Original Paper

The Effects of Demecolcine, Alone or in Combination with Sucrose on Bovine Oocyte Protrusion Rate, MAPK1 Protein Level and C-Mos Gene Expression Level

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Key Words

Demecolcine • Bovine oocyte • C-mos • MAPK1

Abstract

Aims: Our study aims to clarify the effects of demecolcine, alone or in combination with sucrose on bovine oocyte protrusion rate, MAPK1 protein level and c-mos gene expression level. **Methods:** The effects of the demecolcine concentration, treatment duration, and synergistic effects with sucrose solution on the rate of membrane protrusions of bovine oocytes were investigated. Using real-time fluorescent quantitative PCR, western blot analysis, and immunofluorescence assays, the expression of the maternal c-mos gene, the protein level of mitogen-activated protein kinase 1 (MAPK1), and the change in the localization of spindles and nuclei during the demecolcine treatment were analyzed in bovine oocytes. **Results:** Treatment of bovine oocytes with both demecolcine (0.6 µg/mL) and sucrose (0.05 M) for 1 h led to the highest rate of membrane protrusions, and synergistic effects were also observed. Real-time fluorescent quantitative PCR analysis revealed that the demecolcine treatment up-regulated the expression of the maternal c-mos gene. Western blot analysis indicated that the demecolcine treatment enhanced the protein level of MAPK1 in bovine oocytes. Immunofluorescence analysis indicated that the spindles and nuclei were localized at the place of the membrane protrusions. **Conclusions:** The present results suggest that demecolcine might contribute to the activation of the Mos/MAPK pathway and affect spindle structure. These results provide a reference for more efficient generation of enucleated bovine oocytes.

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Introduction

Since Wilmut et al. developed the somatic nuclear transfer technology to clone “Dolly” [1], new cloned animals have been produced continuously. Although somatic nuclear transfer technology is constantly being optimized and improved, cloning efficiency remains low (approximately 3-4%). Cloning technology involves many steps, and a critical role in determining the cloning efficiency is played by the enucleation of oocytes because the success of animal cloning is the result of the continuous embryo transfer of a large number of reconstructed embryos. In addition, the completeness of the enucleation and the amount of aspirated cytoplasm directly affect the development rate of reconstructed embryos. Thus, an improved understanding of the mechanism of enucleation is critical to improving the current enucleation methods.

The methods for the enucleation of oocytes are divided into two categories, chemically assisted enucleation and physical enucleation, based on the involvement of chemicals in the process. Chemically assisted enucleation utilizes certain chemicals to induce membrane protrusions in the oocytes, and the nuclei are removed accurately based on the membrane protrusions. The most frequently used chemicals are demecolcine, cycloheximide [2], and caffeine [3], such chemicals have been applied successfully in the production of cloned cattle [4], pigs [5] and sheep [6]. Compared to physical enucleation, chemically assisted enucleation is more accurate, more complete, involves less aspirated cytoplasm [7] and has been widely applied in the cloning of large livestock because their oocytes contain larger lipid droplets and because it is impossible to visualize the nuclei under a normal microscope [8]. Chemically assisted enucleation induces membrane protrusions in the oocytes, and the nuclei can be removed accurately based on the protrusions.

Demecolcine is able to destroy the 3-dimensional structure of spindles, thus causing chromosome condensation and leading to membrane protrusions. Past studies on the mechanism of enucleation induced by demecolcine in goats indicated that during the process of membrane protrusion, demecolcine affected the activities of maturation-promoting factor (MPF) and MAPK [9], which were directly correlated with the meiosis of the oocytes, zygote formation, and the late stage of embryonic development [10]. Therefore, we investigated the effects of demecolcine concentration, treatment duration, and co-treatment with sucrose on the membrane protrusion rate of bovine oocytes.

Demecolcine was previously shown to affect the activity and phosphorylation of MAPK [3, 9]. However, the action of demecolcine on the upstream regulator factor c-mos has not been reported. Here, we employed real-time fluorescent quantitative PCR, western blot analysis, and immunofluorescence assays to investigate the effects of demecolcine on the expression of the maternal c-mos gene, the protein level of MAPK1 in bovine oocytes, and the change in the localization of spindles and nuclei in bovine oocytes during this process, which is conducive to the further improvement of chemically assisted enucleation methods.

Materials and Methods

The collection and in vitro maturation of bovine oocytes

Ovaries were collected in Jilin Province Changchun Haoyue Halal Meat Co., Ltd, China, stored in saline containing penicillin and streptomycin at 37 °C, and shipped to the laboratory within 3 h after collection. After rinsing the ovaries 3 times with saline, ovaries that had ovarian follicles 2-8 mm in diameter were collected using a 10-mL syringe equipped with a number 18 needle. The follicles were placed into 50-mL centrifuge tubes and incubated for 15 min in a 37 °C water bath. After the precipitation of the oocytes, the supernatant was removed, the cells were resuspended in PVA-TL-HEPES (0.1% polyvinyl alcohol (PVA)), and the suspension was transferred to a 60-mm petri dish to let it stand for 5 min. Under a stereomicroscope, cumulus-oocyte-complexes (COCs) that were surrounded by at least 3 layers of cumulus cells and exhibited dense and evenly distributed cytoplasm were selected. The oocytes were washed 3 times with PVA-TL-HEPES, 3 times with maturation medium (fetal calf serum (10%, v/v), pyruvate (1%), follicle-stimulating

hormone (0.5%), β -estradiol (0.1%) and double antibiotics (1%), and transferred to medium droplets that were previously balanced in an incubator at a density of 10-15 COCs per 100 μ L droplet. In each 60-mm petri dish, 12 droplets were covered with mineral oil and cultured for 24 h at 39 °C and 5% CO₂.

The treatment of bovine oocytes with sucrose, demecolcine, or both

The pretreatment of sucrose could promote the localization of spindles within the MII oocyte [11]. Small hyaline protuberant alabastrum was formed in the cytoplasm of the oocyte treated by sucrose [12]. Therefore, bovine oocytes were treated with sucrose.

Twenty-four hours after the in vitro maturation, the spreading of the cumulus cells surrounding the oocytes was monitored. The COCs were transferred to a solution containing 0.5% hyaluronidase, and the cumulus cells were removed by gently blowing and flushing with a micropipet. The oocytes that had already secreted the first polar body were chosen and washed 3 times with in vitro maturation medium (IVM medium). Some oocytes were transferred into droplets with either 0.05 M or 0.07 M sucrose and were then incubated for 0.5 h or 1 h in a CO₂ incubator; some oocytes were treated with demecolcine at various concentrations (0.4, 0.5, 0.6, 0.7, or 0.8 μ g/mL) for 0.5 h or 1 h; and some oocytes were treated with 0.05 M sucrose in combination with various concentrations of demecolcine for 0.5 h or 1 h. After the treatment, the oocytes were stained with Hoechst 33342 dye (10 μ g/mL) for 15 min, membrane protrusion rate was estimated by direct counting using a microscope during enucleation. The mathematical equation is: Protrusion rate = The number of oocytes with membrane protrusions $\times$ 100% / The number of total oocyte.

Fluorescent quantitative PCR of maternal c-mos gene

Bovine oocytes that had matured for 24 h and had already secreted the first polar body were chosen and divided into 3 groups: a) no treatment, b) treated with 0.05 M sucrose only, and c) treated with both 0.05 M sucrose and 0.6 μ g/mL demecolcine. Total cellular RNA was extracted using TRIzol Reagent (Invitrogen, Beijing, China) from the bovine oocytes of the aforementioned three groups and detected using 1% agarose gel electrophoresis.

Based on the mRNA sequence of the maternal gene *c-mos* retrieved from the NCBI GenBank (Gene ID: 444869), PCR primers were designed (Forward primer: CCAACATCCTCATCAGTGAGC, Reverse primer: GTGATGGCGAAGGAGTAGATG), and the expected amplicon size was 186 bp. The cDNA was generated using a reverse transcription kit (Takara, Dalian, China) and was stored in a -20 °C freezer. The cDNA was later used as a template in fluorescent quantitative PCR. The PCR reaction system included ddH₂O (6.0 μ L), RealMasterMix (10 μ L), primers (1 μ L each at the concentration of 10 pmol/ μ L), and cDNA (2.0 μ L). The PCR reaction was as follows: denaturation at 95 °C for 1 min followed by 45 cycles of 94 °C 20 sec, 61 °C 20 sec, and 72 °C 45 sec. Melting curve was used to prove specificity of RT-PCR amplification. The quantitative analysis of the expression of mRNA of the maternal *c-mos* gene was performed using the relative quantitation method ($2^{-\Delta\Delta Ct}$ method). Each cDNA sample was assayed 3 times.

Detection of the MAPK1 protein level using western blot analysis

The bovine β -actin and MAPK1 (catalog#: sc-154) primary antibodies were purchased from Santa Cruz. The bovine oocytes were treated with 0.6 μ g/mL demecolcine, rinsed 3 times with D-PBS, transferred to 1.5-mL centrifuge tubes, and added to 200 μ L of protein lysis solution. After vigorously shaking, the tubes were centrifuged at 12,000 xg for 6 min at 4 °C. The supernatant was transferred to a new centrifuge tube, and the pellet was discarded. The total protein of the control group was extracted using the same method and stored at -80 °C. Fifty micrograms of the protein samples was mixed with 5x loading buffer, boiled for 10 min in a 105 °C water bath, and subjected to electrophoresis with 12% SDS-PAGE. The proteins were electrotransferred to a nitrocellulose membrane with the pore size of 0.45 μ m. After 2 h of blocking with nonfat milk at room temperature, the membrane was incubated with the primary antibodies against MAPK1 or β -actin at 4 °C overnight, and peroxidase-conjugated goat anti-rabbit IgG antibody (Boster, Wuhan) was then added and incubated at room temperature for 2 h. The X-ray film was exposed using the chemiluminescence method.

We took a photograph for film using gelatun image processing system. The grayscale value of every specific strap in the image was digitized using GIS1000 analysis software. The MAPK1 protein expression difference between the demecolcine treatment group and the control group was analysed using SPSS version 13.0.

Immunofluorescence staining of bovine oocytes

Oocytes that had matured for 24 h and had already secreted the first polar body were chosen, rinsed 3 times with D-PBS, transferred into 4% paraformaldehyde droplets, and fixed for 20 min at room temperature. The oocytes were aspirated using a mouth pipet and washed 3 times with D-PBS for 5 min each. Approximately 200 μ l of 1% Triton X-100 was added to a 24-well culture plate to perform permeabilization of the zona pellucida and the cell membrane of oocytes. After incubation in a CO₂ incubator overnight, the oocytes were aspirated and washed with D-PBS 3 times for 5 min each. The oocytes were then transferred into the blocking solution droplets (1% bovine serum albumin and 0.01% Triton X-100 in dulbecco's phosphate buffered saline (D-PBS)) and incubated in a CO₂ incubator for 4 h. The oocytes were aspirated, washed with D-PBS 3 times for 5 min each, and incubated in anti- α -tubulin primary antibody droplets in a CO₂ incubator for 2 h. The oocytes were aspirated, washed with D-PBS 3 times for 5 min each, and incubated in FITC-labeled secondary antibody droplets in a CO₂ incubator for 2.5 h. The oocytes were aspirated, washed with D-PBS 3 times for 5 min each, and incubated in 10 μ g/mL propidium iodide (PI) staining solution droplets in a CO₂ incubator for 20 min. The results were observed under an inverted fluorescence microscope.

Statistical analysis

The data were expressed in the form of the mean $\pm$ standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) using the SPSS 13.0 software package. The Least Significant Difference was used to test the significance of differences.

Results

SPSS analysis of oocyte membrane protrusion induced by sucrose at various concentrations and at different incubation times revealed that no oocyte membrane protrusion was induced regardless of treatment time (0.5 h or 1 h) or concentration (0.05 M or 0.07 M). Bovine oocytes stained with Hoechst 33342 demonstrated that sucrose alone induced no membrane protrusions.

Table 1. The effect of demecolcine treatment on the membrane protrusion rate of bovine oocytes. Different letters in the same column indicate that the difference between the number is statistically significant ($P < 0.05$)

Concentration of demecolcine (μ g/mL)	No. of oocytes	Demecolcine treatment alone for 0.5 h Membrane protrusion rate (LSM $\pm$ SE)	No. of oocytes	Demecolcine treatment alone for 1 h Membrane protrusion rate (LSM $\pm$ SE)
0.4	42	4.74 $\pm$ 1.71 ^a	40	10.50 $\pm$ 2.09 ^a
0.5	47	4.25 $\pm$ 1.51 ^a	44	9.54 $\pm$ 1.90 ^a
0.6	46	6.52 $\pm$ 1.54 ^b	46	13.62 $\pm$ 1.78 ^b
0.7	48	4.58 $\pm$ 1.74 ^a	43	14.41 $\pm$ 1.94 ^b
0.8	43	23.71 $\pm$ 1.94 ^c	43	24.18 $\pm$ 2.65 ^c

Table 2. The effect of co-treatment with demecolcine and sucrose on the membrane protrusion rate of bovine oocytes. Different letters in the same column indicate that the difference between the values was statistically significant ($P < 0.05$). Bovine oocytes were treated with 0.05M sucrose in combination with various concentrations of demecolcine for 0.5 h or 1 h

Concentration of demecolcine (μ g/mL)	No. of oocytes	Co-treatment with sucrose for 0.5 h Membrane protrusion rate (LSM $\pm$ SE)	No. of oocytes	Co-treatment with sucrose for 1 h Membrane protrusion rate (LSM $\pm$ SE)
0.4	24	13.3 $\pm$ 3.48 ^a	24	21.66 $\pm$ 3.48 ^a
0.5	24	45.83 $\pm$ 5.10 ^b	24	50.83 $\pm$ 3.48 ^b
0.6	25	68.00 $\pm$ 4.89 ^c	25	72.80 $\pm$ 3.34 ^c
0.7	24	54.99 $\pm$ 3.48 ^b	24	51.66 $\pm$ 6.97 ^b
0.8	67	67.41 $\pm$ 1.23 ^c	67	68.63 $\pm$ 2.35 ^c

Fig. 1. The effects of demecolcine treatment and demecolcine and sucrose co-treatment on the membrane protrusion rates of bovine oocytes. A: Oocytes after 1 h of demecolcine treatment alone (0.8 $\mu\text{g}/\text{mL}$). Bar = 50 μm . The yellow arrows indicate the membrane protrusions. B: Hoechst 33342-stained bovine oocytes after 1 h of demecolcine treatment alone (0.8 $\mu\text{g}/\text{mL}$). Bar = 100 μm . C: Bovine oocytes after 1 h of demecolcine and sucrose co-treatment. Bar = 50 μm . The yellow arrow indicates the membrane protrusion, and the blue arrow indicates the first polar body. D: The enlargement of C. Bar = 25 μm .

The effect of demecolcine treatment on the membrane protrusion rate of bovine oocyte is summarized in Table 1. The results demonstrated that when treating for the same period of time, the differences in the membrane protrusion rates were significant after treatment at different demecolcine concentrations ($P < 0.05$). Regardless of the treatment duration (0.5 h or 1 h), treatment with 0.8 $\mu\text{g}/\text{mL}$ demecolcine resulted in the highest cell membrane protrusion rate, and multiple membrane protrusions were observed per oocyte (Fig. 1A), which indicated that demecolcine cytotoxicity to the oocytes is enhanced. At a constant demecolcine concentration, the membrane protrusion rate gradually increased with increasing treatment time. The bovine oocytes stained with Hoechst 33342 after 1 h of demecolcine treatment are shown in Fig. 1B.

The effect of demecolcine and sucrose co-treatment on the membrane protrusion rate of bovine oocytes is shown in Table 2. The results demonstrated that when the treatment time was held constant, treatment with different concentrations of demecolcine resulted in significantly different membrane protrusion rates ($P < 0.05$). Regardless of the treatment duration (0.5 h or 1 h), treatment with 0.6 $\mu\text{g}/\text{mL}$ demecolcine resulted in the highest cell membrane protrusion rates. The oocytes after co-treatment with demecolcine and sucrose for 1 h are shown in Fig. 1C and 1D.

The fluorescent quantitative PCR results of the maternal gene *c-mos* are shown in Fig. 2A. The results demonstrated that the *c-mos* gene expression did not exhibit significant differences between the sucrose treatment group and the untreated control group ($P > 0.05$), whereas significant differences were observed between the demecolcine and sucrose co-treatment group and the other groups ($P < 0.05$). The MAPK1 western blot results are shown in Fig. 2B, demonstrating that the MAPK1 expression level differed significantly between the demecolcine treatment group and the control group ($P < 0.05$).

To assess the correlation of position of chromosomes and membrane protrusions, forty membrane protrusions oocytes treated with demecolcine/sucrose were stained for DNA and microtubules. Co-localisation of chromosomes and membrane protrusions was observed, as shown in Fig. 3. Such a localisation pattern was observed every time. The immunofluorescence staining of the chromosomes, the first polar bodies, and spindles in bovine oocytes clearly demonstrate that the membrane protrusion was caused by chromosomes (Fig. 3). The observation of membrane protrusion demonstrated that the spindles in bovine oocytes were localized at the sites of membrane protrusion.

Fig. 2. Effects of demecolcine treatment on the expression of the maternal gene *c-mos* and MAPK1 protein level in the bovine oocytes. A: The comparison of the relative mRNA expression of *c-mos* among different groups. Oocytes that had matured for 24 h and had already secreted the first polar body were chosen and divided into 3 groups: a) no treatment, b) treated with 0.05 M sucrose only, and c) treated with both 0.05 M sucrose and 0.6 μ g/mL demecolcine. The *c-mos* gene expression did not exhibit significant differences between the sucrose treatment group

and the untreated control group ($P>0.05$). * indicates: significant differences were observed between the demecolcine and sucrose co-treatment group and the other groups ($P<0.05$). B: The comparison of MAPK1 protein expression between the demecolcine treatment group and the control group.

Fig. 3. Immunofluorescence assay. A: Bovine oocytes after PI staining. Bar=200 μ m. B: Bovine oocytes after PI staining. Bar=25 μ m. The yellow arrow indicates the membrane protrusion, also the position of chromosome. The blue arrow indicates the first polar body. C: Bovine oocytes with stained spindle microtubules. Bar =200 μ m. D: Bovine oocytes with stained spindle microtubules. Bar =25 μ m. The yellow arrow indicates the membrane protrusion, also the position of chromosome.

Discussion

Optimization of the demecolcine treatment

Demecolcine is a chemical reagent that can disrupt spindle stability. Treating bovine oocytes with demecolcine at certain concentrations causes irreversible membrane protrusion. The enucleation can be accurately achieved based on the visible membrane protrusion. Vajta et al. obtained high cloning efficiency with a 48% blastocyst rate by treating bovine oocytes with demecolcine [13]. Tani et al. reported that treating bovine oocytes with demecolcine for over 30 min caused protrusions in the cell membrane and enhanced MPF activity in the oocytes during the process [14]. During the demecolcine treatment of porcine oocytes, spindles in the oocytes were destroyed, the chromosomes condensed, and cell

membrane protrusions readily formed [15]. Here, we report that when bovine oocytes were treated for 0.5 h or 1 h with demecolcine, the cell membrane protrusion rate increased with the elongation of treatment duration when the demecolcine concentration was constant. The effect of demecolcine treatment on oocytes did not exhibit a linear correlation with the demecolcine concentrations. When the concentration of demecolcine treatment was 0.8 $\mu\text{g/mL}$, the membrane protrusion rate was the highest, which may be associated with the higher osmotic pressure and elevated membrane brittleness caused by demecolcine treatments. The production of multiple membrane protrusions in the oocytes is disadvantageous for accurate enucleation. In this study, we found that the optimal demecolcine concentration was 0.6 $\mu\text{g/mL}$, whereas Saraiva et al. reported in their study that the optimal concentration was 0.4 $\mu\text{g/mL}$ [16]; this disparity may be attributed to different experimental goals. Saraiva et al. attempted to obtain the best subsequent embryo cleavage rate and reconstructed embryo rate; thus, 0.4 $\mu\text{g/mL}$ demecolcine resulted in the least cytotoxicity to bovine oocytes. The focus of our study was to study the maximum membrane protrusion efficiency of demecolcine to investigate the mechanism underlying the formation of membrane protrusions in bovine oocytes caused by demecolcine.

Saito et al. reported that in the demecolcine treatment of mouse oocytes, the general effect of sucrose was limited to cryopreservation; for example, maltose, sorbitol, and sodium chloride all had the same effect [17]. The study also reported that sucrose treatment alone was not able to induce any cell membrane protrusions; sucrose was unable to pass through the membrane and could only permeate to the cell's exterior along with water in the oocytes, which is helpful for oocyte shrinkage and provides better conditions for the formation of membrane protrusions.

Our results indicated that sucrose and demecolcine co-treatment of the bovine oocytes helped improve the membrane protrusion rate through a synergistic effect. There are several possible explanations for this synergy. First, sucrose provides a hypertonic environment to induce oocyte shrinkage and increases membrane fragility, which is conducive for demecolcine to penetrate into bovine oocytes to exert its effects. Second, during the demecolcine treatment of bovine oocytes, chromosome condensation occurs, and this process requires achieving a certain energy threshold that can be attained by the energy released during the hydrolysis of sucrose into glucose. Third, during the process of electrofusion, the fusion solution requires sucrose; therefore, the co-treatment with sucrose was beneficial to the subsequent electrofusion [18]. Fourth, high concentrations of demecolcine can cause multiple protrusions in the oocyte; the addition of sucrose helps decrease the concentration of demecolcine. In addition, the demecolcine treatment duration should not be excessive so as to alleviate its cytotoxicity to the oocytes.

The effects of demecolcine treatment on the expression of the maternal gene c-mos and MAPK1 protein levels in bovine oocytes

The ERK1/ERK2 MAP kinases (MAPKs) are transiently activated during mitosis, and MAPK activation has been implicated in the spindle assembly checkpoint and in establishing the timing of an unperturbed mitosis [19-22]. Accordingly, MAPK1 protein levels were increased after demecolcine treatment. The proto-oncoprotein Mos has long been recognized as essential for the activation of MEK1 and MAPK1 during oocyte maturation [23-28]. The c-Mos proto-oncogene product, a serine/threonine kinase, is a strong activator of the MAPK pathway, which is important for oocyte maturation [29, 30]. Moreover, Mos and the MAPK cascade have been established as crucial regulators of second meiotic metaphase arrest in mammalian oocytes. Accordingly, the c-mos mRNA expression level was significantly increased after demecolcine treatment. Demecolcine is a microtubule-disrupting agent that depolymerizes microtubules and limits microtubule formation (inactivating spindle fiber formation). Although the mechanism by which demecolcine elicits its effects is unclear, the present results suggest that demecolcine might contribute to the activation of the Mos/MAPK pathway and affect spindle structure. These results provide a reference for more efficient generation of enucleated bovine oocytes.

Abbreviations

MAPK1 (mitogen-activated protein kinase 1); MPF (maturation-promoting factor); PVA (polyvinyl alcohol); COCs (cumulus-oocyte-complexes); IVM (in vitro maturation); D-PBS (dulbecco's phosphate buffered saline); PI (propidium iodide); LSM (the least squares mean); SE (standard error); ANOVA (one-way analysis of variance); MRT (multiple range test).

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