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A COMPARATIVE STUDY ON THE EFFICIENCY OF TWO ENUCLEATION METHODS IN PIG SOMATIC CELL NUCLEAR TRANSFER: EFFECTS OF THE SQUEEZING AND THE ASPIRATION METHODS

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Abstract

In this study, two enucleation methods, the squeezing and the aspiration methods, were compared. The efficiency of these two methods to enucleate pig oocytes and the in vitro and in vivo viability of somatic cell nuclear transfer (SCNT) pig embryos, were evaluated. In the squeezing method, the zona pellucida was partially dissected and a small amount of cytoplasm containing metaphase II (MII) chromosomes and the first polar body (PB) were pushed out. In the aspiration method, the PB and MII chromosomes were aspirated using a beveled micropipette. After injection of fetal fibroblasts into the perivitelline space, reconstructed oocytes were fused and activated electrically, and then cultured in vitro for 6 days or transferred to surrogates. The squeezing method resulted in a higher proportion of degenerated oocytes than the aspiration method (14% vs.5%). The squeezing method took longer to enucleate 100 oocytes (306 minutes) than the aspirating method (113 minutes). Fusion rate (72–78%) and cleavage rate (67%) were not influenced by the enucleation method but blastocyst formation was improved ($P < 0.05$) in oocytes enucleated by the aspiration method (5 vs 9%). When SCNT embryos were transferred to recipients, pregnancy rates to term were similar (27%, 3/11 and 27%, 3/11) in both methods with the birth of 10 piglets/3 litters and 16 piglets/3 litters in the squeezing and the aspiration methods, respectively. Our results indicate that the aspiration method for oocyte enucleation is more efficient than the squeezing method in producing a large number of pig SCNT embryos with normal in vivo viability.

Keywords

Enucleation; In vivo viability; Somatic cell nuclear transfer; Pig

INTRODUCTION

Somatic cell nuclear transfer (SCNT) has been widely used to produce genetically superior animals or transgenic animals for agricultural or biomedical purposes (1,2). In addition, SCNT is a useful tool to study the interaction of the oocyte cytoplasm with the somatic cell nuclei introduced into the enucleated oocytes (3,4). Live offspring have been successfully produced by SCNT in a variety of animals including sheep (5), cattle (6), pigs (7), horses (8) and dogs

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(9) but the efficiency is still very low. Numerous studies have been performed to optimize SCNT procedures in pigs by improving oocyte quality and modifying manipulation methods such as enucleation, cell injection and oocyte activation (10-13).

Several steps are involved in SCNT: removal of nuclear materials from the recipient oocyte, introduction of somatic cell nucleus into the enucleated oocyte, activation of reconstructed oocytes, and in vitro culture or transfer to surrogate animals. Enucleation is a very important factor influencing the developmental ability of SCNT embryos and also the most time-consuming procedure in SCNT (10,14,15). In contrast with other species, large numbers of SCNT porcine embryos (50 to more than 300 embryos) must be transferred to surrogates to produce cloned piglets (10,16,17). During the long manipulation time required to produce a large number of embryos by SCNT, embryos might be exposed to fluctuations in medium pH and temperature, which may be detrimental to subsequent viability of SCNT embryos. Therefore, a simple and efficient method of enucleation and cell injection is necessary in pigs to prepare a large number of SCNT embryos to transfer into surrogates. Various methods of enucleation such as mechanical enucleation by squeezing out or aspirating the first polar body (PB) and metaphase II (MII) chromosomes have been developed and commonly used (16, 18-20). Recently, chemical enucleation using demecolcine or nocodazole (10,21) and inactivation of nuclear DNA by X-ray irradiation (22) have also been developed. Enucleation by the aspiration method is done using a beveled pipette connected to an aspirator, taken out the PB and a small amount of cytoplasm. In the squeezing method, a slit is made in the ZP with a micro needle and then PB and a small amount of cytoplasm containing MII chromosomes are removed by squeezing oocytes with pipettes. In the squeezing method, no aspirator is needed. Each enucleation method has shown successful production of cloned blastocysts or live offspring (16,18,23,24) but there are few reports available in which the efficiencies of enucleation methods in the in vitro and in vivo development of SCNT embryos have been directly compared. The objective of the present study was to compare the overall efficiency of two enucleation methods, the squeezing and the aspiration methods, in the production of SCNT embryos and to examine their effects on the in vitro and in vivo development of SCNT pig embryos.

MATERIALS AND METHODS

Media and Reagents

Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO, USA). The oocyte manipulation medium for SCNT was salt-buffered North Carolina State University (NCSU)-23, which was the same as previously described (16) except for the medium for donor cell suspension. We replaced 10% fetal bovine serum in cell suspension medium with 0.05% (w/v) bovine serum albumin (BSA). The in vitro culture (IVC) medium for embryo development was NCSU-23 medium with 0.4% (w/v) BSA (25), which was modified by replacing glucose with 0.5 mM pyruvate and 5.0 mM lactate (26).

Preparation of Recipient Oocytes

Oocytes obtained from Bomed (Madison, WI, USA) were matured in TCM199 with Earle's salts (Biowhittaker, Walkersville, MD, USA) supplemented with 10% (v/v) porcine follicular fluid, 25 mM HEPES, 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml epidermal growth factor, 25 µg/ml gentamicin, 1 µg/ml insulin, and 5 µg/ml FSH. After maturation culture for 22 h, oocytes were washed in a fresh maturation medium and cultured in HEPES- and FSH-free maturation medium for additional 16–18 hours before manipulation.

Preparation of Donor Cells

Fetal fibroblasts obtained from Day 57 fetus were cultured in a 1:1 mixture of Dulbecco's modified Eagle medium:nutrient mixture F-12 (Cat. No. 12500-039; Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum. The cells were trypsinized, centrifuged, and resuspended in salt-buffered NCSU-23 containing 0.05% (w/v) BSA prior to use.

Enucleation and Cell Injection

After 38–40 h of in vitro maturation (IVM), cumulus-cell-free oocytes were incubated for 15 min in manipulation medium containing 5 µg/ml Hoechst 33342 and 5 µg/ml cytochalasin B. Following incubation, the oocytes were washed twice in a fresh manipulation medium and transferred for enucleation into a manipulation medium drop containing 5 µg/ml cytochalasin B overlaid with warm mineral oil. In the squeezing method, zonae pellucidae were partially dissected with a fine glass needle and the first polar body (PB) and a small amount of cytoplasm containing metaphase chromosomes were pushed out by squeezing the oocyte with the holding pipette and the glass needle. In the aspiration method, the PB and adjacent cytoplasm containing metaphase chromosomes were aspirated using a 16-µm beveled glass pipette (Humagen, Charlottesville, VA, USA). Enucleation was confirmed by briefly exposing oocytes one by one to a shutter-controlled ultraviolet light. Then, a single cell was inserted into the perivitelline space of each oocyte. In the oocytes enucleated by the squeezing method, a donor cell was inserted through the same hole made at the time of enucleation using a blunt-tip pipette. In the aspiration method, the same pipette was used for both, enucleation and cell injection.

Cell Fusion and Activation of Reconstructed Oocytes

Following cell injection, oocyte-cell couplets were placed on a 1-mm fusion chamber overlaid with 1 ml of 280 mM mannitol containing 0.001 mM CaCl₂ and 0.05 mM MgCl₂ as previously described (16). Membrane fusion was induced by applying an alternating current field of 2 V for 2 sec, followed by two pulses of 1.5 kV/cm direct current (DC) for 40 µs using a cell fusion generator (ECM-2001; BTX, Holliston, MA). Oocytes were incubated for 1 h in IVC medium and examined for fusion under a stereomicroscope prior to activation. Fused oocytes were activated by two pulses of 1.2 kV/cm DC for 60 µsec in 290 mM mannitol that contained 0.1 mM CaCl₂ and 0.05 mM MgCl₂.

Embryo Culture

Electrically fused and activated SCNT embryos were thoroughly washed in IVC medium, transferred into 30-µl droplets (13–15 embryos/droplet) of medium under mineral oil, and cultured for 6 days at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂. Cleavage and blastocyst formation were evaluated on Days 2 and 6, respectively (the day of SCNT was designated as Day 0). Total cell number in the blastocysts was assessed using Hoechst 33342 staining under an epifluorescence microscope.

Transfer of SCNT Embryos into Surrogates

On the day of SCNT, reconstructed oocytes were transferred into naturally cycling gilts on the first day of standing estrus. A mid-ventral laparotomy was performed under general anesthesia maintained by nitrous oxide and halothane. The reproductive tract was exposed, and the SCNT embryos (81–151 embryos/recipient) were transferred into one oviduct at the ampullary isthmus junction. Maintenance of pregnancy was aided by using a combined administration of eCG (1,250 IU) injected intramuscularly on Day 11 of the estrous cycle (Day 0 being the first day of standing estrus), and hCG (750 IU) injected intramuscularly 3 days later (Day 14 of the cycle). Pregnancy was diagnosed regularly at 4-week interval by ultrasonography and the day of estrus return was recorded. All cloned piglets were delivered naturally. All experimental

procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Experimental Design and Statistical Analysis

Effects of two enucleation methods on in vitro and in vivo development of SCNT embryos were compared in two independent experiments. In Experiment 1, survival rate after manipulation, time efficiency in the production of SCNT embryos, and in vitro development of SCNT embryos were compared (3 replications). To determine the manipulation efficiency, time from the start of enucleation to the completion of electric fusion was recorded and the number of degenerated oocytes during enucleation and electric fusion was counted. Then, the time efficiency and survival rates were calculated. In Experiment 2, Pregnancy rate, non-return days, and litter size were compared between the two enucleation methods.

Data were analyzed by ANOVA in the Statistical Analysis System (SAS, Version 9.1; Statistical Analysis System Institute, INC., Cary, NC, USA) to determine model effect of each treatment. All percentage data were subjected to arcsine transformation before analysis. The normality of homogeneity of variance was verified by the Levene's test. Data were expressed as mean $\pm$ standard error (SE). A probability level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of Enucleation Methods on the Manipulation Efficiency and In Vitro Development of SCNT Embryos

Manipulation efficiency in the production of SCNT embryos is summarized in Table 1. Survival rate defined as the proportion of oocytes showing normal morphology and cytoplasm content after enucleation and fusion, was higher ($P < 0.05$) in the aspiration method than in the squeezing method (95% vs 86%). The squeezing method needed more ($P < 0.05$) time for reconstructing oocytes than the aspiration method.

No significant differences in fusion rate, embryo cleavage and blastocyst cell number were observed between the two methods (Tables 1 and 2). However, SCNT embryos produced by the aspiration method showed higher ($P < 0.05$) development to the blastocyst stage than those derived from the squeezing method (Table 2).

Effects of Enucleation Methods on In Vivo Development of SCNT Embryos

An average of 118 SCNT embryos was transferred to each of 22 surrogate gilts. Three out of 11 recipients from each group farrowed a total of 10 and 16 piglets in the squeezing and the aspiration methods, respectively (Table 3). The development of SCNT embryos to term based on the total number of transferred embryos was 0.76% for the squeezing method and 1.24% the aspiration method. No significant differences were found in the mean of non-return days, with 34.6 ± 6 (20–64) days for squeezing and 32.6 ± 6 (18–71) days for aspiration. Litter size was also not significantly different between squeezing (3.3 ± 0.7) and aspiration (5.3 ± 3.4) methods.

DISCUSSION

In this study, we tested the effects of squeezing and aspiration methods for oocyte enucleation on oocyte survival rate after manipulation, and in vivo and in vitro development of SCNT pig embryos. Our results demonstrate that in terms of time, the aspiration method for oocyte enucleation is more efficient than the squeezing method in the production of a large number of SCNT embryos having normal in vivo viability.

SCNT consists of a series of oocyte manipulation processes such as removal of nuclear materials from oocytes, injection of donor cell into enucleated oocytes, cell-oocyte fusion, and activation of reconstructed oocytes (13,16). During manipulation, some oocytes degenerate due to damage in ooplasmic membrane generated by mechanical enucleation (27,28). In the present study, more oocytes which were enucleated by the squeezing method degenerated during or after enucleation and electric cell fusion compared to those enucleated by the aspiration method. In the squeezing method, a slit was made in the zona pellucida and a small amount of cytoplasm was pushed out through the hole. Although the size of the hole made in the squeezing method was not directly compared with that in the aspiration method, it was thought in the squeezing method that more damage to the ooplasmic membrane may have occurred due to a large slit in the ZP.

The squeezing method needed at least twice as much time as the aspiration method in making the same number of SCNT embryos. For cell injection into oocytes enucleated by the squeezing method, a slit made during enucleation had to be identified to insert donor cells into the PVS. This procedure was time-consuming and consequently resulted in extended manipulation time. By contrast, in the aspiration method donor cells were inserted into the PVS with a beveled pipette through any part of ZP. We tried to inject donor cells with a beveled pipette into the squeezing-enucleated oocytes but it was difficult because the mechanical pressure on ZP during injection made cytoplasm bulge out through the preexisting hole.

Cell-oocyte fusion, embryo cleavage and blastocyst cell number were not altered by the different enucleation methods in this study. However, the aspiration method significantly improved the blastocyst formation compared to the squeezing method. It is not clear whether the increased blastocyst formation in the aspiration method was due to the direct effect of the enucleation method itself. It has been reported that many factors such as the volume of removed cytoplasm during enucleation, the duration of IVM before enucleation, and the activation method influence the development of SCNT embryos (12,13,29-31). In the squeezing method, oocytes were enucleated in a wide range of IVM duration because of the extended time of enucleation and cell injection, and were maintained outside for a longer time compared to those enucleated by the aspiration method. In addition, a relatively larger amount of cytoplasm had to be pushed out from oocytes for the complete removal of the PB and MII chromosomes when the squeezing method was applied to the oocytes with MII chromosomes distant from the PB. On the other hand, using the bis bensimide staining to identify the MII, it was possible to aspirate the PB and MII chromosomes separately without removing large amount of cytoplasm in the aspiration method. These factors might be responsible for the reduced blastocyst formation in the squeezed group.

In vivo development to term of SCNT pig embryos was not influenced by the enucleation methods tested in this study although a beneficial effect of the aspiration method was found in vitro blastocyst development of SCNT embryos. More SCNT-derived piglets were produced by the aspiration method (16 piglets/3 litters) than by the squeezing method (10 piglets/3 litters) but the difference in the number of piglets was not significantly different. SCNT embryos were transferred to each 11 surrogates to examine the effect of enucleation methods on the piglet production and only 3 of the surrogates farrowed. The number of surrogates used in this study might be too small to clarify the precise effect of the enucleation methods and, therefore, further extensive embryo transfer studies would be needed. However, when taking all factors into consideration including the time required to generate SCNT embryos, the higher in vitro viability, and the trend towards increased in vivo viability, the results of the present study demonstrated that the aspiration method for enucleation of oocytes is more efficient than the squeezing method in the production of large number of SCNT embryos.

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Table 1Efficiency of SCNT using two different enucleation methods¹

Enucleation method	No. of oocytes reconstructed	% (mean±SE) of oocytes survived after reconstruction	Time needed to make 100 SCNT embryos (min, mean±SE)
Squeezing	409	86±2.9 ^{a,2}	307±13 ^a
Aspiration	405	95±0.4 ^b	113±2 ^b

¹Three replicates.^{2ab}Values with different superscripts in the same column differ significantly ($P < 0.05$).

Table 2
In vitro development of cloned pig embryos produced by two different enucleation methods¹

Enucleation method	No. of oocytes reconstructed	No. (% mean±SE ²) of oocytes fused and cultured	% (mean±SE) of embryos developed to ³		Cell number /blastocyst (mean±SE)
			≥ 2-cell	Blastocyst	
Squeezing	350	251 (71±4)	67±7	5±1 ^{a,4}	21±1
Aspiration	385	301 (78±2)	67±5	9±1 ^b	24±1

¹Three replicates.
²Percentage of the oocytes reconstructed.
³Percentage of the oocytes fused.
⁴*ab*Values with different superscripts in the same column differ significantly ($P < 0.05$).

Table 3
In vivo development of cloned pig embryos produced by two different enucleation methods[/]

Enucleation method	No. of recipients	No. of embryos transferred (mean±SE/recipient)	No. (%) of recipients to term	No. of piglets born	Percent development [/]
Squeezing	11	1309 (119±5)	3 (27.3)	10	0.76
Aspiration	11	1292 (117±8)	3 (27.3)	16	1.24

There was no significant difference in the rate of recipients to term, number of piglets born, and percent development between two enucleation methods. The percentage of development based on the farrowed gilts was 2.6 and 4.1 for the squeezing and the aspiration, respectively ($P > 0.05$).

[/]Number of piglets/total number of embryos transferred.