at different stages: A, pig fibroblast cells for door cells (x200), B; 2-cell stage (x300), C; 4-cell stages (x300), D; early morula (x300)**,** E; blastocyst (x300), F; blastocyst under fluorescence investigation (x300)

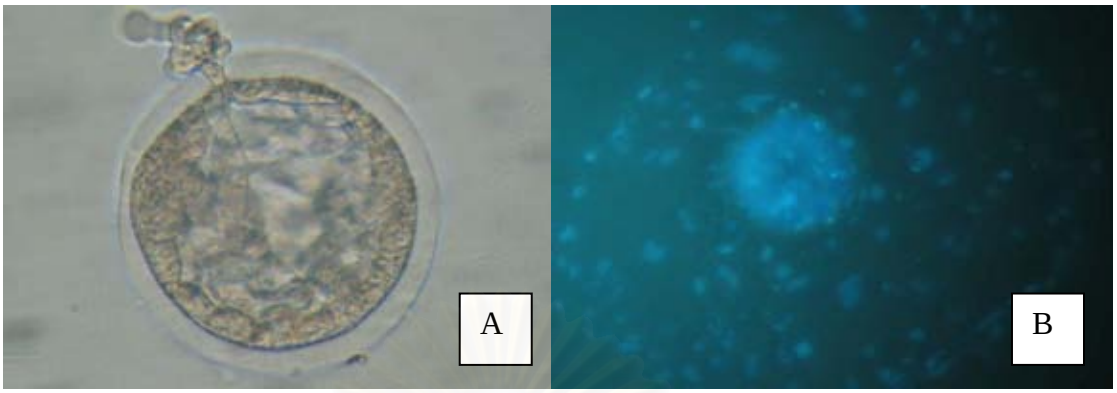

Figure 1.7: Cat-Rabbit cloned blastocyst under inverted microscope (A) (x300) and under fluorescence (B)(x300)

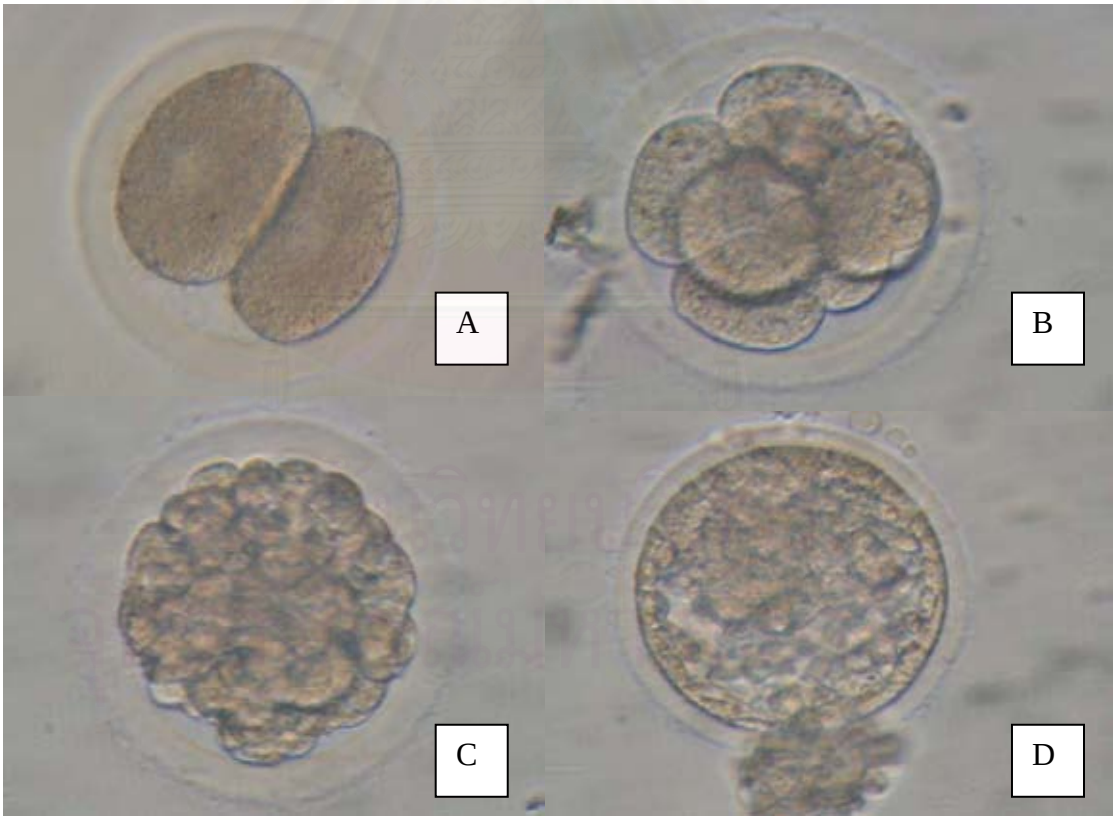

Figure 1.8: Marble cat-Rabbit cloned embryos at different stages: A, 2-cell stage (x300), B; 8-cell stages (x300), C; compact morulat (x300)**,** D; hatched blastocyst (x300)

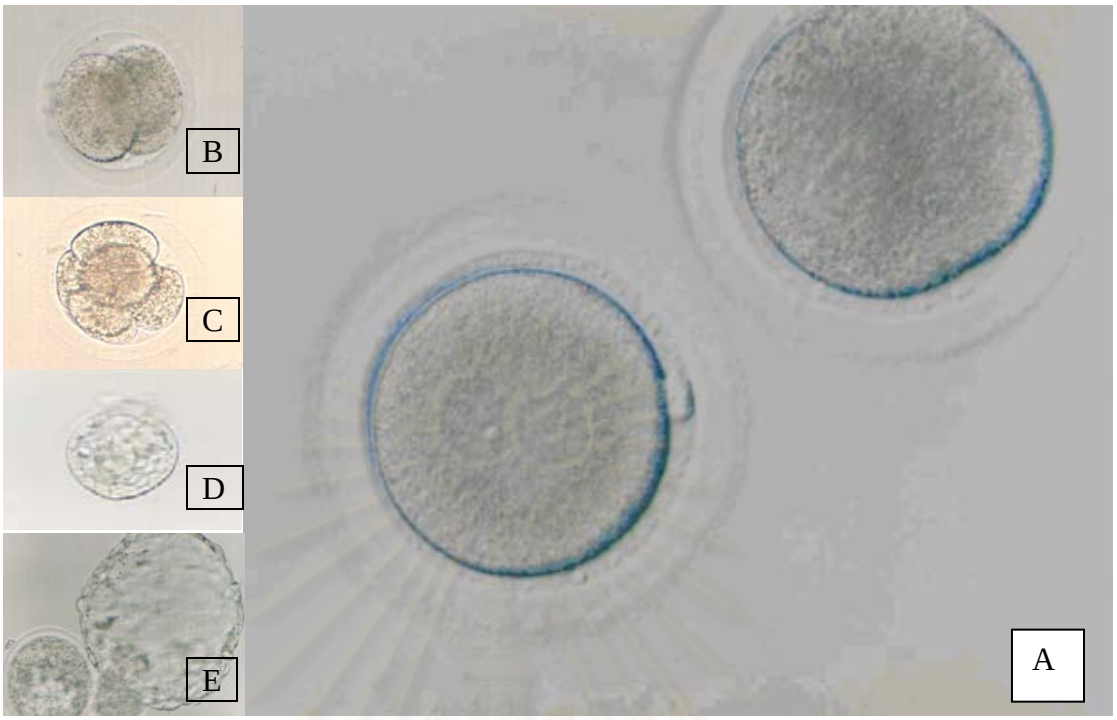

Figure 1.9**:** Elephant-Rabbit cloned embryos at different stages: A, 1-cell stage with two pronuclei formation (x400), B; 2-cell stage (x100), C; 5-cell stages (x100), D; blastocyst (x100)**,** E; hatched blastocyst (x100)

Discussion

With our somatic nuclear transfer technique, cloned rabbit embryos can be produced successfully (Techakumphu et al., 2004). This is a prerequisite technique for this study. The possibility of inter-species somatic cell nuclear transfer using rabbit oocyte as cytoplasm for transfer of somatic nuclei from different kinds of animals was demonstrated in the study. Cloned embryos at late morula and blastocyst stages can be produced by inter-species cloning with fibroblast cells from cattle, buffalo, pig, domestic cat, marble cat and elephant can be cultivated and passaged until kept in frozen form. This may help to establish the tissue bank of endangered species and to enlarge the basic knowledges on reprogramming of foreign nuclei to cytoplasm.

As shown in bovine (Dominko et al., 1999; Hwang et al., 2001; Kitiyanant et al., 2001; Li et al., 2002), rabbit cytoplasm of oocyte also support dedifferentiation and early development of introduced various type of nuclei, regardless of chromosome number or species. Seventy to 80% of inter-species cloned embryos can put in culture, which means about 20-30% were lysed after reconstruction, similarly to control group (rabbit-rabbit). This lysis percentage was lower than our previous study (Techakumphu et al., 2004). The limitation of exposure time to fluorescence during enucleation is one of key factors to reduce the lysis, that was performed in the study.

The donor cells presumptive starvation stage, G0 stage were used in the study. Data from Wilmut and colleagues (1997) indicated that using G0 stage nuclear donor provided more benefit to be reprogrammed than other phases of cell cycle.

There are two objectives of this study, the first one was to investigate the early embryonic development of cattle, buffalo and pig transferred into rabbit. As we know that the blocking stage in culture medium occurs at 4 to 8-cell stage in pig, at 8 to 16-cell stage in cattle and swamp buffalo. Related to our study, it was demonstrated that inter-species cloned embryos (cattle-rabbit, buffalo-rabbit, pig-rabbit) can develop beyond 16 cell stage in simple medium. Then, it can be suggested that rabbit cytoplasm can help to overcome the blockage of cattle, buffalo, pig embryos which is not seen in intraspecies cloning. However, it was remarked that no blastocyst was produced when buffalo nuclei used as donor cell. Kitiyanant et al., (2000) showed that buffalo-cattle hybrid embryos can develop to blastocyst with the rate of 39%, not different from cattle-cattle cloned embryos (33%). The differences of culture system, activation, fusion protocol and recipient oocyte source may be explained the different observation.

Secondly, the study of early stage embryonic development in wild animal such as marble cat and Asian elephant is impossible, thus using inter-species somatic cell nuclear transfer technique is valuable tool for embryo chronology studies and embryo production *in vitro* for a genetic conservation. According to IUCN, World Conservation Union, 5,485 animal species reported as threatened, including 180 species of mammals cited by Loi et al., 2001). A marble cat and an elephant are both wild animals considering as threatened or endangered species in Thailand. A marble cat is one from 36 of 37 species of Felidae is at risk of extinction. Around ten marble cats are still alive in National Zoological Park of Thailand, while only 2-3,000 heads of elephants persist and tends to decline from human destruction of natural ecosystems. This will affect to biodiversity in the country in the future.

Up to now, 4 species offspring derived from inter-species cloning born in a couple year ago including of gaur (Lanza et al., 2000), mouflon (Loi et al., 2001), banteng (Jansen et al., 2004) and African wild cat (Gomez et al., 2004). Normally, using recipient oocytes from close related species of donor nucleus is preferred, however, oocytes of wildlife especially marble cat or elephant are difficult to obtain. Cattle oocytes are good recipient cytoplasm for inter-species cloning, many attempts have been done intensively in past century such as in rat, sheep, pig (Dominko et al., 1999), buffalo (Kitiyanant et al., 2001), gaur (Lanza et al., 2000; Hammer et al., 2001), monkey (Dominko et al., 1999; Simerly et al., article in press), chicken (Tae et al., 2004), whale (Ikumi et al., 2003), and human (Chang et al., 2003). Using rabbit oocytes, recipient cytoplasm for inter-species cloning is also favorable, which was easy to obtain by administrating of gonadotropin hormone (Techakumphu et al., 2004) and a high number of matured oocytes (>10-40 oocytes) per rabbit were collected by oviductal flushing (data was not shown). Besides, several cloned mammalian embryos derived from rabbit oocytes have been reported such as panda (Chen et al., 2002; Li et al., 2002), cat (Wen et al., 2003), cattle (Techakumphu et al., 2005) and human (Chen et al., 2003). We hypothesized that marble cat, elephant genetics from somatic cell (fibroblast) could be modified in rabbit ooplasm. The experiment indicated that

marble cat and elephant cloned embryos developed comparable to rabbit cloned embryos, control group. This study revealed that marble cat or elephant nucleus can be reprogrammed in enucleated rabbit oocytes and developed to morula and blastocyst stage. The developmental capacity of marble cat-rabbit or elephant-rabbit (inter-species) and rabbit-rabbit (intra-species) cloned embryos was similar, which had blastocyst rate, 4%. We found that rabbit ooplasm was competent to dedifferentiate marbled cat or elephant donor nucleus, was not species specific. Accordingly, our study emphasized that rabbit oocytes are suitable for study of inter-species cloning as the universal recipient cytoplasm. Up to now, there is no report of inters-species cloned offspring when using a species possessing a high different chromosome numbers except the cloned intra-species, cat and wild cat (felidae) (Gomez et al., 2004) or cattle and wild cattle (bovidae) (Lanza et al., 2000). The use of somatic cell nuclear transfer to produce embryonic stem cells (ES cell) was envisaged as a means to produce cells or tissues for human autologous transfer (Chen et al., 2003). It was showed that embryonic stem cells by the transfer of human somatic nuclei into rabbit oocytes. The ntES cells maintain the capability of sustained growth in an undifferentiated stage, form embryoid bodies, which, on further induction, give rise to cell types such as neuron and muscle, as well as mixed cell populations that express markers representative of all three germ layers. However, this technique is underdebating in our country, and it seemed not to be allowed (personal communication with Prof. Pramuan Virutamasen).

The fusion-activation program is one of factor involving the cloning success. In our study, the fusion-activation program was followed in related to oocyte source. The result of comparison of electrical fusion protocol was studied in in elephant-rabbit clone indicated that inducing 3 DC pulses of 3.2 kV/cm at interval 20μs (E1) had superior fusion efficiency and cleavage rate which had 70.5% fusion rate and 65% cleavage rate, whereas inducing 2 DC pulses of 1.4 kV/cm at interval 80μs (E2) had 52.2% fusion rate and 47% cleavage rate. However, the competence of cloned embryos developed to blastocyst stage that derived from both fusion protocols provided the similar rate (E1: 7% vs. E2: 3%). Our fusion rates in each fusion protocol were similar to previous reports. E1 fusion protocol is common used for rabbit-rabbit somatic cell nuclear transfer (Chesne et al., 2002), following those protocol, the high fusion rate (73.5-91.3%) of rabbit-rabbit nuclear transfer units was obtained (Techakumphu et al., 2004). E2 fusion protocol was used for panda-rabbit (Chen et al., 2002) and rabbit-rabbit cloned embryos production (Wen et al., 2003), provided 57.2% vs. 63.1% fusion rate, respectively. According to the present results, we therefore suggested using E1 protocol as a fusion protocol of elephant-rabbit cloned embryos production, which provided a high number of fused and cleaved embryos. This study also showed in the first report that ear skin from stillborn elephant can be used for tissue culture and fibroblast cells derived can be used as donor nucleus for somatic cell nuclear transfer. Healthy cloned mouflon born on 2001 from granulose cells collected from female mouflons found dead in the pasture (Loi et al., 2001), shows obviously that inter-species cloning is valuable tool for preserving genetic biodiversity.

In conclusion, rabbit oocytes can be served as recipient cytoplasm for donor cells from different kinds of animals. The outcome of research is the basic knowledges on

reprogramming of foreign nuclei to cytoplasm, establishing the genetic banking and offering an opportunity to genetic rescue of endangered mammal. The next step of research will focus on the inter-species of cloning by using pig and cattle recipient oocytes and the study of totally differentiated cells such as liver cells or white blood cells on cloning success.

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CHAPTER 2

THE IN VITRO DEVELOPMENTAL OF INTER-SPECIES CLONED EMBRYOS, RECONTRUCTED USING PIG AND SWAMP BUFFALO OOCYTES

Abstract

The objective of the study was to investigate the development of intra- and inter-species cloned embryos derived from pig and swamp buffalo oocytes. The interaction between donor nuclei-recipient oocytes was observed. Pig and swamp buffalo ovaries were collected from slaughter house. The oocytes were selected for *in vitro* maturation and matured oocytes were used recipient cytoplasm. The pig and swamp buffalo's donor cells were obtained from ear stripping. Fetal rabbit fibroblasts used as a donor cell for interspecies cloning in the experiment. The development of pig-pig cloned embryos at cleavage (2-cell), premorula (8-16 cell), morula and blastocyst stages was 78%, 25.5%, 5.5% and 0%, respectively. The number of rabbit-pig cloned embryos reached the cleavage stage was similar with pig-pig cloned embryos. However, only 5.4% of rabbit-pig cloned embryos reached premorula stage. In addition, they could not develop beyond premorula stage. Only parthenogenic embryos were competence to develop to blastocyst stage. The developmental capacity of swamp buffalo-swamp buffalo, rabbit- swamp buffalo cloned embryos and parthenogenic embryos at premorula stages was 15%, 31.3% and 14.3%, respectively. In conclusion, the pig and buffalo oocytes are possibly be used as recipient cytoplasm for producing intra- and inter-species cloned embryos. However, the developmental rates of the cloned embryos are limited.

Key words: interspecies, cloned embryo, pig, swamp buffalo

Introduction

In the previous study, it was demonstrated that rabbit oocyte can be served as recipient oocytes for donor nuclei from different species as cow, swamp buffalo, pig or domestic cat, wild cat and elephant in nuclear transfer process. In addition, many attempts have been done to observe the capacity of bovine oocytes to reprogram the donor cells of several species including rats, sheep, pigs (Dominko et al., 1999), buffaloes (Kitiyanant et al., 2001), gaurs (Lanza et al., 2000; Hammer et al., 2001), monkeys (Dominko et al., 1999; Simerly et al., 2004), chickens (Tae et al., 2004), whales (Ikumi et al., 2003), and humans (Chang et al., 2003). In Thailand, among animal model, pig and buffalo can be served as oocyte resources for in vitro fertilization and nuclear transfer programs. Then, if the avaibility of the oocyte source, it will be benefit for intra- and inter-species nuclear transfer studies in the future. No study has been performed by using pig or swamp buffalo oocytes as recipient oocytes in nuclear transfer. The objective was to investigate interspecies somatic cell nuclear transfer using donor rabbit nuclei to pig or swamp buffalo oocytes.

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Materials and Methods

Chemicals

Chemicals were purchased Sigma Chemical Co. (St Louis MO), unless otherwise stated.

Establishment of donor cell line

Fibroblast from ear stripping of pig (Exp.I) and swamp buffalo (Exp.II) were used as donor cells. Fetal rabbit fibroblasts used as a donor cell for interspecies cloning in both experiments. Ear tissues were cut into pieces and cultured in Dulbecco's modified eagle medium (DMEM), supplemented with 20% FCS (Gibco, USA) in a 5% CO_2 incubator at 38.5°C. The fibroblast cells were subcultured at 70-80% confluence and cells at passage 5 were used as donor cells in starvation status. The cell starvation was performed by culture in DMEM, supplemented with 0.5% FCS for 5 days before usage.

Preparation of recipient oocytes

Pig and swamp buffalo ovaries were collected from slaughterhouse and transported within 2-4 h in room temperature to the laboratory. Immature oocytes were aspirated from 3-7 mm in diameter follicles. They were cultured in tissue culture medium (M199) supplemented with 10% FCS, 10 µg/ml luteinizing hormone and 10 µg/ml follicle stimulating hormone, at 38.5°C, under 5%CO_2, for 48 h in pig oocytes (Fig. 2.1) or 19-24 h in swamp buffalo oocytes. Matured oocytes which were identified as presenting the 1st polar body, were collected for nuclear transfer process.

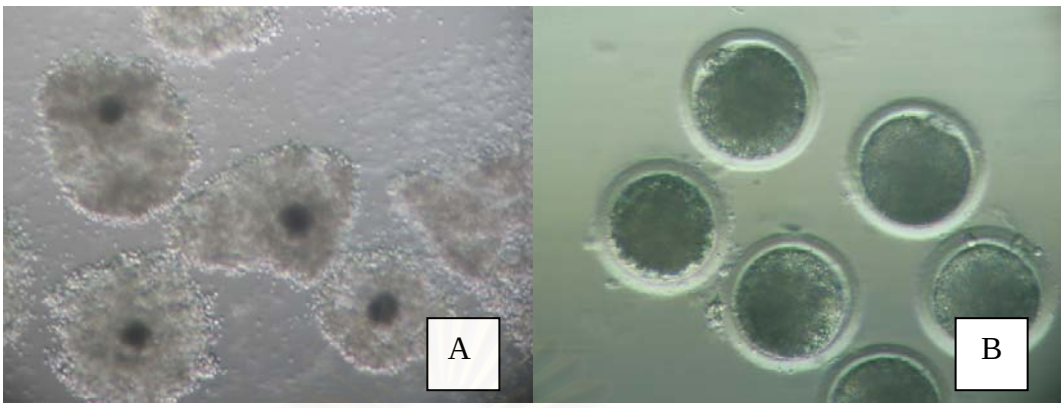

Figure 2.1: Pig matured oocytes before decoronization (A)(x100) and after (B) served as recipient oocytes (x200)

The enucleation and nuclear transfer

The procedure of enucleation and nuclear transfer has been described previously (Techakumphu et al., 2004). Briefly, the metaphase II (MII) plate and the 1st polar body were removed with small amount of cytoplasm by micromanipulation technique. Enucleation was performed under an inverted microscope. The enucleated oocytes were confirmed by staining with 10 µg/ml Hoechst 33342 and investigated under fluorescence microscope.

For nuclear transfer, a single donor cell was introduced in the perivitelline space in close contact with the plasma membrane of an enucleated oocyte as shown in Fig. 2.2.

Fusion and activation

The reconstructed oocytes were transferred to a fusion chamber containing 100 µl of fusion medium (0.3 M mannitol with 0.1 mM Ca^{2+} and Mg^{2+}). Fusion was performed by inducing electrical pulses; 3 DC 1.5 kV/cm, 3 pulses, 60 µs in pig oocytes (modified from Polejaeva et al., 2000) and 2 DC 2.1 kV/cm for 20 µs in buffalo oocytes (modified from Saikhun et al., 2002). The reconstructed oocytes were then washed in M199 supplemented with 10% FCS 3 times and incubated in the same medium for 1 h, at 38.5 ^{o}C, in a humidified air containing 5 % CO_2. The reconstructed oocytes were checked for fusion efficiency with the stereomicroscope. The fused pig oocytes were activated by the same electrical pulses and incubated in 10 µg/ml of cycloheximide and 10 µg/ml of cytochalacin B, at 38.5^{o}C, in a humidified air containing 5 % CO_2, for 3-4. In swamp buffalo, the fused couplets were activated by exposure 5 µM ionophore, at room temperature, for 6 min and subsequent cultured in 10 µg/ml of cycloheximide and 5 µg/ml of cytochalacin B, at 38.5 ^{o}C, in humidified air containing 5 % CO_2, for 5 h.

The activated oocytes were cultured in drop of the culture medium (NCSU23 for the pig oocyte and SOF for the swamp buffalo oocytes), supplemented with 10% FCS covered with mineral oil, at 38.5^{o}C, in 5% CO_2 in air.

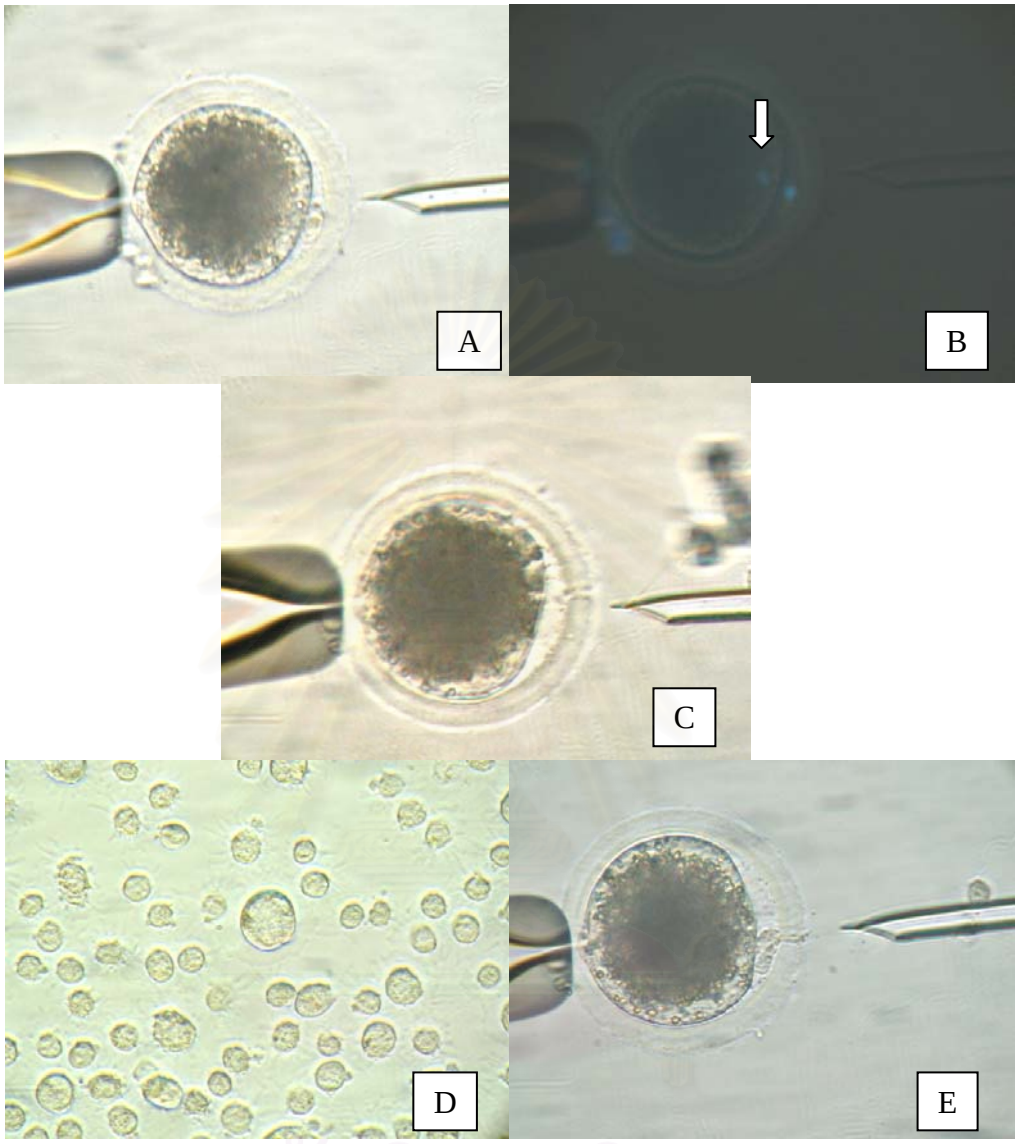

Figure 2.2: MII chromosome (arrow) of matured oocyte was located; before (A) and after Hoechst staining (B), The MII chromosome was removed by micropipetting (C), and trypsinized donor cells (D) were transferred to perivitelline space which contacting the enucleated oocyte (E) (x 200)

Embryo culture and assessment of development

The embryo development was observed and recorded every 24h under a microscope for 7 days.

Experimental design

Experiment I: Nine replicates were done to investigate the development of intra- and inter-species cloned embryos (pig-pig and rabbit-pig) derived from matured pig oocytes. The parthenogenic embryos served as control.

Experiment II: Six replicates were done to investigate the development of intra- and inter-species cloned embryos (swamp buffalo-swamp buffalo and rabbit-swamp buffalo) derived from matured swamp buffalo oocytes. The parthenogenic embryos served as control.

Statistical analysis

Differences in the percentages of the development of cloned embryos were determined by chi-square analysis.

Result

Experiment I: *In vitro development of intra-, inter-species cloned and parthenogenic embryos derived from pig oocytes*

The maturation rate of pig oocytes cultured *in vitro* was 54.6% (n=920). The fusion efficiency of pig-pig couplets was greater than those of rabbit-pig couplets, was 72.3% and 69%, respectively. The development of pig-pig cloned embryos (n=55) at cleavage, premorula, morula and blastocyst stages was 78%, 25.5%, 5.5% and 0%, respectively (Fig. 2.3). The number of rabbit-pig cloned embryos reached the cleavage stage was similar with pig-pig cloned embryos. However, only 5.4% of rabbit-pig cloned embryos reached premorula stage (Fig. 2.4). In addition, they could not develop beyond premorula stage. Only parthenogenic embryos (n=71) were competence to develop to blastocyst stage (9.8%) (Fig. 2.5). (Table 2.1)

Table 2.1: Comparison of the development of intra-, inter-species cloned and pathenogenic embryos derived from pig oocytes

Donor cell	n	fused	cleavage	8-16 cell	morula	blastocyst
- Oocyte				n (%)		
Pig-Pig	76	55 (72.3)	43 (78) [a]	14 (25.5) [a]	3 (5.5)[a]	0[a]
Rabbit-Pig	81	56 (69)	44 (78.6) [a]	3 (5.4) [b]	0[a]	0[a]
Parthenote	71	-	59 (83) [b]	22 (31) [a]	22 (31)[b]	7 (9.8)[b]

[a, b] Values with different superscripts within the same column differ significantly ($P<0.05$)

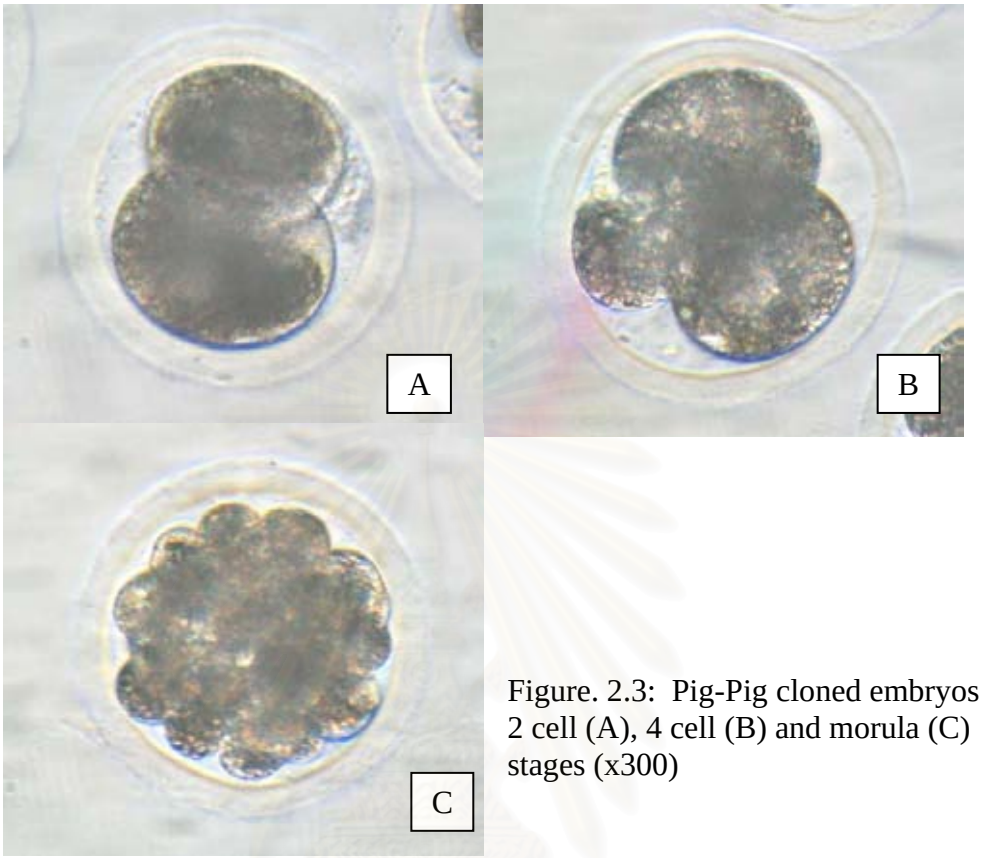

Figure. 2.3: Pig-Pig cloned embryos at; 2 cell (A), 4 cell (B) and morula (C) stages (x300)

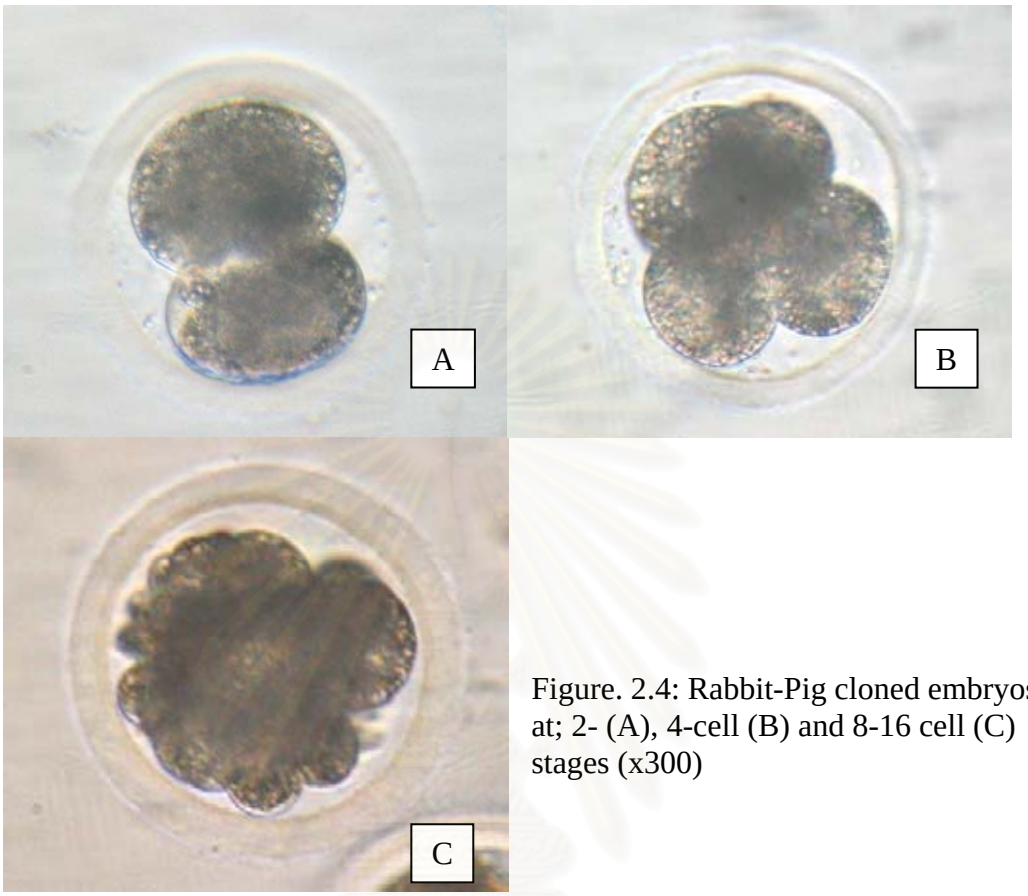

Figure. 2.4: Rabbit-Pig cloned embryos at; 2- (A), 4-cell (B) and 8-16 cell (C) stages (x300)

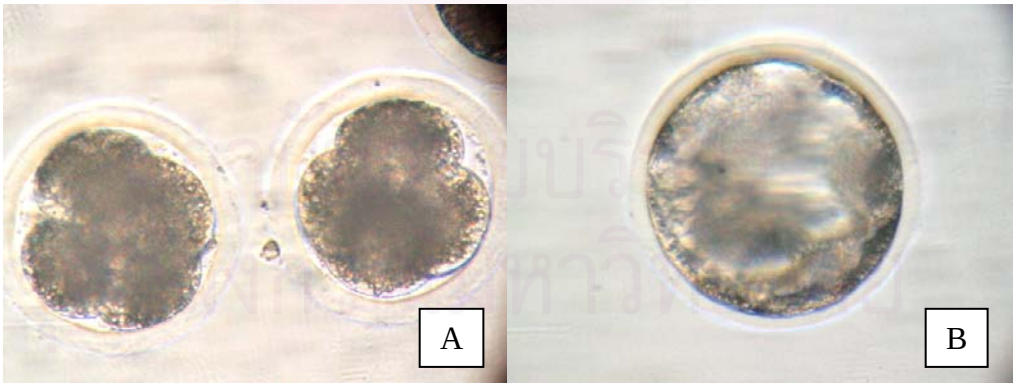

Figure 2.5: Parthenogenic pig embryos at cleavage (A) and blastocyst (B) stages (x 200)

Experiment II: *In vitro development of intra-, inter-species cloned and pathenogenic embryos derived from swamp buffalo oocytes*

The maturation rate of swamp buffalo oocytes cultured *in vitro* was 43% (n=315). The fusion efficiency of swamp buffalo-swamp buffalo (n=45) and rabbit-swamp buffalo (n=29) couplets were 44% and 55% respectively. The developmental capacity of swamp buffalo-swamp buffalo (n=20) (Fig. 2.6), rabbit- swamp buffalo (n=16) (Fig.2.7) cloned embryos and parthenogenic embryos (n=35) at cleavage and premorula stages was 15%, 31.3% and 14.3%, respectively. (Table 2.2)

Table 2.2: Comparison of the development of intra-, inter-species cloned and pathenogenic embryos derived from swamp buffalo oocytes

Donor cell - Oocyte	n	fused	cleavage	8-16 cell	morula	blastocyst
		n (%)				
SB-SB	45	20 (44)	3 (15)	3 (15)	0	0
Rabbit-SB	29	16 (55)	5 (31.3)	5 (31.3)	0	0
Parthenote	35	-	5 (14.3)	5 (14.3)	0	0

SB: swamp buffalo, [a, b] Values with different superscripts within the same column differ significantly ($P<0.05$)

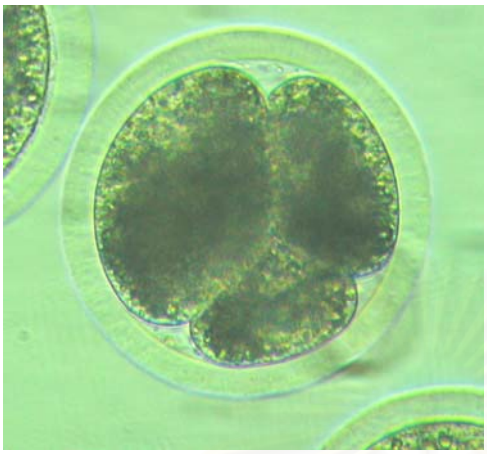

Figure 2.6: Swamp Buffalo-Swamp Buffalo cloned embryos at cleavage stage (x 300)

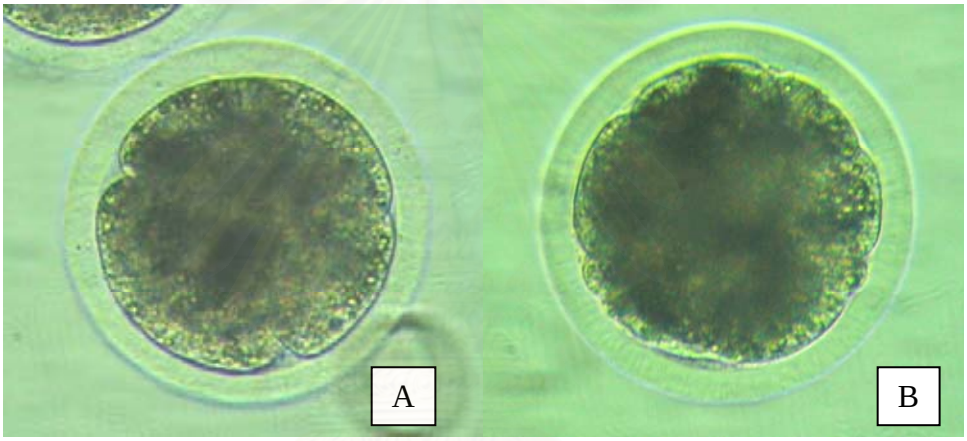

Figure 2.7: Rabbit-Swamp Buffalo cloned embryos at 2- (A) and 8-16 cell (B) stages (x 300)

(Sousa et al., 2002).

For inter-species cloning using pig oocytes, the study demonstrated the feasibility of producing rabbit cloned embryos. However, it is likely to be species specific between the donor nuclei and ooplasm because only intra-species cloned embryos could be developed beyond the premorula stage. These suggest that pig oocytes are not suitable for interspecies (rabbit-pig) somatic cell nuclear transfer. Nevertheless, our study showed an opportunity to produce pig-pig cloned embryos which opens the window for the application of gene-targeting technology, thus allowing for genetic modifications, including gene knock out of the donor cell (Polejaeva et al., 2000). These are subsequently being applied for xenotransplantation, the production of pharmaceutical proteins and enhancement of pig breeding programs (Betthauser et al., 2000). The fibroblasts of a sow transgenic for several important genes such as porcine lactoferrin, human factor IX (Lee et al., 2003), human accelerating factor (DAF, CD55) and N-acetylglucosaminyltransferase III (GnT-III) (Fujimura et al., 2004) had been studied. Lived born cloned transgenic piglets were produced by the

whole-cell intracytoplasmic injection method (Lee et al., 2003). Unfortunately, the surviving pigs suddenly when less 6 months of age due to heart failure, a condition dubbed “adult clone sudden death syndrome”. These deaths have underscored the dangers and risks of supplying organs from cloned animals. Accordingly, many attempts should be made to clarify the factors affecting the development of cloned offspring, so cloned products are more safe and practically for use in medical field in the future.

Bovine oocytes are named as “universal recipient cytoplasm”, to support the development of nuclei from other species that may benefit for embryology studies. Many attempts have been done to observe the capacity of bovine oocytes to reprogram the donor cells from several species including rats, sheeps, pigs (Dominko et al., 1999), buffaloes (Kitiyanant et al., 2001), gaurs (Lanza et al., 2000; Hammer et al., 2001), monkeys (Dominko et al., 1999; Simerly et al., 2004), whales (Ikumi et al., 2003), humans (Chang et al., 2003), yak and dog (Murakami et al., 2005). Because buffaloes are considered closely related to bovines, we hypothesized that buffalo oocytes can be also used as a universal host cytoplast for somatic cell nuclear transfer. The study demonstrates the possibility to produce interspecies cloned embryos from buffalo oocytes. However, the low developmental capacity of cloned embryos was recorded. This may due to the quality of the oocytes collected from the slaughter house. The interspecies cloned embryos were more competent at developing than the intraspecies, imply that species specific event between the donor nuclei and the recipient oocytes did not occur in the study. One possible reason that influences cloned embryo development is donor cell line. Rabbit cell lines were collected from a foetus but those of swamp buffalo were obtained from adult animal. The finding is corresponding to previous report in buffalo-bovine cloning, that fetal fibroblast gave a greater percentage of cloned embryo development than adult somatic cells (Saikhun et al., 2002).

In conclusion, the pig and buffalo oocytes are possibly be used as recipient cytoplasm for producing intra- and inter-species cloned embryos. However, the developmental rates of the cloned embryos are limited.

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CHAPTER 3

THE IN VITRO DEVELOPMENT OF CLONED RABBIT EMBRYOS DERIVED FROM VARIOUS PRIMARY SOMATIC CELL TYPES

Abstract

Rabbit nuclear transfer embryos were reconstructed by electrofusion of various donor cells types, liver, kidney, lung, cumulus and fibroblast cells, with recipient enucleated metaphase II (MII) ooplasm. The fusion rate of reconstructed oocyte with freshly lung cells is significant higher ($p<0.05$) than those cumulus, foetal fibroblast, kidney and liver cells respectively (62.0, 47.5, 47.4, 32.4 and 0.0 %), however, the cleavage rate were not significant different among the donor cell types except the liver cells. Only 2.5% of oocyte reconstructed from cumulus cells could develop to the blastocyst stage. This study showed that the primary cells from lung and kidney can be reprogrammed similar to fresh cumulus and foetal fibroblast cells by nuclear transfer technique. The procedure to improve the development of those reconstructed oocyte by various donor cell type should be investigated.

Key words: donor cell type, nuclear transfer, rabbit oocyte

Introduction

Since Dolly, the first cloned animal, had been cloned by using the somatic cells from the mammalian gland (Wilmut et al., 1997), there were several species have been successfully cloned such as mice (Wakayama et al., 1998), cows (Cibelli et al., 1998., Vignon et al., 1998), goat (Baguisi et al., 1999), pig (Onishi et al., 2002), cat (Shin et al., 2002), rabbit (Chesne et al., 2002), mule (Woods et al., 2003), horse (Galli et al., 2003), rat (Zhou et al., 2003) and dog (Lee et al., 2005). These animals usually were cloned by using fibroblast cells recovered from skin or muscle a donor cell. In rabbit, our previous studies (Techakumphu et al., 2004), showed the success of cloned embryos production by two sources of donor cells, cumulus and cultured fibroblast cells. It is suggested that the donor cell type could affect the efficiency of development of cloned embryo (Tian, 2005; Kato et al., 1998, Wakayama et al., 1998). In bovine, it have been proved that various cell types such as oviduct, heart, liver, or kidney can be used as the donor cells and cloned life offspring can be obtained, however the result was different (Kato et al., 2000). No study has been done by using other differentiated cells of internal organ such as liver, kidney and lung for nuclear transfer in rabbit. This study was designed to examine *in vitro* development of the cloned embryos derived from various other donor cell types under certain nuclear transfer protocols.

Materials and Methods

Chemicals

Chemicals were purchased Sigma Chemical Co. (St Louis, MO), unless otherwise stated.

Preparation of donor cells

The foetal fibroblast, cumulus cells, liver, kidney and lung cells from rabbit were used as donor cells. The foetal fibroblast cells come from the frozen stock (-196^{o}C) since the 1st year project in 2004 while cumulus cells were stripped by pipetting from ovulated matured ooocytes after incubation in 0.1% hyluronidase for 15 min. For other kinds of cell preparation, the liver, kidney and lung were immediately removed from rabbit after humane killing by CO_2 suffocation. The organs were washed to be free of blood with phosphate buffer saline (PBS) and kept in normal saline with antibiotics, pennicilline and streptomycin. Subsequently, they were washed several times with 0.9% NaCl to remove clotting blood. For the liver, it was digested by perfusion of collagenase solution. When digestion is complete, the liver is cut open, and the hepatocytes are dispersed in phosphate buffer solution, washed, and suspended in growth medium. Fresh hepatocytes were used in the experiment.

For kidney and lung cell recovery, the inner tissue was isolated by removal of outer membrane and placed on petri dish, and minced it in small pieces. These small pieces of tissue were rinsed with 20 ml of 0.25% trypsin EDTA into conical flask (250 ml) and stirred at room temperature for 15 min. for cell separation. The cells were twice filtered through sterile gauses into sterile beaker (250 ml) for separating cells from tissue. The cells were from the filtered solution with 5 ml fetal calf serum

supernatant was removed and the precipitant was washed with PBS (w/o Ca^{2+}, Mg^{2+}) three times. Three ml. of DMEM +20% FCS were added to the pellet and single cells were separated by pipetting, isolation and incubated in the same media at 5% CO_2 in air for 1-2 days before use.

Preparation of recipient oocytes

The ovulated mature oocytes (Figure 3.1) recovered from a mature New Zealand White rabbit doe after superovulation, were used as recipient oocytes as described previously (Techakumphu et al., 2004). The oocytes were incubated in TCM-199 supplemented with 0.1% hyaluronidase, for 15 min in 5% CO_2 at 38.5^{o}C. The cumulus cells were removed from the oocytes by gentle pipetting. Later, the cumulus freed oocytes were incubated in TCM-199 + 2.5M Hepes supplemented with 7µg/ml cytochalasin B for 30 min.

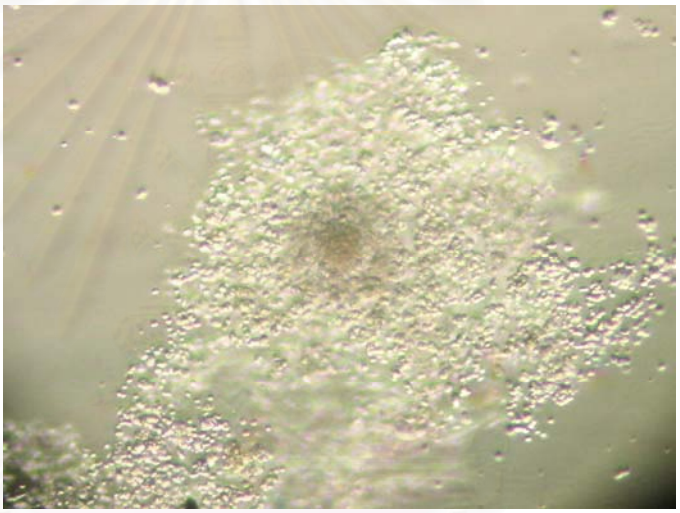

Figure 3.1**:** Rabbit matured oocytes, collected by oviductal flushing (x100)

Enucleation and nuclear transfer

The procedures of enucleation and nuclear transfer have been described previously (Techakumphu, et al., 2004). Briefly, the metaphase II plate and the first polar body together with small amount of cytoplasm, were removed by a micromanipulation technique. Enucleation were performed under an inverted microscope and the successful enucleation was confirmed after staining with 5 µg/ml Hoechst 33342 and investigated under fluorescence microscope.

For nuclear transfer, a single donor cell was introduced in the perivitilline space in close contact with the plasma membrane of an enucleated oocyte as shown in figure. 3.2.

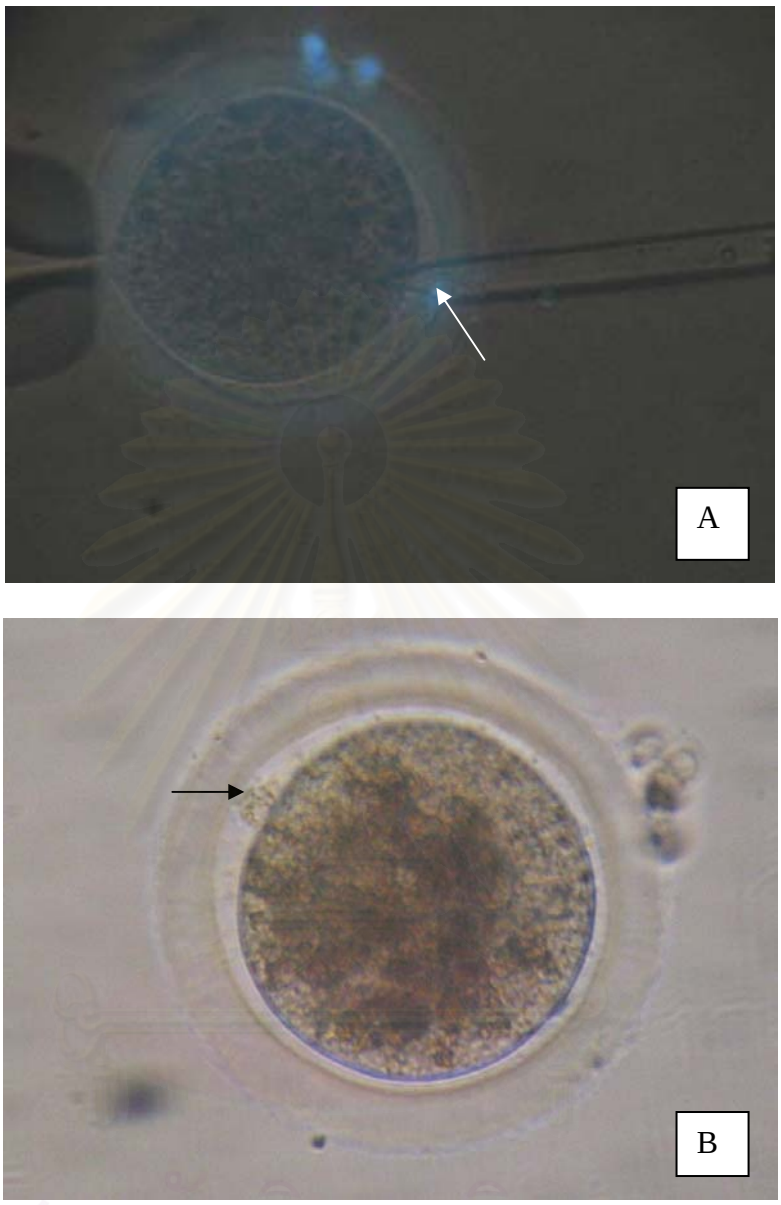

Figure 3.2: Enucleation was performed by genetic materials aspiration (arrow) (A) (x200). A single donor cell (arrow) was placed into perivittelline space contacting with the rabbit enucleated oocytes (B) (x 300)

Fusion and activation

The reconstructed oocytes were transferred to a fusion chamber containing 100 µg of fusion medium (0.3 M mannitol with 0.1 mM Ca^{2+} and Mg^{2+}). Fusion was induced by 3.2 kV/cm, 3 pulses, 20 µs (Chesne et al., 2002). The reconstructed oocytes were then washed in M199 (Gibco, USA) supplemented with 10% FBS (Gibco, USA) three times and incubated in the same medium for 1 h at 38.5 ^{o}C in a humidified air containing 5% CO_2. The reconstructed oocytes were checked for fusion with the stereomicroscope and the fused oocytes were activated by the same

electrical pulses and incubated in 5 μg/ml of cyclohexamide and 2mM of 6-DMAP for 1 h.

The activated oocytes were washed in TCM-199+10% FCS for at least three times before transferring them into a small culture drop of synthetic oviductal fluid (SOF) or B2 culture medium (Menezo, France) covered with mineral oil, at 38.5^{o}C, in 5% CO_2 in air.

Embryo culture and assessment of development

The embryo development was observed and recorded for cleavage up to blastocyst stage every 24 h under a microscope.

Statistical analysis

Differences in the percentages of oocytes developing to a particular stage between electrical pulses and type of donor cell will be determined by chi-square analysis.

Result

From 278 enucleated oocytes, 32.3% (n=90) fused with the donor cells and 20.5% of these oocytes (n= 57) developed into cleavage stage. The highest fusion rate was found in lung cell group while they were around 47.5, 47.4 and 32.4% in cumulus, fibroblast, kidney cell groups. No fusion was found in liver cell group. The fusion and developmental rate to blastocyst stage of the cloned embryos derived from various donor cell types has shown in the table 3.1. Only cloned oocytes fused with cumulus cells developed to blastocyst.

Table 3.1: Comparison of the development of cloned embryos derived from various cell types

Donor cell type	n	fused/ cultured	cleavage	8-16 cell	morula	blastocyst
		n (%)				
liver	82	0 (0) [d]	0(0) [b]	0(0) [b]	0(0)	0(0)
kidney	68	22(32.4) [c]	18(26.5) [a]	8(11.8) [a]	0(0)	0(0)
lung	50	31(62.0) [a]	15(30.0) [a]	5(10.0) [a]	0(0)	0(0)
cumulus	40	19(47.5) [b]	13(32.5) [a]	5(12.5) [a]	1(2.5)	1(2.5)
fibroblast	38	18(47.4) [b]	11(28.9) [a]	8(21.1) [a]	0(0)	0(0)
total	278	90(32.3)	57(20.5)	26(9.4)	1(0.4)	1(0.4)

[a, b, c, d] Values with different superscripts within the same column differ significantly (P<0.05)

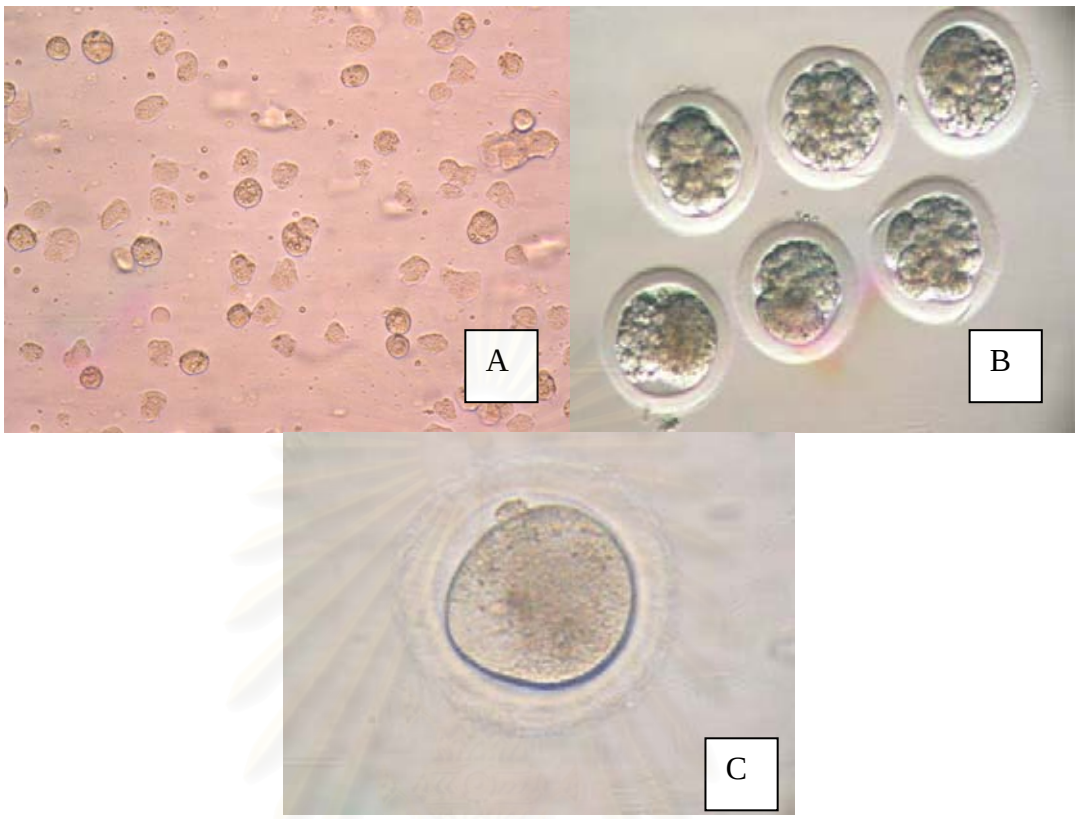

Figure 3.3: Rabbit liver cells (A), Fragmented embryos (B), and non-fused reconstructed unit (C) (x 200)

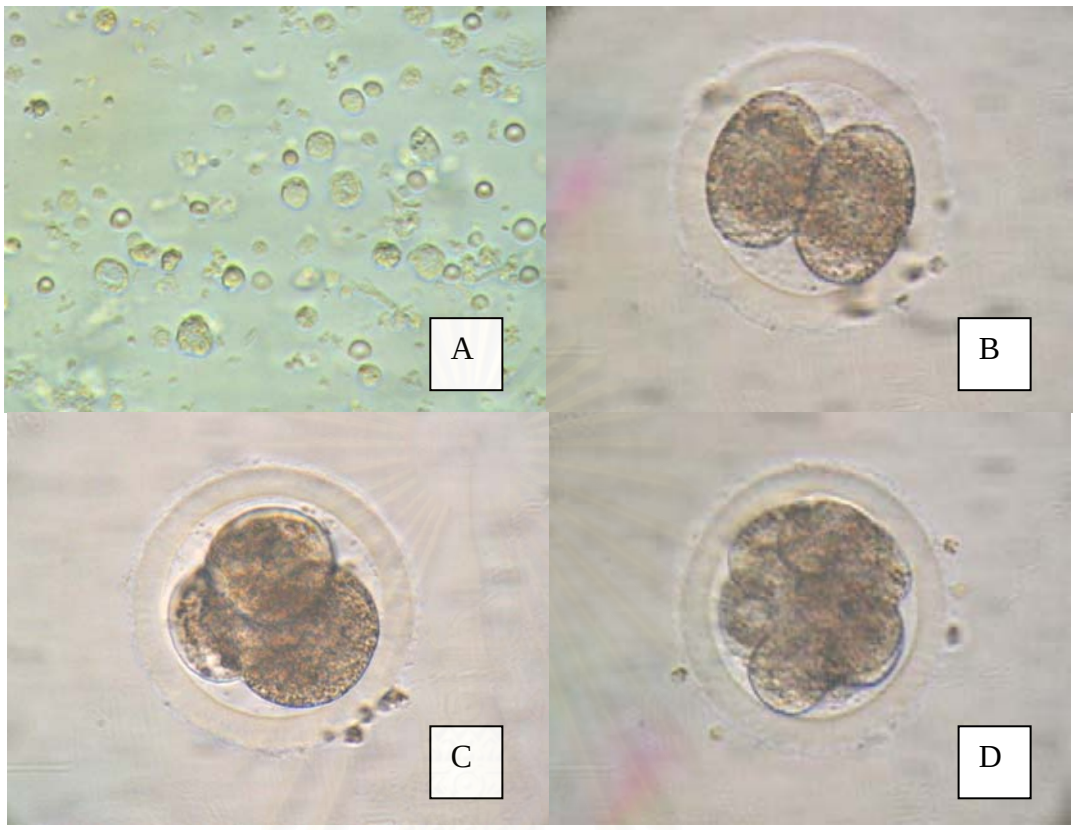

Figure 3.4: Rabbit lung cells (A), rabbit cloned embryos at; 2-cell stage(B), 4-cell (C) and 8-cell stages (D) (x 200)

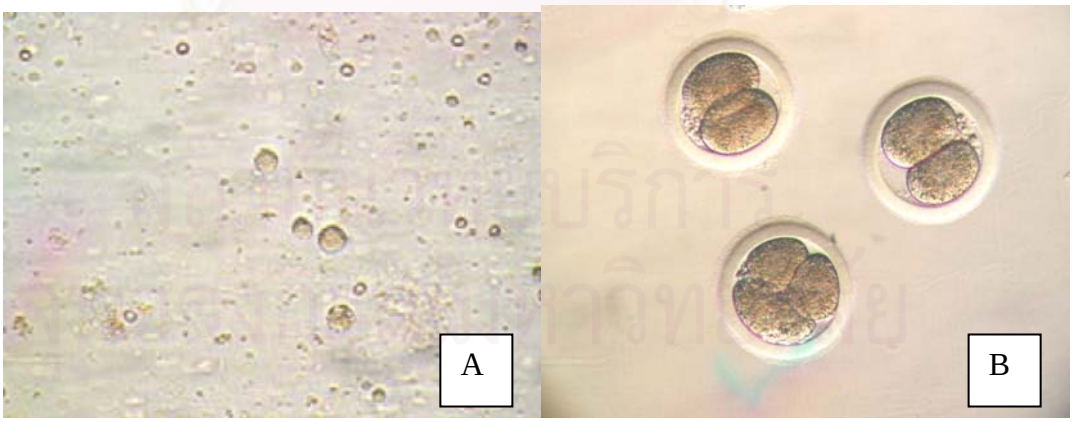

Figure 3.5: Rabbit lung cells (A), rabbit cloned embryos at 2-4 cell stage (B) (x 100)

Discussion

This work was undertaken in order to study the developmental competence of nuclear transfer into rabbit oocytes using donor nuclei from various cell types. As previous study reported by Techakumphu et al. (2004) showed that rabbit oocyte could support the development of cloned rabbit embryos *in vitro* using cumulus or fibroblast cell as a donor cell. In normal practice, fibroblast cells recovered and cultivated from skin or muscle were used as donor nuclei for somatic cell cloning. Only few experiments were performed by using other kinds of donor nuclei. In the literatures, showed that many somatic cell types, including mammary epithelial cells, ovarian cumulus cells, fibroblast cells from skin and internal organs, various internal organ cells, Sertoli cells (Ogura et al., 2001), macrophage and blood leukocytes (Galli et al., 1999; Hochedlinger et al., 2002) have been successfully utilized for nuclear transfer. However, these differentiated cells of internal organ such as liver, kidney and lung never been studied in rabbit. In this study, the fresh cells obtained from liver, kidney and lung were used immediately after preparation. Our results showed that the donor cell type can significantly affect the *in vitro* development of cloned rabbit embryos. This observation is correlated to Tian (2005), who observed the difference of cloning competence *in vitro* and *in vivo* of three cell types: ovarian cumulus, mammary epithelial and skin fibroblast cells from the same donor. The same observation conducted by Wakayama et al. (1998) using microinjection technique to produce clone mice using nuclei from somatic cells, Sertoli, neuronal and cumulus cells. They observed that some enucleated oocytes receiving Sertoli or neuronal neuronal nuclei developed *in vitro* and implanted following transfer, but non developed beyond 8.5 days post coitum; however, a high percentage of enucleated oocytes receiving cumulus nuclei developed *in vitro*. Kato et al. (2000) also compared cells from the liver, testis, skin, ear along with cumulus and oviductal cells and concluded that cumulus and oviduct epithelial cells are the most suitable for nuclear donors. The cumulus cells used as donor cells in the study provide a superior development compared to the others, which agreed with Forsberg et al. (2002) and Tian (2005) who found that cumulus cells are the most effective cell type for cloning both *in vitro* and full-term development. The highest calving rate 15.2% was obtained using cumulus cells while fetal genital ridge cells and fibroblast cells produced a 9% calving rate (Forsberg et al., 2002).

It is remarked that the liver cells are not fused with cytoplasmic membrane of recipient oocyte compared to other cell types. It is disagreed with Kato et al.(2000) in cattle which more than 50% of liver cells were fused with cytoplasm of recipient oocyte. It is plausible that the failure of fusion comes from unsuitable electrical pulses which could not break liver cell membrane or nature of liver cell membrane. While kidney cells provided a similar fusion rate to those of cumulus and fibroblast cells. Interestingly, the fusion rate of using the lung cells was highest compared to other donor cell types but there were not significant different of cleavage rate among donor cells types. More research on the appropriate gamme of electrical pulse for fusion and activation on different kinds of cells should be performed.

It is noted that the reprogramming process and subsequent cleavage development can happen by using kidney, lung and other two frequently used cell type, fresh cumulus and fibroblast cells. However, the blastocyst development from all donor cell types was very low. From our result suggested that non-passaged kidney and lung cells have a low potential to generate premorula compared to fibroblasts. There are two possible explanations, firstly, the cell cycle of the donor cells has a major influence on the reprogramming process and subsequent blastocyst development (Stice et al., 1993) and secondly, primary cells are less homogenous in cell populations (Kubota et al., 2000), which might result in overall poorer development. Nevertheless, this study showed that the primary cells from lung and kidney can be reprogrammed similar to fresh cumulus and foetal fibroblast cells using nuclear transfer technique. The procedure to improve the development of those reconstructed oocyte by various donor cell type should be investigated in the future.

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