Original Article

Cloned Asian Elephant (*Elephas maximus*) Embryos Reconstructed from Rabbit Recipient Oocytes

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Abstract

The information about reproductive biology, especially embryo development of Asian elephant (*Elephas maximus*) generated from naturally fertilization is lacking. In the present study, somatic cell nuclear transfer (SCNT) was applied as an alternative way to produce the elephant embryos. The fibroblasts derived from ear skin of Asian elephant and rabbit were used as the donor cells and rabbit oocytes were used as the recipient cytoplasm. The objectives of the study were 1) to find the optimal conditions for fusion and activation by using electrical pulses (experiment I) and 2) to investigate the *in vitro* development of cloned Asian elephant in comparison to clone rabbit embryos (experiment II). Enucleation was accomplished by aspiration of the first polar body and the metaphase II plate together with a small amount of cytoplasm. The donor cells were transferred into the perivitelline space of the enucleated oocytes. In experiment I, sixty-one of elephant-rabbit reconstructed units were fused by electrical pulses E1 (3.2 kV/cm, 3 pulses, 20 µs) and sixty-nine units were fused by E2 (2.0 kV/cm, 2 pulses, 20 µs) in 0.3 M mannitol with 0.1 mM Ca^{2+} and Mg^{2+}. The fused units were activated by using the same electrical pulses and incubated in activation medium for 1 h. Subsequently, the activated embryos were cultured in B2 medium containing 2.5% fetal calf serum and the developmental rate was observed daily for 7 days. The results showed that the fusion and cleavage rates of elephant-rabbit cloned embryos fused and activated by E1 were significantly higher than E2 ($p<0.05$). Electrical pluses program E1 was selected for further investigation in experiment II. The fusion and activation rated of elephant-rabbit units displayed significantly higher than rabbit-rabbit units ($p<0.05$). However, by comparison of cloned embryo development between elephant- and rabbit-rabbit units, the development from cleavage throughout the blastocyst stage of elephant-rabbit cloned units was similar to those of rabbit-rabbit units. In conclusion, fusion and activation protocol of E1 is suitable for elephant-rabbit SCNT and the elephant nuclei could be reprogrammed and developed to blastocyst stage in enucleated rabbit oocytes. The present study provides the fundamental knowledge for further investigation of conservation and therapeutic aims, including cloned elephant embryo development *in vivo* after transfer, rescuing valuable elephant and establishment of elephant embryonic stem cells.

Keywords: Asian elephant, interspecies somatic cell nuclear transfer, rabbit oocyte

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บทคัดย่อ

การสร้างโคลนตัวอ่อนช้างจากโอโอไซต์กระต่าย

มงคล เตชะกำพุ[1] รัฐจักร รังสิวิวัฒน์[1,2] ปราณี นำชัยศรีค้า[2] อัมพิกา ทองภักดี[1,3]*

ข้อมูลเกี่ยวกับชีววิทยาของระบบสืบพันธุ์ โดยเฉพาะการพัฒนาของตัวอ่อนของช้างเอเชีย (*Elephas maximus*) ที่เกิดจากการปฏิสนธิตามธรรมชาตินั้นยังไม่มีรายงาน การศึกษานี้จึงนำเทคโนโลยีการย้ายฝากนิวเคลียสมาใช้จึงเป็นวิธีทางเลือกในการสร้างตัวอ่อนของช้าง ซึ่งใช้เซลล์ไฟโบรบลาสต์ที่แยกได้จากใบหูช้างเป็นเซลล์ต้นแบบและใช้โอโอไซต์ของกระต่ายเป็นเซลล์ตัวรับ การศึกษานี้มีวัตถุประสงค์ ในการทดลองที่ 1 เพื่อหาสภาวะที่เหมาะสมของการเชื่อมติดของเซลล์ไฟโบรบลาสต์ของช้างกับโอโอไซต์กระต่ายและการกระตุ้นให้พัฒนาไปเป็นตัวอ่อนด้วยกระแสไฟฟ้า และการทดลองที่ 2 เพื่อศึกษาการพัฒนาภายนอกร่างกายของโคลนตัวอ่อนช้าง เปรียบเทียบกับโคลนตัวอ่อนกระต่าย นำเอานิวเคลียสของโอโอไซต์กระต่ายออกโดยดึงเอาโพลาร์ บอดี้ที่ 1 และแผ่นนิวเคลียสระยะเมทาเฟส ทู ออก ฉีดเซลล์ต้นแบบของช้างเข้าบริเวณระหว่างเปลือกและขอบไซโตพลาสมของโอโอไซต์ที่ได้นำเอานิวเคลียสออกไปแล้ว ในการทดลองที่ 1 เซลล์โอโอไซต์กระต่าย ที่ได้รับการฉีดเซลล์ต้นแบบช้าง จำนวน 61 ใบ ถูกเชื่อมเข้ากับเซลล์โอโอไซต์กระต่ายด้วยกระแสไฟฟ้าโปรแกรมที่ 1 (E1) ขนาด 3.2 กิโลโวลต์/ซม. จำนวน 3 ครั้ง นาน 20 ไมโครเซกัน และอีก 69 ใบถูกเชื่อมด้วยโปรแกรมที่ 2 (E2) ขนาด 2.0 กิโลโวลต์/ซม. จำนวน 2 ครั้ง นาน 20 ไมโครเซกัน ในน้ำยาแมนิทอล 0.3 โมลาร์ และ 0.1 มิลลิโมลาร์ ของแคลเซียมและแมกนิเซียม หลังจากนั้นกระตุ้นด้วยกระแสไฟฟ้าและเลี้ยงในน้ำยากระตุ้นตัวอ่อนนาน 1 ชม. เลี้ยงตัวอ่อนที่ถูกกระตุ้นในน้ำยาเลี้ยงตัวอ่อนชนิด บี 2 ที่มีซีรั่มโค 2.5% ตรวจการพัฒนาของตัวอ่อนทุกวันติดต่อกัน 7 วัน ผลการศึกษาพบว่าอัตราการหลอมรวมกันของเซลล์ต้นแบบช้างและโอโอไซต์กระต่าย การแบ่งตัวของโคลนตัวอ่อนช้าง-กระต่าย ด้วย E1 สูงกว่าวิธี E2 อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) เมื่อเลือกกระแสไฟฟ้าโปรแกรมที่ 2 ในการทดลองที่ 2 พบว่าอัตราการหลอมรวมและอัตราการถูกกระตุ้นด้วยกระแสไฟฟ้าของเซลล์ต้นแบบช้างและโอโอไซต์กระต่ายสูงกว่าเซลล์ต้นแบบกระต่าย และโอโอไซต์กระต่ายอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) อย่างไรก็ตามเมื่อเปรียบเทียบอัตราการพัฒนาตั้งแต่ระยะแบ่งตัวจนถึงระยะบลาสโตซิส พบว่าโคลนตัวอ่อนช้างมีการพัฒนาไม่แตกต่างจากโคลนตัวอ่อนกระต่าย การศึกษานี้ได้ข้อสรุปว่าการหลอมเซลล์ต้นแบบและการกระตุ้นด้วยกระแสไฟฟ้าโปรแกรมที่ 1 (E1) เหมาะสมในการทำการย้ายฝากนิวเคลียสของเซลล์ต้นแบบช้างเข้าสู่โอโอไซต์กระต่าย และเซลล์ต้นแบบช้างสามารถพัฒนาเป็นตัวอ่อนจนถึงระยะบลาสโตซิสในโอโอไซต์กระต่ายได้ การศึกษานี้เป็นการสร้างองค์ความรู้พื้นฐานสำหรับการศึกษาอื่นๆ ต่อไปในหลายๆด้านทั้งการอนุรักษ์และทางการแพทย์ เช่น การพัฒนาของตัวอ่อนช้างภายในร่างกายภายหลังการย้ายฝาก การช่วยรักษาพันธุกรรมช้างที่มีคุณค่า และการสร้างเซลล์ต้นกำเนิดจากตัวอ่อนช้าง

คำสำคัญ: ช้างเอเชีย การย้ายฝากตัวอ่อนข้ามชนิดสัตว์จากเซลล์ร่างกาย โอโอไซต์กระต่าย

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Introduction

Asian elephant (*Elephas maximus*) formerly ranged from West Asia along Iranian coast in the Indian subcontinent, eastwards into South-East Asia. The population of Asian elephant tends to decrease due to poaching, habitat loss, degradation and fragmentation. Due to the rapid progress of biotechnology such as artificial insemination (AI), *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT), those techniques may be applied in order to preserve an endangered species (Siriaroonrat et al., 2009; Thongphakdee et al., 2006; Thongtip et al., 2009). However, performing SCNT or cloning research in endangered species is impractical because of the unavailability of oocytes and surrogate animals. In theory, it is possible to expect a successful production of cloned embryo by transferring the donor cell of one species into the enucleated oocyte of another species, so called interspecies SCNT. We recently prove this principle as shown by the successful intergeneric cloning when producing marbled cat and flat-head cat embryos from domestic cat oocytes (Thongpakdee et al., 2010), this demonstrated the feasibility of using interspecies SCNT for wildlife embryo production. In addition, previous reports of the birth of gaur (Lanza et al., 2000), mouflon (Loi et al., 2001), banteng (Jansen et al., 2004) and African wildcat (Gómez et al., 2004) produced by SCNT also demonstrated that

interspecies SCNT technique could be applied for wildlife preservation. In fact, the embryo of Asian elephant generated from naturally fertilization is impractically obtained. Thus SCNT might be an alternative way to be applied in order to achieve the elephant embryos. However, the limitation of studies of interspecies SCNT in Asian elephant is not only caused by the unavailability issue of the recipient oocytes as the source but also the lack of information about early stages of embryo development, which have never been reported. Since rabbit oocyte collection are not complicate to achieve and are considered as the universal recipient oocytes for several mammalian species (Dominko et al., 1999; Thongphakdee et al., 2006), rabbit oocyte was selected to be used as the recipient cytoplasm throughout this study. The objectives of the study were 1) to find the optimal conditions of electrical pulses used for fusion and activation of elephant donor cells and rabbit oocytes (Experiment I) and 2) to investigate the in vitro development of cloned Asian elephant embryos produced from rabbit recipient oocytes (Experiment II).

Materials and Methods

Culture of the donor cells: All chemicals were purchased from Sigma Co. (St Louis, USA) unless otherwise stated. The procedure for isolation and culture of rabbit and elephant fibroblast was performed as described in the previous report (Vignon et al., 1998). Briefly, ear skin of stillborn Asian elephant (*Elephas maximus*) and rabbit were cut into small pieces and digested with 0.25% trypsin (Gibco, Grand Island, USA) for 30 min at 37°C. Digested cells and tissue were cultured in Dulbecco's modified eagle medium (DMEM; Gibco, USA) supplemented with 20% fetal bovine serum (FBS; BioClot GmbH, Germany) under 5% CO_2 at 37°C until it would reach 70-80% of confluency. The fibroblast cells were trypsinized, continuously cultured and cells at passage No. 5 were used as the donor cells.

Preparation of recipient oocytes: Mature oocytes were recovered from the oviducts of New Zealand White rabbit after superovulation (Techakumphu et al., 2002). The oocytes were incubated in TCM-199 supplemented with 0.1% hyaluronidase for 15 min in 5% CO_2 at 38.5°C. Then cumulus cells were removed from the oocytes by gentle pipetting. Before removing the nucleus from the oocytes, the cumulus freed oocytes were incubated in TCM-199, contained 2.5 M Hepes, 7 µg/ml cytochalasin B for 30 min.

Enucleation and nuclear transfer: Metaphase II plate and first polar body together with small amount of cytoplasm were removed from the oocyte by micromanipulation technique as described by Chesne et al. (2002). Enucleation was performed under an inverted microscope and the success of enucleation was confirmed by exposing the oocytes previously stained with 5 µg/ml of Hoechst 33342 with the UV light. For nuclear transfer, single donor cell was injected into the peri-vitilline space while ensuring close contact to the plasma membrane of an enucleated oocyte.

Fusion and activation: 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+}). The electrical pulses either E1: 3.2 kV/cm, 3 pulses, 20 µs (Chesne et al., 2002) or E2: 2.0 kV/cm, 2 pulses, 20 µs (Chen et al., 2002) were applied. The reconstructed oocytes were then washed for three times in M199 (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and incubated in the same medium for 1 h at 38.5°C in a humidified air containing 5% CO_2. The success of fusion of reconstructed oocytes was observed under the stereo microscope. Fused oocytes were activated by the same electrical pulses as for fusion, then incubated in 5 µg/ml of cyclohexamide and 2mM of 6-DMAP for 1 h. The activated oocytes were washed in TCM-199 supplemented with 10% FBS at least three times before being cultured.

Embryo culture and assessment of embryo development: To assess the chromatin remodeling, reconstructed oocytes were stained with Hoechst 33342. The appearance of swollen nuclei or distinct pseudo-pronuclei in recontructed oocytes was considered as the evidence of successful activation. The activated oocytes were then cultured in B2 medium containing 2.5% of FBS. The development as well as the chronology of the cloned embryos was investigated daily for up to 7 days.

Experimental design

Experiment I: In order to find the suitable electrical pluses for fusing and subsequent activation of the Asian elephant fibroblast into the rabbit cytoplasm, the electrical pulses program E1 and E2 were tested. The experiments were performed after at least 5 replications to achieve the sufficient number of reconstructed oocytes for statistical comparison.

Experiment II: The program of electrical pulses that shows superior results for fusion and activation in experiment I was then selected for the experiment II. The fusion and activation rate as well as the embryo developmental rate from cleavage throughout blastocyst stages were compared between rabbit oocyte reconstructed with Asian elephant- and rabbit fibroblasts.

Statistical analysis: Differences in the percentages of fusion, activation as well as oocyte reaching a particular stage between electrical pulses and type of donor cells were determined by chi-square analysis and considered significant at $p<0.05$.

Results

The result in Table 1 clearly demonstrated that, using electrical pulses program E1, the number of elephant-donor cell fused to rabbit-oocyte and reached the cleavage stage was significantly higher ($p<0.05$) than when using program E2. Furthermore, elephant-rabbit cloned embryos fused and activated

by either program E1 or E2 were able to develop into blastocyst stage with no significant difference.

The result in the Table 2 showed that after fusing with rabbit oocytes, elephant-donor cells displayed significantly higher (p<0.05) efficiency than rabbit donor cell (51.3 VS 41.7%, respectively). However, no difference of developmental ability of clone-embryos from cleavage up to blastocyst stage was observed between using elephant- and rabbit fibroblast. Therefore, elephant-rabbit cloned embryos display similarity of morphology with rabbit-rabbit cloned embryos (Figure 1).

Table 1 Fusion efficiency and developmental ability of elephant-rabbit cloned embryos after being fused and activated by different electrical pulses

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

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

Table 2 Developmental ability of elephant-rabbit cloned embryos compared to rabbit-rabbit cloned embryos

Donor cell	Recons-tructed n	Fused/Culture	Cleavage	2-8 cells	>8-16 cells	M	B
		n (%)					
Elephant	160	82(51.3)[a]	44(53.7)[a]	27(32.9)[a]	11(13.4) [a]	2(2.4) [a]	4(4.9) [a]
Rabbit	132	55(41.7) [b]	29(52.7)[a]	16(29.1) [a]	8(14.5)[a]	3(5.5) [a]	3(5.5) [a]

M: morula, B: blastocyst [a, b] Values with different superscripts within the same column differ significantly (p<0.05)

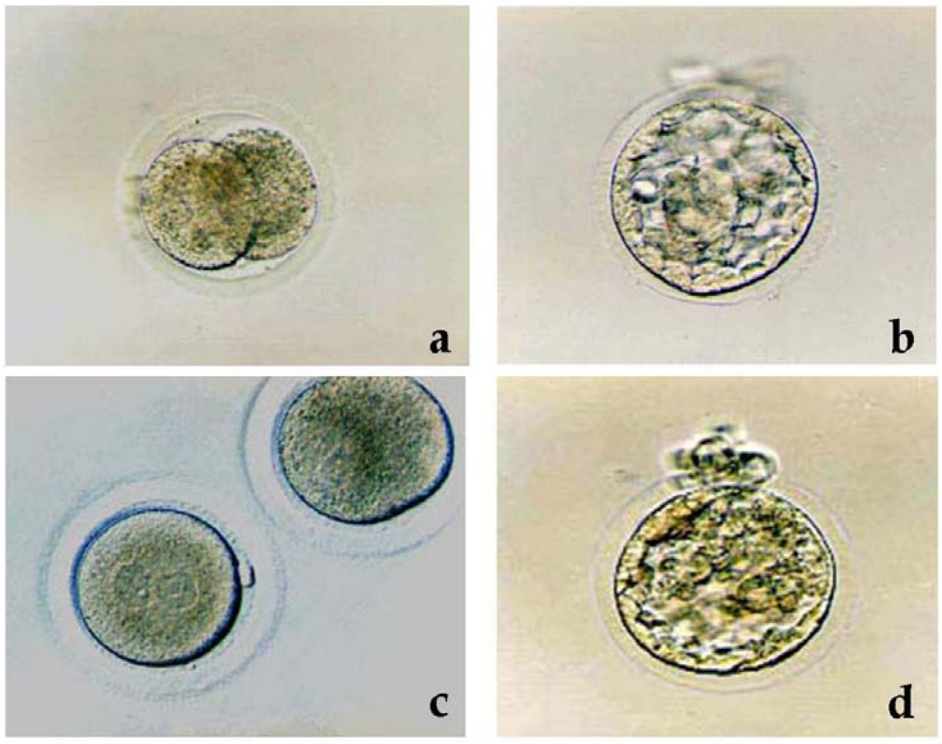

Figure 1. Morphological characteristic of elephant-rabbit cloned embryos at two-cell (a) and blastocyst stages (b), and rabbit-rabbit cloned embryos at pseudopronuclei (c) and blastocyst stages (d). 400x

Discussion

In the present study, we demonstrated the possibility of producing cloned-elephant embryos by using interspecies SCNT. Surprisingly, the success rate of producing blastocyst from elephant-rabbit cloned embryos was comparable to rabbit-rabbit cloned embryos. This indicates that SCNT is a promising and valuable technique for embryo production. This success would be beneficial for conservation issue as well as for using these cloned embryos for biological studies. In fact, using recipient oocytes from closely related species to donor nucleus is preferable for interspecies SCNT. However, oocyte

of wildlife especially elephant is impractical to obtain. From the previous reports, bovine oocytes are able to remodel and reprogram other species nuclei including rat, sheep, pig (Dominko et al., 1999), buffalo (Kitiyanant et al., 2001), gaur (Hammer et al., 2001; Lanza et al., 2000), monkey (Dominko et al., 1999), whale (Ikumi et al., 2003), and human (Chang et al., 2003) as demonstrated by the cloned embryos that were generated. Besides that, the use of rabbit oocyte as the recipient cytoplasm for interspecies SCNT is also favorable. A sufficient amount of matured oocytes (approximately 10 to 40 oocytes per rabbit) can be expected by administrating 21 mg of follicle stimulating hormone (FSH) and 100 IU of human chorionic gonadotropin (hCG) (Techakumphu et al., 2002). In addition, 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), bovine (Techakumphu et al., 2005), marbled cat (Thongkpakdee et al., 2006), flat-headed cat (Thongkpakdee et al., 2007) and human (Chen et al., 2003). The results in the present study proved that elephant somatic cells could be remodeled in rabbit cytoplasm and developed to blastocyst stage with similar developmental success to cloned rabbit embryos. Accordingly, our results emphasized that rabbit oocyte is the universal recipient cytoplasm for interspecies SCNT.

The electrical fusion and activation protocol influence the success of cloned embryo production. To our knowledge, there is no report of a specific protocol for elephant cloning. Accordingly, the suitable cloning protocol is a prerequisite to extrapolation. The induction of 3 DC pulses of 3.2 kV/cm at interval of 20 μs (E1) provided a higher fusion and cleavage rate than induction of 2 DC pulses of 2 kV/cm at interval of 20 μs (E2). However, the percentage of cloned embryos fused and activated by E1 and E2 was not significantly different in blastocyst formation. Fusion rates of each fusion protocol in the present study were similar to the fusion protocol that used for rabbit-rabbit SCNT (Chesne et al., 2002). Following those protocol, high fusion rate (73.5-91.3%) of rabbit-rabbit nuclear transfer units was obtained (Techakumphu et al., 2003). E2 fusion protocol was used for panda-rabbit (Chen et al., 2002) and rabbit-rabbit cloned embryos production (Wen et al., 2003). It provided 57.2% vs. 63.1% fusion rate, respectively. According to the present results, using E1 as a fusion/activation protocol for elephant-rabbit cloned embryos production provided a high fusion efficiency and cleavage rate. This study also found that fibroblast cells could be obtained from the ear skin of stillbirth elephant, and used as donor nucleus for SCNT. Accordingly, interspecies SCNT is a beneficial tool to obtain valuable genetic from both live and dead animals. These would be useful for maintaining genetic diversity and investigate the field of reproductive biology, including the development of pre-implantation embryos.

In conclusion, E1 fusion and activation protocol is more suitable for elephant-rabbit SCNT than counterpart E2. The nuclei of Asian elephant fibroblast could be reprogrammed in enucleated rabbit oocyte and developed to blastocyst stage. The present study provides the fundamental knowledge for further investigation in several aspects for conservation and therapeutic aim, including the *in vivo* development of cloned elephant embryo after transfer, rescuing mammoth from frozen cells and establishment of elephant embryonic stem cells.

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