The use of demecolcine for enucleation of bovine oocytes

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ABSTRACT. Nuclear transfer requires removing all genetic materials associated with the chromosomes of recipient oocyte. This Study was designed to further explore the pharmacological role of Demecolcine (DM) on assisting enucleation in animal somatic cell nuclear transfer. The *in vitro* matured bovine oocytes were incubated with DM at different concentration, or at a fixed DM concentration for additional different hours, then the oocytes extrusion cones rate (ECR) were determined in the inverted microscope. The highest ECR (61.90%) was measured from the treatment with 0.5μg/mL demecolcine. The time-dependent manner of the development of the extrusion cones, in 2hr groups were significantly higher ($P<0.05$) than in 0hr, 0.5hr, 1hr and 2.5hr groups. The highest ECR were found *in vitro* matured 18hr groups (73.86%), which was significantly higher ($P<0.05$) than that observed in 14hr, 16hr groups, and 20hr groups. However, the Granualar cell existence during maturation can influence on PR and embryos development rate. The *in vitro* matured 18hr bovine oocytes with granualar cell were added to DM, which were significantly higher ($P<0.05$) than that of control groups. Meanwhile, we evaluated the effects of DM on the cleavage rate of *in vitro* matured oocytes. The results showed that the IVM medium with or without DM, the IVF embryos rate of cleavage, of blastocysts, and average cell number of blastocysts between the two groups were not significantly different from each other. This simple, chemically assisted method to remove maternal chromosomes makes it possible to produce a large number of nuclear-transferred eggs and to efficiently produce cloned bovines.

KEY WORDS : bovine, assisted-enucleation, Demecolcine, nuclear transfer, oocyte

INTRODUCTION

Since the birth of Dolly in 1997 (WILMUT et al., 1997), a number of studies have reported the production of cloned animals (WILMUt et al., 1997; CAMPBELL, 2002; LI et al., 2004; TIAN et al., 2003; WILMUT & PATERSON, 2003). These successes revealed the extraordinary capacity of the oocyte to erase the process of genome cell differentiation and to reprogram the genetic information to produce a new individual. This process is attainable by nuclear transfer, which consists of the replacement of the maternal genetic material of the oocyte by the genetic information of donor cells after nuclear transfer. Unfortunately, even if cloning is possible in these few experiments, nuclear transfer is a very complex, time consuming, poorly understood and inefficient process. In fact, the efficiency of nuclear transfer has been estimated to be between 1 and 2% of all oocytes used (POLEJAEVA et al., 2000). The reasons for this low efficiency can be attributed to the source and quality of oocytes, the preparation of the recipient cytoplasm, the donor cell type, the synchronization of the cell cycle of both, recipient cytoplasm and donor cells, the failure to reprogram the transplanted nucleus and finally the failure of artificial activation methods. Clearly, much research on cloning remains to be done.

One of the major steps involved in the nuclear transfer procedure is the removal of genetic material from the recipient oocyte (enucleation). It is clear that this procedure also removes important cytoplasmic components, which may reduce cytoplast viability. In routine nuclear transfer procedures, one third or more of the ooplasm is frequently removed that would presumably result in a corresponding decrease in the total cell number of cloned blastocysts. This procedure requires time and limits the number of oocytes available for cloning. In present study, our aim was to evaluate the utility of demecolcine to assisted-enucleation of bovine oocytes. In addition, the developmental competence of resulting cytoplasts was examined in nuclear transfer experiments using fibroblast as nuclear donors. We examined the developmental competence of DM treatment using IVF.

MATERIALS AND METHODS

1. Chemicals and materials

Dishes for oocyte culture were purchased from Corning/Coster Company (NY, USA), and all chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

2. Cell Cultures

Primary cultures of bovine ear skin fibroblasts were established from tissue samples of a 3-week-old Holstein cow. Briefly, the tissues were cut into small pieces and dispersed by exposure to 0.1% (w/v) trypsin (Gibco, Grand Island, NY). The cell suspension was then transferred into 10cm culture dishes containing Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (Hyclone FBS; Biochrom, Berlin, Germany), 2mM L-glutamine, 0.1mM 2-mercaptoethanol, 2mM non-essential amino acids (Sigma, St.

Louis, MO), 100IU/mL penicillin and 100μg/mL streptomycin (passage 0). The cells were cultured until subconfluence (usually 2–3 days) at 37°C in a humidified atmosphere of 5% CO_2 in air and then frozen in DMEM with 10% DMSO and 20% FBS.

3. Preparation of Donor Cells

Frozen-thawed adult ear fibroblast cells less than four passages were used as donor cells, which were plated into a four-well culture dish, and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin until confluent. These cells were then subjected to serum starvation (0.5% FBS in DMEM) for 5–8 days as described by (WILMUT et al., 1997). Immediately prior to somatic cell nuclear transfer (SCNT), donor cells were collected after trypsinization and then resuspended in DMEM containing 0.5% FBS.

4. Oocyte Collection and Removal of Cumulus Cells

Bovine ovaries were collected immediately after slaughter and brought to the laboratory in normal saline at 25-32°C within 4hr after removal. Cumulus-oocyte complexes (COCs) aspirated from small antral follicles were washed three times with Phosphate buffer fluid supplemented with 5% FBS, 0.2mM sodium pyruvate (Sigma) and 50μg/mL gentamycin sulfate (Sigma). The oocytes were then cultured for 18 to 20hr in HEPES buffered TCM 199 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FBS, 0.02units/mL FSH (from porcine pituitary, Sigma), 1μg/mL estradiol 17 ß 0.2mM sodium pyruvate, and 50μg/mL gentamycin sulfate under a humidified atmosphere of 5% CO_2 in air at 38.5°C. Cumulus cells were removed by vortexing the COCs in 0.2% hyaluronidase (Type 1-S, Sigma) in Ca^{2+}- and Mg^{2+}- free Dulbecco's PBS. To visualize DNA, oocytes were stained in 5μg/mL Hoechst 33342 for 10min and observed with a Nikon inverted microscope (Eclipse TE300; Nikon, Tokyo, Japan) under normal and/or ultraviolet (UV) light.

5. Enucleation, Somatic Cell Microinjection

In the groups treated with DM media, bovine oocytes were enucleated according to a modified protocol. Briefly, as shown in Fig.1, eggs with extrusion cones membrane were moved to medium supplemented with 5mg/mL cytochalasin B (CB) and 0.5mg/mL demecolcine and then oocytes were fixed to the holding pipette and the extrusion cones membrane was removed by aspiration using a pipette with a diameter of 22μm. After enucleation, the donor cell was introduced through the same pipette in the zona pellucida and wedged between the zona pellucida and the cytoplast membrane to facilitate close membrane contact for subsequent fusion. After injection, reconstructed embryos were cultured in SOFaa medium until fusion.

6. Fusion, Activation, and Embryo Culture

Fusion was done by a double electric pulse of 2.1kV/cm for 10msec using a Zimmermann Cell Fusion Instrument (Bachofer, Reutlingen, Germany). Reconstructed embryos were cultured in SOFaa (NaCl 107.63mM, KCl 7.16mM, KH_2P0_4 1.19mM, $MgSO_4$ 1.51mM, $CaCl_2.2H_20$ 1.78mM, Sodium lactate 5.35mM, $NaHCO_3$ 25.00mM, Na-pyruvtae 7.27mM, L-Glutamine 0.20mM, BME amino acdis 45.0μL/mL, MEM amino acids 5.0μL/mL, tri-Sodium-citrate 0.34mM, Myo-inositol 2.77mM, Gentamycine 50.0μg/mL, Phenol-red 10μg/mL) medium supplemented with 2mg/mL BSA. Only fused embryos were used for the activation experiments to avoid incorrect interpretation of the PB extrusion.

The reconstructed embryos were activated with 5μM ionomycin in PBS for 5min, followed by exposure to 30mg/mL BSA for 4min in order to stop the activation. To maintain the low level of MPF, the eggs were further incubated in IVC medium containing 2μM DMAP for 4hr. The reconstructed embryos were cultured in 0.5mL SOFaa, supplemented with 5% FBS, in 4-well dishes overlaid with paraffin oil at 38.5°C in a humidified atmosphere with 5% CO_2. Morphological survival and cleavage rates were recorded. All cleaved embryos were further cultured in SOFaa medium supplemented with 2mg/mL BSA for 7d.

7. *In Vitro* Fertilization

After maturation for 20hr, oocytes with expanded cumulus complexes were removed from maturation medium, washed three times in TL Hepes (Bio-Whittaker, Walkersville, MD, USA), aliquoted into groups of 15-20 and washed three times in fertilization medium, and then transferred into a 50μL drop of fertilization medium in a petri dish (Becton Dickinson, Franklin Lakes, NJ, USA) and placed under mineral oil in 5% CO_2 in humidified air at 38.5°C. Semen from the same bull was used in this study, and was thawed at 38°C for 1min. The sperm was washed three times by centrifugation at 453×g for 8min in sperm wash medium. Following the final wash, the sperm motility and concentration were determined. Sperm pellets were re-suspended in sperm wash medium to a volume of 1mL. Sperm suspension was added to each fertilization drop, giving a total concentration of 1.0×10^6 spermatozoa/mL. Oocytes and sperm were incubated together in 5% CO_2 in humidified air at 39°C for 6hr before *in vitro* culture.

8. Experimental Design

Experiment 1: This experiment was designed to define the optimal concentration of DM for initiating extrusion formation of bovine oocytes. Oocytes at the MII stage were cultured in SOFaa plus 10% FBS containing 0; 0.4; 0.5 or 0.6μg/mL at 38.5°C for 2hr. Projections were recorded and enucleated under an inverted microscope.

Experiment 2: This experiment was designed to investigate whether exposure time has any effect on the formation of extrusion cones. Oocytes were incubated 0; 0.5; 1; 1.5; 2 or 2.5hr, respectively.

Experiment 3: This experiment was designed to examine the effects of oocyte maturation time on the formation of extrusion cones. Oocytes were matured 14hr, 16hr, 18hr and 20hr, respectively and then incubated 2hr with 0.5μg/mL DM .The treatment was similar to experiment 1.

Experiment 4: This experiment was designed to check if granualar cell existence during maturation can influence oocytes maturation if DM existed in the media. DM was added into the media when oocytes were cultured at 14, 16 or 18hr, and oocytes of all groups were cultured for 20hr.

Experiment 5: This experiment was designed to check the effect of oocyte developmental capacity in the presence or absence of DM. DM was added to oocytes maturated for 18hr and IVF at 20hr.

9. Statistical Analysis

The formation, cleavage and blastocysts rates were compared by χ^2 analysis. Differences at $P<0.05$ were considered significant.

RESULTS

After DM treatment, the oocytes had a membrane extrusion cone in which the chromosomes were located, and the extrusion cone was close to Pb 1 (Fig. 1A, B). The maternal chromosomes were easily aspirated and only about 5% volume of cytoplasm was removed. The metaphase II oocytes were treated with different concentrations of demecolcine (ranging from 0.4 to 0.6μg/mL) for 2hr, the extrusion cone was affected in a dose-dependent manner as shown in Table I. On examination under UV light, the extrusion cone gathered with chromosomes in all of the oocytes (100%) (Fig.1A, B). Recipient cytoplasts for NT experiments were prepared by demecolcine treatment. NT embryos were produced by fusion with fibroblast cells less than four passages.

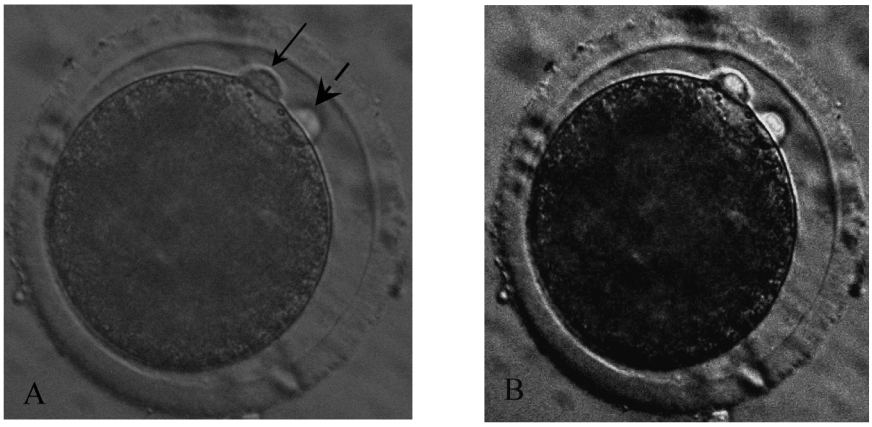

Fig. 1. – An oocyte with an extrusion cone following 0.5h of demecolcine treatment. Arrow shows the extrusion cones, and arrowhead indicates the first polar body (a). The condensed chromosome mass can be seen in the extrusion cones under fluorescent field (b) after Hoechst stain.

The oocytes with extrusion cone were successfully enucleated. As shown in Table 2, the bovine oocytes matured *in vitro* treated with 0.5μg/mL demecolcine were examined 0hr to 2.5hr, the ECR in group 2hr was still significantly higher than those in groups 0.5hr, 1hr and 2.5hr, individually ($P>0.05$). When we examined the effects of oocyte maturation time on the formation of extrusion cone, we found that the ECR of maturation 18hr was still significantly higher than those in groups maturation 14hr and 16hr, individually (Table 3). After 12-18hr maturation in vitro, COCs were further cultured for 2hr in the same solution supplemented by 0.5μg/mL demecolcine. The cumulus cells of all groups were removed at 20hr of culture by vortexing the COCs in 0.2% hyaluronidase in Ca^{2+}- and Mg^{2+}- free Dulbecco's PBS. The ECR in group maturating for 18hr was significantly higher than those in groups maturating 14hr and 16hr, individually (Table 4).

As shown in Table 5, the proportions of IVF that developed into blastocysts were not significantly different among groups using DM treatment.

TABLE 1

DM concentration influences ECR

DM (μg/mL)	No. of oocytes	ECR%	No. of reconstructed embryos	rate of cleavage%	rate of blastocyst%
0	67	0.04^a(3/67)	□	□	□
0.4	66	54.54^b(36/66)	32	78.13^a(25/32)	12.00^a(3/25)
0.5	84	61.90^c(52/84)	48	79.17^a(38/48)	13.16^a(5/38)
0.6	78	44.87^b(35/78)	33	78.79^a(26/33)	11.54^a(5/26)

Within columns, values with different superscripts differ significantly ($P<0.05$). "□"means no results. The following tables are the same.

TABLE 2

Influence of DM incubation time on ECR

Time (hr)	No. of oocytes	ECR%	No. of reconstructed embryos	rate of cleavage%	rate of blastocysts%
0	56	0.05[a] (3/56)	□	□	□
0.5	74	56.76[b](42/74)	40	82.50[a](33/40)	9.09[a](3/33)
1	49	67.34[b](33/49)	33	78.79[a](26/33)	7.69[a](2/26)
2	81	76.54[c](62/81)	61	77.05[a](47/61)	6.38 [a](3/47)
2.5	68	66.18[b](45/68)	43	76.74[a](33/43)	6.06 [a](2/33)

TABLE 3

Oocyte maturation time influence on ECR

Maturation time(hr)	No. of oocytes	ECR%	No. of reconstructed embryos	rate of cleavage%	rate of blastocysts%
14	18	11.11[a](2/18	7	14.29[a](1/7□	□
16	45	33.33[b](15/45)	14	50.00[b](7/14)	□
18	88	73.86c(65/88)	65	89.23[c](58/65)	13.79[a](8/58)
20	75	65.33[c](49/75)	48	81.25[c](39/48)	10.26[a](4/39)

TABLE 4

Granular cell existence and add DM influence on ECR

Maturation time(hr)	No. of oocytes	ECR%	No. of reconstructed embryos	rate of cleavage%	rate of blastocysts%
12	15	0.13[a](2/15)	□	□	□
14	42	42.86[b](18/42)	18	55.56[a](10/18)	□
16	86	73.26[c](63/86)	54	68.52[b](37/54)	13.33[a]5/37
18	73	80.8259/73	50	86.00[c](43/50)	18.60[a]8/43

TABLE 5

Effect of DM on embryonic development of and *in vitro* fertilized bovine oocytes

group	No. of oocytes	rate of cleavage %	rate of blastocysts %	Number of blastocyst cells
Control	95	83.16[a](79/95)	39.24[a](31/79)	117[a]
Add to DM	87	81.61[a](71/87)	36.62[a](26/71)	113[a]

DISCUSSION

This study provided encouraging results to efficiently assisted-enucleate bovine oocyte. The oocyte enucleation procedure is crucially important to cloning efficiency by eliminating any genetic contribution of the recipient cytoplasm, and for excluding the possibility of parthenogenesis (Dominko et al., 2000; Li et al., 2004). Traditionally, mammalian oocyte cytoplasts are prepared by physically removing nuclear chromatin by micromanipulation techniques in preparation to receive the donor genome (Wilmut et al., 1997; Cibelli et al., 1998). Enucleated oocytes arrested at MII are subsequently ''reconstructed'' by the addition of the donor karyoplast, typically using either electrofusion (Wilmut et al., 1997) or microinjection techniques (Wakayama et al., 1998).

Enucleated oocytes were stained with Hoechst and exposure to ultra violet may cause damages of cytoplasmic organelles and mitochondrial DNA, and result in low cloning efficiency (Bell et al., 1997; Dominko et al., 2000). Chemically assisted enucleation is probably a better procedure for animal cloning. Recently, with colcemid assisted enucleation, cloned rabbit fetus (Yin et al., 2002a), pigs (Yin et al., 2002b), and mice (Gasparrini et al., 2003) were produced. More than 70% of bovine oocytes treated with colcemid had a membrane bleb in which the condensed maternal chromosomes are located and are easily removed by aspiration. Enucleation rate is very high (96%), which insures that the maternal genetic

material is removed, and eliminates the possibility of the involvement of maternal genetic residual in NT embryos.

Demecolcine is a specific microtubule inhibitor that binds to tubulin dimers and prevents microtubule polymerization, thus resulting in the loss of the dynamic spindle microtubules. As previously demonstrated in the mouse, transient treatment of pre-activated oocytes with this tubulin-binding agent allows the enucleation of oocytes by the expulsion of the entire chromosome complement within the PBs (BAGUISI & OVERSTROM, 2000; IBANEZ et al., 2003). The removal of chromosomes from activated eggs at the telophase stage is also effective (IBANEZ et al., 2003), but decreased maturation promoting factor activity might decrease the viability of nuclear-transferred eggs with somatic cells (TANI et al., 2001). Matured pig eggs treated with demecolcine had a membrane extrusion cone in which the condensed chromosome mass was located. Similar to porcine oocyte by an actin-rich domain after DM treatment, bovine meiotic chromosomes are also observed within an extrusion cone. Although the mechanisms of action of demecolcine are not clear, the appearance of the extrusion cone might be related to the condensation of maternal chromosomes. When eggs were treated with demecolcine for 0.5; 1; 2 and 2.5hr, the proportions of eggs with condensed chromosomes were 56.76%, 67.34%, 76.54%, and 66.18%, respectively. Most of eggs with condensed chromosomes had an extrusion cones membrane. When oocytes were incubated without demecolcine, extrusion cones were present in only 0.05% of oocytes, and the size of these cones was considerably smaller than when the agents were used. Demecolcine incubation causes a significant increase in the number of oocytes with extrusion cones formation started at 0.5hr. All of these oocytes could be success-fully enucleated, and the reliability of enucleation was 100%. The DM assisted enucleation procedure made the NT work more rapid, because the only sophisticated instrument required, the inverted fluorescent microscope, was no longer required. According to our knowledge, the procedure described in this work is the most efficient and accurate published enucleation method for bovine oocytes. The high efficiency achieved in our experiments was not only the consequence of incubation with demecolcine, but was due to *in vitro* matured oocytes with granualar cell were added to DM. Demecolcine incubation without granualar cell also induced extrusion, but the proportion of oocytes with cones was small, and reached only 64%. However, a significant increase in the number of oocytes with extrusion cones was observed with granualar cell (73.86% vs 80.82%). The viablity of NT embryos produced by using without granualar cell of oocytes in DM incubation was generally lower than that of with granualar cell produced NT embryos (13.79% vs 18.60%). Previous work demonstrated that contact of the cumulus-enclosed oocyte with an intact, undisturbed granulosa layer provides the greatest continuity linking literature reports together. The granulosa surface and cell-to-cell interaction with the oocyte is potentially the important factor in meiotic regulation. (TSAFRIRI et al., 1976; CHANNING & TSAFRIRI, 1997), yet the same researchers obtained almost complete inhibition using co-culture of porcine oocytes and granulose monolayers (TSAFRIRI & CHANNING, 1975). Rat granulosa monolayers are reported to cause inhibition of rat oocytes but only after the granulosa cells have been cultured 24hr prior to the addition of oocytes (TSAFRIRI, 1978). This suggests the need for regeneration of cell surface receptors. The different molecular weights reported for OMI (TSAFRIRI et al.,1976; JAGIELLO et al.,1977; STONE et al.,1978) indicate that fragments of a larger molecule are appearing in fluids causing inhibition of oocyte maturation; such fragments perhaps derives from portions of cell surface constituents. OHNO & SMITH (1964) described the precocious maturation past the dictyotene stage and the ensuing degeneration of primary oocytes that fail to develop when into intimate contacts with follicle cells in foetal calf ovaries. This close association between the oocyte and surrounding follicle cells is continued throughout all stages of oocytic development until preovulatory changes occur (GILULA et al., 1978). A more unified approach to the regulation of oocyte meiosis is provided by viewing it as a problem concerning cell surface interactions or contacts. This is an approach that encompasses not just control in antral follicles but that extends back to the original contact between follicle cells and oocytes at the time of primordial follicle formation.

In conclusion, the present study shows that assisted enucleation can be accomplished in bovine oocytes using demecolcine.

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