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Effects of Lower Osmotic Pressure to Granulosa Cell on Reconstructed Embryos Development and Apoptosis in Bovine Nuclear Transfer by Intraplasmic Injection*

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Abstract: This study was performed to determine lower osmotic pressure (0.075 M KCl) duration optimal for breaking granulosa cell membrane and to determine its effect on reconstructed embryos formation, reconstructed embryos developmental potential and apoptosis in blastocysts in bovine nuclear transfer by intraplasmic injection. Enucleated bovine oocytes were divided into five groups according to holding time of 0.075 M KCl to granulosa cells: control (0), 30, 60, 90 and 180 sec. There were significant differences in reconstructed embryos formation between different holding time of 0.075 M KCl and control ($p<0.05$) and control had low rate of reconstructed embryos (27.4% versus 41.7, 36.4, 41.0 and 36.0%). The 180 sec experiment group had significant low rate of cleaved embryos and blastocysts ($p<0.05$). The 90 and 180 sec groups had significant high rate of apoptotic blastomere and they were 10.4 and 11.6% ($p<0.05$), respectively. These results suggested that lower osmotic pressure to bovine granulosa cells could accelerate the formation of reconstructed embryos in nuclear transfer by intraplasmic injection and long holding time of lower osmotic pressure had disadvantageous effect on blastocysts through resulting in low blastocysts rate and high level of apoptosis in blastocysts.

Key words: Nuclear transfer, bovine, granulosa cells, lower osmotic pressure

INTRODUCTION

The transfer of mammalian somatic nuclei into enucleated oocytes has been the subject of much investigation in the last few years and the technique offered many applications in agriculture, endangered species preservation and transgenic animal production. Despite the successes of cloning, however, overall cloning efficiency has remained low (Wilmut *et al*., 2002; Simerly *et al*., 2003). Cell fusion method and intracytoplasmic direct nuclear injection are currently used in nuclear transfer. The cell fusion method has been used to generate cloned cattle (Cibelli *et al*., 1998; Kubota *et al*., 2000), sheep (Fletcher *et al*., 2007), goats (Baguisi *et al*., 1999), pigs (Dai *et al*., 2002; Bondioli *et al*., 2001) and horse (Lagutina *et al*., 2005; Galli *et al*., 2003). However, cell fusion method require the manipulation of the fusion (somatic donor cells and recipient oocytes), which are labor intensive. Furthermore, the area of membrane contact between a somatic donor cell and an oocyte is thought to be relatively limited in somatic nuclear transfer with induced fusion by electrical pulse (Piotrowska *et al*., 2000; Du *et al*., 2002). Direct injection of nuclei into the cytoplasm of oocytes has been used to successfully generated mice and pigs (Wakayama *et al*., 1998; Onishi *et al*., 2000; Lee *et al*., 2003). As compared to cell fusion method, intracytoplasmic microinjection has fewer

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experiment steps and avoid limited contact of somatic cells and oocytes. Breakage of donor cells membrane is crucial to intracytoplasmic microinjection. Pipette with a sharp, beveled tip (inner diameter 7-25 mm) was commonly used for breaking somatic cell membrane in intracytoplasmic microinjection (Wakayama *et al*., 1998; Onishi *et al*., 2000; Lee *et al*., 2003; Choi *et al*., 2002). Somatic cells were aspirated in and out of the injection pipette until the cell membrane broken (Wakayama *et al*., 1998; Choi *et al*., 2002). However, it takes long time and incomplete breakage of donor cell membrane may persist in the oocytes resulting in failure of transfer. Therefore, how to make somatic cells membrane breakage should be a subject of in intraplasmic injection. Lower osmotic pressure was commonly used in facilitating cell membrane disruption. Litter work has been done on facilitating cell membrane breakage in nuclear transfer and the effect of breaking grannulosa cells membrane on the efficiency of nuclear transfer is unclear, especially on cattle. Apoptosis is cells selfdestruction under physiological control and it plays a crucial role in normal embryo development by eliminating both unnecessary and abnormal cells (Levy *et al*., 2001). Whether donor cells membrane breakage through lower osmotic pressure has the effect on the occurrence of apoptosis in blastocysts is unclear in intraplasmic injection.

In the present study, two experiments were designed to determine duration of 0.075 M KCl to bovine granulosa cells and to study its effect on subsequent reconstructed embryos developmental potential and the apoptosis in blastocysts. Apoptosis was assessed by terminal deoxynucleotidyl transferase mediated d-UTP nick end-labeling (TUNEL).

MATERIALS AND METHODS

This study was conducted from 2005 to 2007 in Wuhan, China and ovaries of cattle were collected from local slaughter house.

Recipient Oocytes Collection and *in vitro* Maturation

Ovaries were transported to the laboratory within 1-3 h at 30°C in Dulbecco's Phosphate-Buffered Saline (DPBS) containing 100 IU mL^{-1} streptomycin and 100 IU mL^{-1} penicillin from local slaughterhouse. Cumulus-Oocyte Complexes (COCs) were recovered from 3-8 mm follicles in diameter with a 12 mL disposable syringe fitted with an 18-gauge needle. The COCs with evenly granulated ooplasm surrounded by several layers (three or more dense layers) of compact cumulus cells were selected for use. Groups of 10-15 oocytes were placed in 100 µL droplets of IVM medium under mineral oil (Sigma). Briefly, the maturation medium was TCM 199 with Earle's salts supplemented with 10% (v/v) fetal calf serum (FCS, Sigma), 25 mM Hepes (Sigma), 0.25 mM sodium pyruvate, 10 µg mL^{-1} FSH, 10 µg mL^{-1} LH, 1 µg mL^{-1} estradiol, 100 IU mL^{-1} streptomycin and 100 IU mL^{-1} penicillin. The COCs were matured for 19 h at 38.5°C in an atmosphere of 5% CO_2.

Preparation of Somatic Donor Cells

Adult granulosa cells were isolated from follicular fluid after removal of the COCs. Granulosa cells were washed twice by centrifugation at 300xg for 2 min in Dulbecco's modified Eagle's medium (DMEM) and subsequently seeded onto 100 mm plastic culture dishes at a density of 100,000 cm^{-2}. Seeded cells were subsequently cultured in DMEM with 10% (v/v) FCS and 2 mM glutamine in 5% CO_2 in humidified air at 38.5°C. Once a granulosa cell layer was established (2-3 day), cells were grown in 100 mm culture plates until they reached 95% confluency. After three subpassages, cells were trypsinized with 1% (v/v) trypsin-EDTA solution and then exposed to a cryoprotectant solution consisting of DMEM, 20% calf serum and 5% dimethylsulfoxide (DMSO) in liquid nitrogen. To

prepare adult fibroblasts as donors for nuclear transfer, the preserved cells were thawed and cultured in 100 mm culture plates in 10 mL DMEM containing 10% (v/v) FCS until the cells reached confluency. Granulosa cells were used as nuclear donors at passage three of culture. Before using the cells in nuclear transfer experiments, granulose cells were induced into quiescence by serum starvation (0.05% FCS) for 2-3 days.

Enucleation and Injection

Oocytes were enucleated after 19 h of maturation culture. Cumulus cells were removed mechanically by gentle pipetting in 1% (w/v) hyaluronidase solution and only oocytes with excellent morphology and a visible polar body were selected for this experiment. Prior to enucleation, oocytes were placed for 15 min in Hepes-buffered M199 with 7.5 μg mL^{-1} cytochalasin B (sigma) and 5 μg mL^{-1} Hoechst 33342 (sigma). Groups of oocytes were enucleated in Hepes-buffered M199 with 7.5 μg mL^{-1} cytochalasin B using pipette with a sharp, beveled tip (inner diameter 20 mm). Only oocytes in which the removal of both the polar body and metaphase nucleus was confirmed by observation under UV light were included in the experiment. Granulosa cells were trypsinized, washed by centrifugation and than resuspended in 0.075 M KCl lower osmotic pressure medium for 30, 60, 90 or 180 sec after granulosa cells were thawed and cultured to confluency. After washing three times in M199 by centrifugation, Granulosa cells were resuspended in injection medium of TL-Hepes containing 10% (w/v) polyvinylpyrrolidone. Whole-cell injection was conducted using the procedure similar to the intracytoplasmic sperm injection (ICSI). Granulosa cells were combined with enucleated oocytes in 7.5 μg mL^{-1} cytochalasin B in Hepes-buffered M 199 and injected into an enucleated oocytes via the injection pipette (inner diameter 10-13 mm) with a sharp and beveled tip. Reconstructed Embryos were then returned to M199+10% FCS for 2 h at 38.5°C under 5% CO_2 in air.

Activation of Reconstructed Embryos

Chemical activation was performed within 2 h of injection by a 4 min incubation in 5 μM ionomycin (sigma) and then the reconstructed embryos were cultured in M199 containing 2 mM 6-dimethylaminopurine (DMAP, sigma) for 3 h. After washing in M199, the reconstructed embryos were cultured in M199 + 5% FCS for 20 h.

***In vitro* Culture of Reconstructed Embryos**

After activation, the reconstructed embryos were subsequently transferred to development culture drops of M199 and co-cultured with granulosa cells for 7 days. Embryos were culture at 38.5°C under 5% CO_2 in air. The rates of cleaved embryos and blastocysts development were examined at day 2 and 7, respectively.

Detection of Apoptosis by the TUNEL Assay

Apoptosis in blastocysts was detected by the TUNEL assay using a *in situ* Apoptosis Detection Kit (Takara Bio Inc., Shiga-ken, Japan) (Jang *et al*., 2004). Blastocysts were fixed in PBS containing 4% (v/v) paraformaldehyde for 30 min at 39°C and placed in a Permeabilization Buffer from the Kit for 10 min at 4°C. After washing in PBS containing 1 mg mL^{-1} BSA, blastocysts were treated with a terminal deoxynucleotidyl transferase-labeling buffer for 2 h at 39°C. The blastocystes were then placed in 70 μL fluorescein isothiocyanate solution for 60 min at 39°C and treated with 50 μg mL^{-1} propidium iodide for 30 min at room temperature. Blastocysts were mounted on glass slides after being treated with 0.2 M diazabicyclo-ctane in PBS

supplemented with 50% (v/v) glycerol and were examined under an epifluorescence inverted microscope. Apoptotic blastomeres appeared as yellow, fragmented and condensed nuclei, while normal blastomeres as red color.

Experimental Design

Experiment 1: The objective of this experiment was to investigate the effect of duration of 0.075 M KCl to bovine granulosa cells on reconstructed embryos formation and subsequent reconstructed embryos developmental potential. Enucleated oocytes were divided into five groups according to different durations of 0.075 M KCl to granulosa cells: control (No. 0.075 M KCl), 30, 60, 90 and 180 sec. There were 242, 236, 227, 236 and 241 enucleated oocytes in the treatment of 30, 60, 90, 180 sec and control, respectively.

Experiment 2: The objective of this experiment was to investigate the effect of 0.075 M KCl to bovine granulosa cells on the apoptosis in blastocysts. There were 12, 12, 12, 7 and 12 blastocysts in the treatment of 30, 60, 90, 180 sec and control, respectively.

Statistical Analysis

The results were pooled and then tested by Chi-square analysis in experiment 1 and 2. Differences at a probability $p<0.05$ were considered significant. Each experiment was repeated three or four times.

RESULTS AND DISCUSSION

A total of 1182 bovine oocytes were enucleated and 431 were successfully reconstructed. There were significant differences between different holding time of 0.075 M KCl and control in reconstructed embryos formation ($p<0.05$) and control had low rate of oocytes reconstructed (27.4%). The 180 sec experiment group had significant low rate of cleaved embryos and blastocysts than other groups (23.5 and 8.2%, respectively, $p<0.05$) (Table 1).

The 90 and 180 sec groups had higher rate of apoptotic blastomeres than other groups and they were 10.4 and 11.6%, respectively. The differences were significant as shown in Table 2.

Table 1: The effects of duration of 0.075 M KCl to bovine granulosa cells on subsequent reconstructed embryos developmental potential

Duration (sec)	No. of oocytes enucleated	No. of[c] reconstructed embryos (%)	No. of[d] cleaved embryos (%)	No. of[d] blastocysts (%)
30	242	101 (41.7)[a]	31 (30.7)[a]	17 (16.8)[a]
60	236	86 (36.4)[a]	29 (33.7)[a]	18 (20.9)[a]
90	227	93 (41.0)[a]	33 (35.5)[a]	17 (18.3)[a]
180	236	85 (36.0)[a]	20 (23.5)[b]	7 (8.2)[b]
Control	241	66 (27.4)[b]	20 (30.3)[a]	12 (18.2)[a]

[a,b]Superscripts indicate that the mean values in the same column are significantly different ($p<0.05$), [c]based on the number of enucleated oocytes, [d]Based on the number of reconstructed embryos

Table 2: The effect of duration of 0.075 M KCl to bovine granulosa cell on apoptosis in blastocysts

Duration (sec)	No. of blastocysts	Total cell No. in blastocysts	No. of apoptotic blastomeres (%)
30	12	1073	90 (8.4)[a]
60	12	1112	98 (8.8)[a]
90	12	1083	113 (10.4)[b]
180	7	627	73 (11.6)[b]
Control	12	1109	87 (7.8)[a]

Within columns, values with different superscripts are significantly different ($p<0.05$)

Formation the couplet of oocyte cytoplast with the donor cell is crucial not only to electrofusion but also to intraplasmic injection. Although electrofusion was widely used for inducing oocytes activation (Dominko *et al*., 1999), there were many unsolved problem (Zimmermann and Vienken, 1982). In contrast to the method of electrofusion, intraplasmic injection can avoid their disadvantageous aspects. Studies of nuclear transfer were successful by intraplasmic injection (Wakayama *et al*., 1998; Onishi *et al*., 2000; Lee *et al*., 2003; Choi *et al*., 2002), supporting the view that whole-cell injection was conducted using the procedure similar to ICSI. However, litter work was done in intraplasmic injection because of concerns that incomplete breakage of donor cell membrane may persist in the oocytes resulting in failure of transfer. Therefore, it is important to make membrane breakdown and pore formation before whole-cell injection and assess the effects of breaking the membrane on the efficiency of nuclear transfer by intraplasmic injection.

In recent years, a great deal of attention has been focused on the permeability of biological membrane. A variety of factors and methods have been studied on membrane disruption (Karoonuthaisiri *et al*., 2003; Logisz *et al*., 2005) and lower osmotic pressure was one of many methods causing cell membrane disruption. In the study of the effect of 0.075 M KCl to somatic donor cells on the formation of reconstructed embryos and subsequent reconstructed embryos developmental potential, these data showed that 0.075 M KCl had important effect on the rate of reconstructed embryos. Higher rates of reconstructed embryos were observed in 30, 60, 90 and 180 sec treatments as compared to control. Based on this, we though that lower osmotic pressure played a crucial role in the formation of reconstructed embryos. Cells viability depends strongly on the osmotic pressure of the medium and plasma membrane breakdown and pore formation can be induced by lower osmotic pressure of the medium. In this study, 0.75 M KCl affected the formation of reconstructed embryos through breaking granulosa cells membrane. Cell membrane is capable of withstanding pressure to some extent and low rate of reconstructed embryos of control might be caused by incomplete breakage of cell membrane. However, there were no significant differences in the formation of reconstructed embryos among different holding time of lower osmotic pressure. It was more likely that, granulosa cell membrane could be broke easily with lower osmotic pressure and low lever of holding time of lower osmotic pressure was enough to membrane disruption. Experiment treatments (control, 30, 60 and 90 sec) had similar effects on the rates of reconstructed embryos developed to cleavage and blastocysts and 180 sec treatment had significant low rate of cleaved embryos and blastocysts than other groups. It suggested that low lever of holding time of lower osmotic pressure to granulosa cells had no directly effect on blastocysts formation and their blastocysts formation was mainly associated with the developmental competence of the reconstructed embryos. Besides accelerating the formation of reconstructed embryos, high lever of holding time of lower osmotic pressure had potential disadvantageous effects on reconstruced embryos developmental potential.

Besides the rate of blastocysts development, another approach to evaluate embryos developmental potential is to observe the apoptosis in blastocyst using the TUNEL assay (Brison and Schultz, 1997). In the present study, there were significant differences in the incidence of apoptosis in blastocysts between 90, 180 sec experiment groups and other groups. Apoptosis depends on the development stage of embryos and occurs at different frequencies in different strains. Apoptosis is also affected by a wide variety of non physiological stimuli of *in vitro* culture conditions (Kamjoo *et al*., 2002; Navarro *et al*., 2004). Present study and earlier studies showed that different holding time of lower osmotic pressure resulted in the difference of apoptosis in blastocyst and high level of apoptosis in blastocysts was coupled to long holding time of lower osmotic pressure. It was more likely that, besides breaking granulosa cells membrane, long time of lower osmotic pressure to granulosa cells had potential disadvantageous effect on subsequent reconstructed embryos developmental potential. In this process, long holding time of lower osmotic pressure might result in some disadvantageous alterability of cell nuclei.

In conclusion, these results suggested that lower osmotic pressure to bovine granulosa cells accelerated the formation of reconstructed embryos in nuclear transfer of intraplasmic injection and long holding time of lower osmotic pressure had disadvantageous effects on blastocysts through resulting in low blastocysts rate and high level of apoptosis in blastocysts. The outcome of this study should contribute to establish a steady and efficient cloning system of intraplasmic injection in the future.

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