Kasetsart J. (Nat. Sci.) 44 : 610 - 620 (2010)

Production of Cloned Asian Elephant Embryos Using an Interspecies Somatic Cell Nuclear Transfer (iSCNT) Technique

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ABSTRACT

This study was carried out to investigate the feasibility of using interspecies somatic cell nuclear transfer (iSCNT) techniques in Asian elephants (*Elephas maximus*), using elephant fibroblasts as donor cells and rabbit oocytes as the recipient cytoplasts. Elephant fibroblasts were collected post-mortem from the ear skin of a female Asian elephant and cultured in vitro. Monolayer fibroblasts were trypsinized and used for SCNT. The comparative study showed that the blastocyst rates from iSCNT were significantly lower (P<0.05) than for rabbit SCNT and rabbit parthenogenesis (19.4, 56.7, and 70.3%, respectively). The study tried to improve the efficiency of iSCNT by using phytohemagglutinin-P (PHA-P) to increase the fusion rate and nocodazole to synchronize donor cells in the G1 stage of the cell cycle. The fusion rate of elephant-rabbit couples treated with PHA-P was significantly increased (47.5%) compared with the non-treated group (26.2%; *P*<0.05). The cloned embryos could develop into blastocysts (27.8%), which was significantly (*P*<0.05) higher than for the non-treated cells group (14.4%). By using this protocol, Asian elephant blastocysts can be produced by iSCNT of fibroblast cells into rabbit cytoplasts with the use of nocodazole and PHA-P. The blastocyst rate of cloned elephant embryos using rabbit cytoplasts was more than 25% and may have potential in further studies on elephant preservation.

Keywords: iSCNT, Asian elephant fibroblast, rabbit cytoplast, PHA-P, nocodazole

INTRODUCTION

Elephants are exceptionally charismatic megavertibrates; the Asian (*Elephas maximus*) and African (*Loxodonta africana*) species are listed respectively as endangered and threatened by the World Conservation Union. Many researchers have been trying to study semen collection, evaluation, cryopreservation and artificial insemination to conserve this species (Thongtip *et al*., 2004). However, in female elephants, it is possible to study their hormonal profile to detect the estrous cycle and pregnancy diagnosis (Brown *et al*., 1999). Other assisted reproduction biotechnologies, such as in vitro embryo production, embryo transfer and cloning techniques, are not easily applied to the rescue of the elephant species.

The production of cloned embryos by SCNT of post-mortem endangered animal

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Received date : 23/11/09 Accepted date : 02/03/10

fibroblasts holds considerable potential. As oocytes from endangered species are scarce, domestic species oocytes are often used as cytoplasts for iSCNT. Since the cytoplast plays an important role in reprogramming and the ability to receive nuclei from other species, cytoplasm may be correlated to the embryonic stage of maternal to zygotic transition in the species. Several studies have shown that rabbit cytoplasts can support the early development of embryos produced by iSCNT from various species, including pandas (Jiang *et al*., 2004), monkeys (Yang *et al*., 2004), pigs (Chen *et al*., 2005), capra-ibexes (Jiang *et al*., 2005), cows (Zhang *et al*., 2004) and humans (Chen *et al*., 2003). The feasibility of iSCNT and blastocyst formation is possible after transfer of somatic cells into oocytes that have been obtained from a closely related species. The incomplete nuclear reprogramming and the low blastocyst rate of iSCNT are still big problems.

To improve the efficiency of SCNT, cell cycle coordination between the oocyte and donor cell must be refined. The coordination between the donor nucleus and recipient cytoplasm requires that the introduced nucleus be prevented from undergoing premature chromosome condensation that is caused by reducing the level of the meiosis-promoting factor in the recipient cytoplasm, or that donor nuclei are synchronized in the G1/S phase of the cell cycle, which precludes their ability to re-replicate DNA (Piotrowska *et al*., 2000).

The present study investigated the development of cloned Asian elephant embryos produced by iSCNT using enucleated rabbit oocytes as recipient cytoplasts and compared the quality of cloned elephant embryos derived from iSCNT with cloned rabbit and parthenogenetic embryos.

MATERIALS AND METHODS

Animals and chemicals

The protocols for animal care and handling were approved by the Animal Experiments Committee of the Graduate School, Chiang Mai University, Thailand. Unless otherwise stated, all chemicals used were purchased from Sigma Chemical Co.

Preparation of recipient oocytes

Female mixed-breed rabbits (age 5-10 months) were used as oocyt donors. Each rabbit was injected intramuscularly with 150 IU human menopause gonadotropin (hMG; IVF-M®, Korea) and intravenously with 100 IU human choriogonadotropin (hCG; IVF-M®, Korea) 96 h apart. The superovulated animals were operated on 14-15 h after the administration of hCG and the oocytes were flushed from the oviduct using tissue culture medium 199 (TCM-199) with HEPES buffer containing 1% fetal bovine serum (FBS). The cumulus cells were removed from the oocytes by gently pipetting in TCM-199 containing 0.1% hyaluronidase. The cumulus-free oocytes with an extruded first polar body and metaphase II (M II) chromosomes were selected for further experiments.

Preparation of donor cells

Ear skin tissue from a dead young female elephant (2 years of age) and abdominal skin tissue from the superovulated rabbit were collected and stored in normal saline supplemented with penicillin 1,000 IU/ml and streptomycin 1 mg/ml at 4°C within 4 h after collection. The tissue was washed three times in collection medium, sliced into small pieces and cultured in a 35-mm Petri dish, covered with 2 mL of tissue culture media, containing Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS, penicillin and streptomycin at 38.5°C in 5% CO_2 humidified atmosphere. After a confluent fibroblast

monolayer was formed around the piece of tissue, the cultured cells were trypsinized, washed twice with PBS and resuspended in a 35-mm Petri dish containing DMEM supplement with 10% FBS, penicillin and streptomycin at 38.5°C in 5% CO_2 humidified atmosphere.

Isolation of individual fibroblasts: Cultivated elephant and rabbit fibroblast cells were harvested by washing with Ca^{2+}- and Mg^{2+}- free PBS, then treated with 0.25% trypsin and 0.01% EDTA solution for 1 min. Cells were collected in complete cell culture medium and pelletized by centrifugation at 140 g for 5 min, then reseeded and an individual cell was used as a donor cell for iSCNT and SCNT within 1 h. Elephant and rabbit fibroblast cells at passage 4-10 were used as nuclear donors.

Synchronization of elephant fibroblasts: The elephant fibroblast monolayer was treated with 1 μg/mL nocodazole in DMEM supplemented with 10% FBS at 38.5°C in 5% CO_2 for 12-16 h (Figure 1A) to arrest the cells in the M phase of the cell cycle, washed with PBS (free magnesium and calcium) and then cultured in DMEM supplemented with 10% FBS for 2 h to arrest them in G1/S transition. The treated elephant fibroblasts were trypsinzed for iSCNT within 1 h.

Fusion and activation

The nuclear transfer (NT) methods were performed as described by Yin *et al*. (2002), Briefly, the cumulus-cell-free oocytes were cultured in TCM-199 medium containing 6.0 μg/mL of and 7.5 μg/mL of cytochalasin B at 38.5°C,

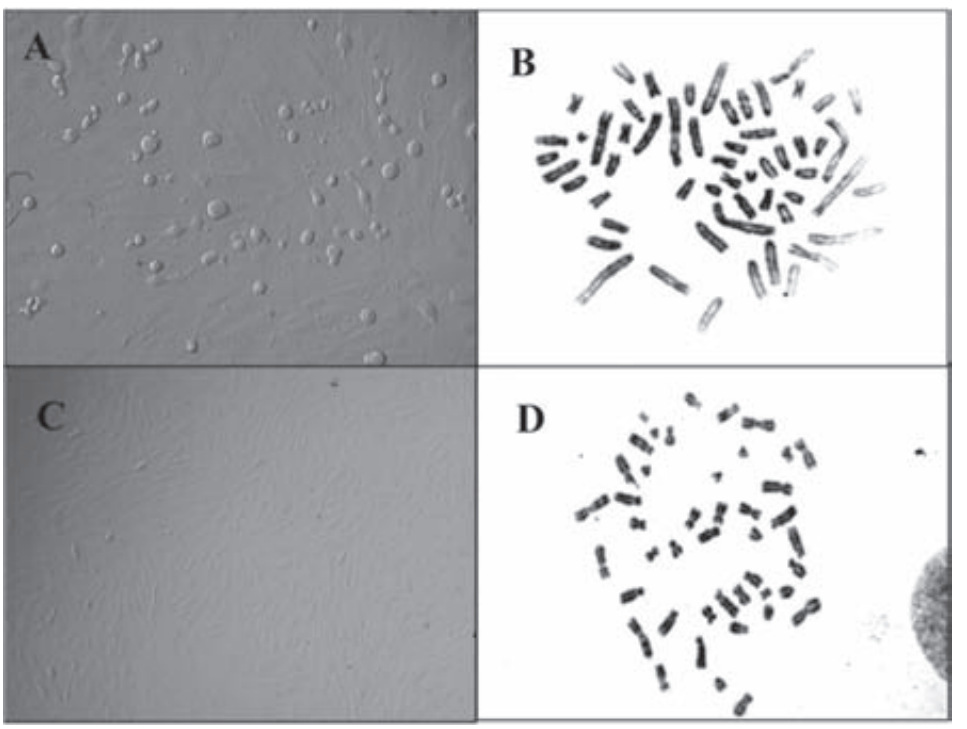

Figure 1 The karyotyping of elephant and rabbit fibroblast cells. **A:** the nocodazole treated elephant fibroblasts monolayer (magnification 200x), **B:** normal chromosomes 2n=56 of elephant fibroblast cell (magnification 1000x), **C:** confluent of rabbit fibroblast monolayer (magnification 100x) and **D:** Normal chromosomes 2n=44 of rabbit fibroblast cell (magnification 1000x).

under 5% CO_2 humidified air for between 40 min to 2 h. The zona pellucida of each oocyte was dissected near the first polar body and a small amount (less than 10%) of the cytoplasm containing the meiotic spindle was squeezed out (Figure 2). Subsequently, an individual elephant or rabbit fibroblast (donor) cell was inserted into the perivitelline space of the enucleated rabbit oocyte (cytoplast) as shown in 2D. The cell-cytoplast complex was transferred into a fusion solution containing 0.25 M mannitol, 0.5 mM $MgSO_4$ and 0.1 mM $CaCl_2$ and placed between two electrodes in a cell fusion chamber of an electrofusion apparatus overlaid with this solution. The couples were fused with double direct current (DC) pulses of 1.4 kV/cm for 80 μs. The couples were rested in TCM-199 supplemented with 10% FBS for 30 min. After that, fused oocytes were activated with double DC pulses of 1.2 kV/cm for 25 μs.

To increase the efficacy of the fused cell-cytoplast complex in iSCNT, the cell-cytoplast complex was incubated in 300 μg/mL PHA-P for 10 min (Du *et al*., 2006) before fusion.

Parthenogenetic activation (PA)

Rabbit cumulus-free oocytes were equilibrated into a fusion solution containing 0.25 M mannitol, 0.5 mM $MgSO_4$ and 0.1 mM $CaCl_2$ and placed between two electrodes in a cell fusion chamber of an electrofusion apparatus overlaid with this solution. The oocytes were electrically

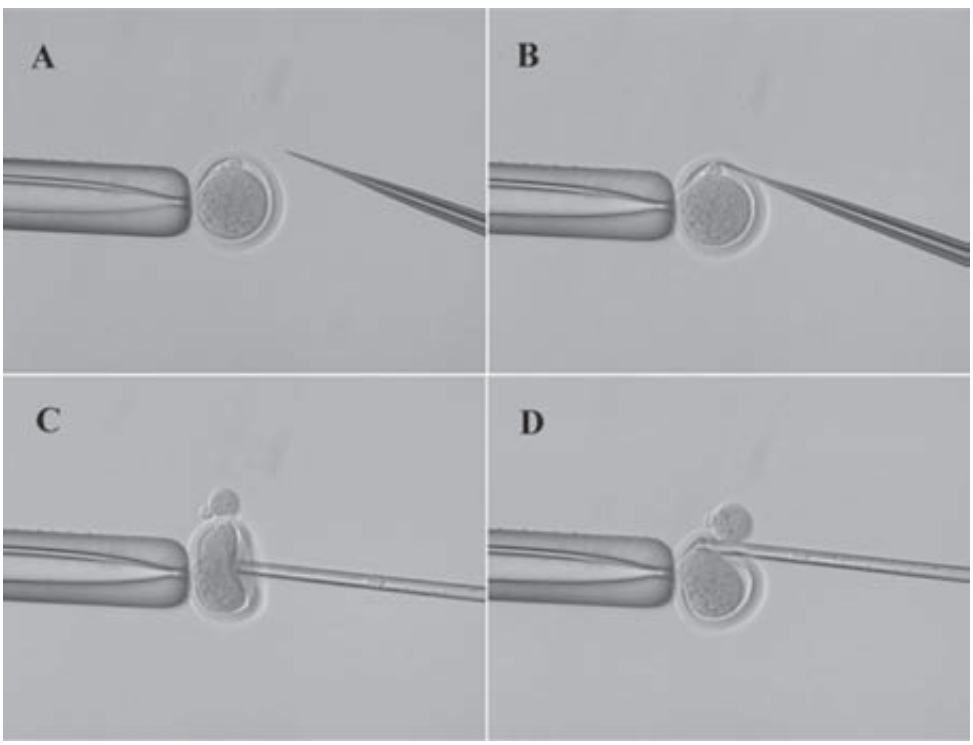

Figure 2 The single steps of enucleation and cell insertion. **A:** the rabbit cumulus free-oocyte with PB1 and extruded cytoplasm with M II, **B** and **C:** the zona pellucida of the oocyte was dissected near the first polar body and the cytoplasm containing the M II spindle was squeezed out; and **D:** elephant fibroblast cell was inserted in the perivitelline space of the rabbit cytoplast (magnification 200x).

activated with two DC pulses of 1.4 kV/cm for 80 μs and rested in TCM-199 supplemented with 10% FBS for 30 min. After that, oocytes were repeat activated with two DC pulses of 1.2 kV/cm for 25 μs.

In vitro culture and evaluation of reconstructed embryos

The iSCNT, SCNT-reconstructed embryos were activated by incubation for 3 h in a culture medium containing 2 mM 6-dimethylaminopurine (6-DMAP) at 38.5°C in humidified atmosphere containing 5% CO_2. The rabbit PA oocytes were activated under the same conditions and used as a control for the reconstructed embryonic development. Elephant iSCNT, rabbit SCNT and PA embryos were cultured in Knockout-Dulbecco's minimum essential medium containing 10% FBS and penicillin-streptomycin at 38.5°C in humidified atmosphere containing 5% CO_2 for 5 d. Half volumes of fresh medium were replaced every 48 h.

The quality of the iSCNT, SCNT and PA embryos was determined after 120 h of culture. The total cell number of blastocysts was counted by differential staining (Thouas *et al.*, 2001). Blastocysts were incubated in 100 μL of BSA-free Hepes buffered MTF medium with 1% Triton-X 100 and 100 μg/mL propidium iodide for 10 s or until the trophectoderm visibly changed color to red and shrank slightly, as monitored visually using a microscope, and then fixed in 500 μL of absolute ethanol with 25 μg/mL bisbenzimide (Hoechst 33342) and stored at 4°C overnight. The inner cell mass (ICM) appeared green, the trophectoderm (TE) was red and total cell numbers of blastocysts were counted under a fluorescence microscope.

Chromosomal analysis

Chromosome preparation of fibroblast cells and iSCNT embryos were each undertaken as previously described by Parkanyi *et al.* (2004). Briefly, elephant and rabbit fibroblast cells were cultured with 0.6 μg/mL of for 4 h prior to the cells being trypsinized. After 30 min of hypotonic treatment with 0.075 M potassium chloride and fixation in methanol:acetic acid, 3:1, the resuspended cells were spread on frozen glass slides, air-dried, and stained with 10% Giemsa solution. The chromosomes on the stained slide were evaluated under a microscope. The chromosome analysis was carried out using 15 G-banded metaphase spreads for the structural evaluation for elephant (Suwattana *et al.*, 2000; Houch *et al.*, 2001) and rabbit (Parkanyi *et al.*, 2004), as shown in Figures 1B and 1D.

Ten elephant-rabbit iSCNT blastocysts were randomly selected to evaluate the chromosomes by karyotyping with G-banding metaphase spreads as described by Suwattana *et al.* (2000) and Houch *et al.* (2001).

Statistical analysis

The fusion rate and developmental competence of cloned embryos were analyzed statistically using a Chi-square test. The numbers of ICM, TE and total cell numbers of blastocysts were analyzed statistically using an ANOVA test in SPSS version 14 and differences at $P<0.05$ were considered significant.

RESULTS

In vitro development of elephant iSCNT, rabbit SCNT and rabbit PA embryos

Elephant iSCNT and rabbit SCNT embryos were developed on the same timeline up to the blastocyst stage. They reached the 2-cell, 4-cell, morula and blastocyst stages at 24, 48, 96 and 120 h, respectively. However, the rabbit PA embryos were early developed to 4-cell, 8-cell, early blastocyst and expanded blastocyst stages at 24, 48, 96 and 120 h, respectively (Figure 3). The in vitro development of iSCNT, SCNT and PA embryos is shown in Table 1. Fusion rates were not different between elephant SC-rabbit oocytes

and rabbit SC-rabbit oocytes (45.6 versus 52.3%, P>0.05). However, the developmental rate at various stages of the cloned elephant iSCNT embryos was statistically different (P<0.05) from that of the rabbit SCNT and PA embryos. Development to the blastocyst stage of elephant iSCNT (19.4%) was significantly (P<0.05) lower than the rabbit SCNT and PA embryos (56.7 and 70.3%, respectively).

The numbers of ICM, TE and total cell numbers of elephant iSCNT blastocysts, rabbit SCNT and PA blastocysts are presented in Table 2. The numbers of ICM, TE and total cell numbers of blastocysts in the iSCNT group were not

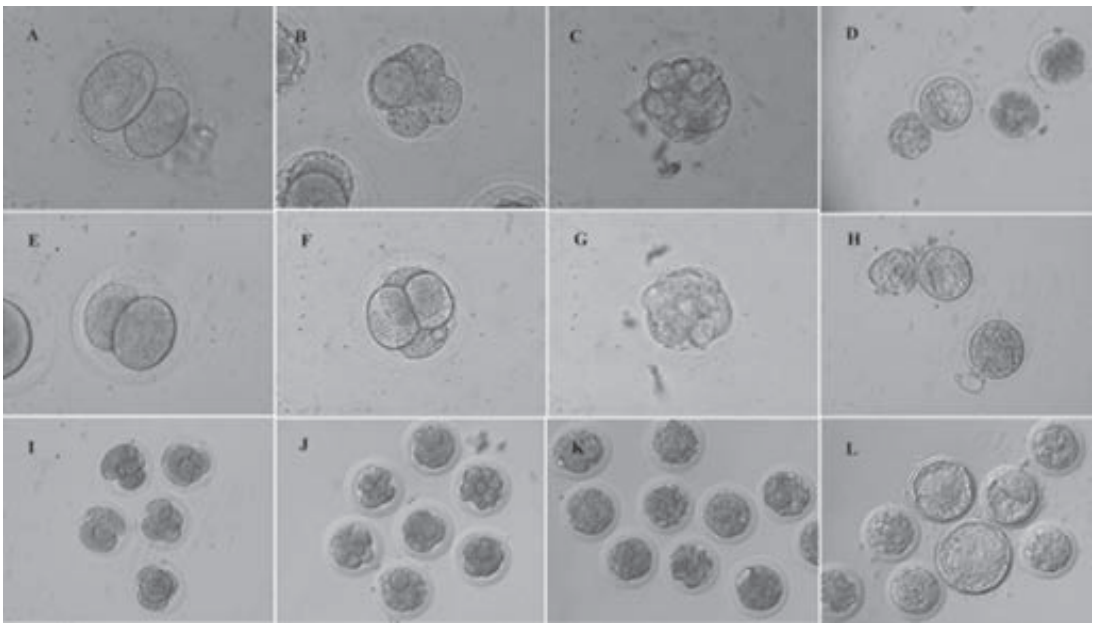

Figure 3 Elephant iSCNT (A-D), rabbit SCNT (E-H) and rabbit PA (I-L) embryos at 24, 48, 96 and 120 h, respectively, after in vitro embryo culture.

Table 1 The fusion rate and developmental competence of embryos between elephant-rabbit iSCNT, rabbit-rabbit SCNT and rabbit PA.

Treatment	No. of oocytes	No. of embryos fused (%)	No. of cleavage embryos (%)	No. of morula (%)	No. of blastocysts (%)
Elephant – rabbit NT	158	72 (45.6) [a]	51 (70.9) [c]	28 (38.9) [b]	14 (19.4) [c]
Rabbit – rabbit NT	128	67 (52.3) [a]	59 (88.1) [b]	43 (64.2) [a]	38 (56.7) [b]
Rabbit PA	148	-	137 (92.6) [a]	116 (78.4) [a]	104 (70.3) [a]

Values with different superscripts within each column are significantly different ($P< 0.05$).

Fusion rate = fused oocytes/reconstructed oocytes.

Developmental rate of cloned embryos at different stages = number of embryos/number of fused embryos.

different (P>0.05) from those in the rabbit PA, though both of them were lower than in the rabbit SCNT embryos.

***In vitro* development of elephant iSCNT with PHA-P treatment**

The *in vitro* development of elephant iSCNT embryos derived from the elephant fibroblast cell-rabbit cytoplast complex was treated with PHA-P before fusion, as shown in Table 3. PHA-P was used to increase the fusion rate of couples of elephant fibroblast and rabbit cytoplast. The fusion rate of the PHA-P treated group was significantly (P<0.05) higher (47.5%) when compared with the non-treated group (26.2%). The developmental competence was not different (P>0.05) between the PHA-P treatment and the non-treatment groups.

Nuclear transfer with elephant fibroblast treated with nocodazole

The *in vitro* development of elephant iSCNT derived from elephant fibroblast cells treated with nocodazole was compared with the no nocodazole-treated group and the results are presented in Table 4. The iSCNT embryos derived from elephant fibroblast treated with nocodazole had a significantly (P<0.05) higher rate of development to the morula and blastocyst stages than those derived from the no nocodazole-treated embryos.

Chromosomal analysis of iSCNT embryos and donor cells

Karyotyping of the elephant fibroblasts used as donor cells to produce elephant-rabbit iSCNT embryos showed a set of normal diploid chromosomes (2n= 56). Similar chromosome numbers were also present in all the elephant iSCNT blastocysts (n=10) randomly selected for karyotyping analysis by the G-banding technique (Figure 4).

Table 2 Numbers of blastocysts from elephant-rabbit iSCNT, rabbit-rabbit SCNT and rabbit PA.

Treatment	Number of blastocysts	Number of ICM ± SEM	Number of TE ± SEM	Total cell number ± SEM
Elephant –rabbit NT	20	23.0 ± 0.7 [b]	71.6 ± 4.0 [b]	94.7 ± 4.5 [b]
Rabbit –rabbit NT	20	34.8 ± 1.8 [a]	102.2 ± 5.5 [a]	137.0 ± 6.5 [a]
Rabbit PA	20	27.7 ± 1.1 [b]	76.8 ± 3.6 [b]	104.5 ± 4.2 [b]

Values with different superscripts within each column are significantly different ($P< 0.05$).

Table 3 Effect of PHA-P on fusion rate and developmental competence of elephant-rabbit somatic cell nuclear transfer.

Treatment	No. of oocytes	No. of oocytes fused (%)	No. of cleavage embryos (%)	No. of morula (%)	No. of blastocysts (%)
+ PHA-P	198	94 (47.5) [a]	82 (87.2) [a]	40 (42.6) [a]	24 (25.5) [a]
No PHA-P	206	54 (26.2) [b]	42 (77.8) [a]	16 (29.6) [a]	14 (25.9) [a]

Values with different superscripts within each column are significantly different ($P< 0.05$).

Fusion rate = fused oocytes/reconstructed oocytes.

Developmental rate of cloned embryos at different stages = number of embryos/number of fused embryos.

Table 4 Effect of elephant fibroblast donor cells treated or not treated with nocodazole on developmental competence of elephant-rabbit iSCNT.

Treatment	No. of oocytes	No. of embryos fused (%)	No. of cleavage embryos (%)	No. of morula (%)	No. of blastocysts (%)
+ nocodazole	195	97 (49.7) [a]	83 (85.7) [a]	45 (46.4) [a]	27 (27.8)[a]
No nocodazole	197	90 (45.7) [a]	76 (84.4) [a]	21 (23.3) [b]	13 (14.4)[b]

Values with different superscripts within each column are significantly different ($P< 0.05$).

Fusion rate = fused oocytes/reconstructed oocytes.

Developmental rate of cloned embryos at different stages = number of embryos/number of fused embryos.

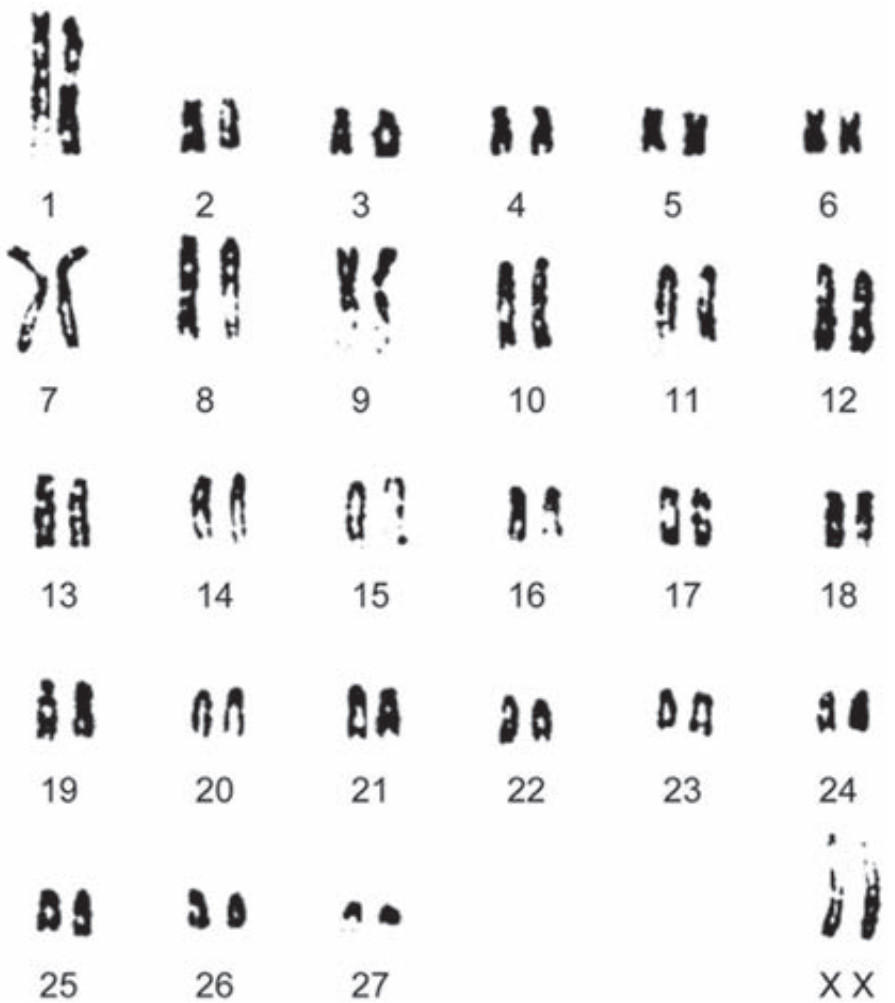

Figure 4 The G-banding karyotype of elephant-rabbit iSCNT embryos, normal chromosomes 2n=56 (XX).

DISCUSSION

This is the first report on SCNT of elephant reconstructed embryos using rabbit cytoplast. The study demonstrated that elephant somatic cell nuclei can be reprogrammed to develop to the blastocyst stage at least and the success rate was high when compared with other iSCNT techniques using rabbit cytoplast (Jiang *et al*., 2005; Zhao *et al*., 2006). But the low development to the blastocyst stage and the cell number of blastocysts of iSCNT, when compared with rabbit SCNT and PA, may be a result of inefficient nuclear reprogramming, mitochondrial heteroplasmy and incompatibilities between the donor nucleus and recipient cytoplast. Asian elephant fibroblast cells could be reprogrammed in rabbit cytoplasts, but there was a low number of fusion couples.

To improve the efficiency of eletrofusing elephant fibroblast cells into rabbit oocytes, PHA-P was used (Zhang *et al*., 2008). PHA has been widely used in nuclear transfer with humans (Tesarik *et al*., 2000) and in bovine oocytes (Keefer *et al*., 1994; Du *et al*., 2006) to increase the fusion rate and developmental competency of NT embryos. The current experiment demonstrated that PHA-P at 300 μg/mL for 10 min was efficient in increasing the fusion rate and was harmless to the oocytes as shown by the equal development of blastocysts from the treated and non-treated cells.

The cell cycle of the donor cells has a major influence on the reprogramming process and subsequent blastocyst development. The present study showed that the donor nuclei were synchronized in the G1 phase of the cell cycle by nocodazole, similar to the method of serum starvation. Synchronized donor embryonic cells with nocodazole were used in the nuclear transfer, which could improve the development of bovine embryos (Tanaka *et al*., 1995; Samake and Smith, 1996). The mechanism of the cells in the G0/G1 stage assures the normal (2n) ploidy of the cloned embryos (Agalioti *et al*., 2000). The present study found that elephant fibroblasts should be induced in the G1 stage of the cell cycle by treatment with nocodazole, before being used in iSCNT.

There are still remains technical to establish iSCNT and further work of molecular (DNA methylation, histone acetylation) and cellular mechanisms are needed to determine interspecies embryos express proper reprogramming factors for other somatic nuclei after iSCNT. It seems clear that a deeper understanding of epigenetic reprogramming will be needed before iSCNT is successful and pleuripotent stem cells are produced.

CONCLUSION

Elephant embryos can be produced by iSCNT of Asian elephant fibroblast cells into rabbit cytoplasts. These achievements will open up a new way to use more widely elephant iSCNT embryos for derived elephant stem cells.

ACKNOWLEDGEMENTS

This work was mainly supported by the Chiang-Mai Biotech Center and partly by research grants from the Graduate School, Chiang Mai University. The authors are especially grateful to the Royal Initiate Projects for support in providing specimens. Thanks are recorded also to Amorn Santhi and Jamjit Doungpunta for their support and assistance in the laboratory.

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