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Effects of Demecolcine and Sucrose on the Incidence of Cytoplasmic Protrusions Containing Chromosomes in Pig Oocytes Matured *In Vitro*

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Abstract. The present study was carried out to examine whether demecolcine and sucrose affect the formation of a cytoplasmic protrusion containing chromosomes in pig oocytes independently or in combination. In the presence of 20 mM sucrose, the rates of oocytes with a cytoplasmic protrusion after culture for 60 min with 0.2–1.0 $\mu\text{g}/\text{ml}$ demecolcine were significantly higher than those with 0.01–0.05 $\mu\text{g}/\text{ml}$ demecolcine. When oocytes were cultured for 15 min in the presence of 0.2 $\mu\text{g}/\text{ml}$ demecolcine and 20 mM sucrose, 35.1% of them extruded a cytoplasmic protrusion; this rate was significantly lower than those of oocytes cultured for 30–90 min. In the presence of 0.2 $\mu\text{g}/\text{ml}$ demecolcine, significantly fewer oocytes extruded a cytoplasmic protrusion after culture for 30 min with 160 mM sucrose than with 0–80 mM sucrose. Significantly more oocytes extruded a cytoplasmic protrusion after culture for 30 min with 0.2 $\mu\text{g}/\text{ml}$ demecolcine than without it, regardless of the presence or absence of 20 mM sucrose. In 88.9–100% of the oocytes, the cytoplasmic protrusions contained chromosomes with no significant differences among the different concentrations of demecolcine and sucrose and among the different treatment times. The results of the present study show that the cytoplasmic protrusion containing chromosomes in the pig oocyte is attributable to demecolcine, but sucrose does not affect its formation.

Key words: Cytoplasmic protrusion, Demecolcine, Oocyte, Pig, Sucrose

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Several research groups have succeeded in producing cloned piglets by transfer of somatic cells into enucleated oocytes [1–11]. However, the efficiency of cloning, when measured as development to offspring as a proportion of embryos transferred into recipient females, is less than 1% in most of the previous reports, and widespread use of this technology in a number of animal agriculture and biomedical applications is limited. One of the important factors that affect cloning efficiency is removal of maternal chromosomes from the oocytes. Although the chromosomes of mouse and rat oocytes can be observed under an inverted microscope, the chromosomes of rabbit, sheep, goat, bovine and pig oocytes are difficult to observe due to the large lipid content in them [12]. In these species, therefore, the position of the chromosomes is usually indirectly determined by the location of the first polar body or directly observed under ultraviolet light after staining the oocytes with a DNA-specific dye such as Hoechst 33342. However, polar bodies often migrate from their place of origin and do not always remain in proximity to chromosomes [13–16]. Meanwhile, DNA dyes and ultraviolet light exposure have been reported to have detrimental effects on oocytes and their developmental potential [15, 17, 18].

Recently, Yin *et al.* [12] reported that brief treatment of *in vitro*-matured pig oocytes with demecolcine, a microtubule disrupter, and sucrose induces a cytoplasmic protrusion containing chromosomes into the perivitelline space that can be easily removed by aspiration. This chemically assisted enucleation of oocytes has

been applied to the production of cloned pigs [12, 19] and miniature pigs [20] derived from somatic cells. In the presence of sucrose, the concentration of demecolcine affects the cytoplasmic protrusion of pig oocytes [19]. On the other hand, it has been reported that hypertonic treatment with sucrose induces formation of a protrusion around the chromosome and spindle area in mouse [21, 22] and bovine [23] oocytes, suggesting that the hypertonicity induced by sucrose might also be essential for the cytoplasmic protrusion in pig oocytes [19]. However, there are no reports that have tested this hypothesis. Clarification of the roles of demecolcine and sucrose in the formation of a cytoplasmic protrusion is important for optimization of chemically assisted enucleation of pig oocytes.

Therefore, the present study examined whether demecolcine and sucrose affect formation of a cytoplasmic protrusion containing chromosomes in pig oocytes independently or in combination.

Materials and Methods

In vitro maturation of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in saline at 35 C. The follicular contents were recovered by aspiration from follicles (2–5 mm in diameter) using an 18-gauge needle (Terumo, Tokyo, Japan) and a 5-ml disposable syringe (Nipro, Osaka, Japan). The cumulus-oocyte complexes (COCs) were gathered from the follicular contents and washed twice with HEPES-buffered Tyrode-lactate-pyruvate-polyvinyl alcohol (PVA) and the maturation medium, respectively. Only COCs possessing a compact cumulus mass and evenly granulated ooplasm were selected. COCs in groups of 20–

Table 1. The incidence of cytoplasmic protrusions in pig oocytes treated with different concentrations of demecolcine^a

Concentration of demecolcine ($\mu\text{g/ml}$)	No. of oocytes examined	No. (%) ^b of oocytes with a cytoplasmic protrusion	No. (%) ^c of oocytes with a cytoplasmic protrusion containing chromosomes
0.01	90	31 (34.4) ^d	30 (96.8)
0.05	90	38 (42.2) ^{d,e}	38 (100)
0.1	89	45 (50.6) ^{d,e,f}	45 (100)
0.2	100	68 (68.0) ^f	68 (100)
0.5	75	47 (62.7) ^f	47 (100)
1.0	105	69 (65.7) ^f	69 (100)
1.5	77	43 (55.8) ^{e,f}	43 (100)
2.0	77	41 (53.2) ^{d,e,f}	41 (100)

^aOocytes were cultured for 60 min in the presence of 20 mM sucrose. The osmolarity of each medium was 314 mOsm. Experiments were repeated three times. ^bPercentage per oocytes examined. ^cPercentage per oocytes with a cytoplasmic protrusion. ^{d,e,f}Values with different superscripts are significantly different ($P < 0.05$).

30 were transferred to a droplet of the maturation medium (200 μl) under paraffin oil (Nacalai Tesque, Kyoto, Japan) in a 35-mm polystyrene dish (Becton Dickinson, Franklin Lakes, NJ, USA) and cultured at 38.5 °C in an atmosphere of 5% CO_2 in air. The maturation medium consisted of 90% (v:v) TCM-199 with Earle's salts (Gibco BRL, Grand Island, NY, USA) supplemented with 0.91 mM sodium pyruvate (Sigma-Aldrich Chemical, St. Louis, MO, USA), 3.05 mM D-glucose (Wako Pure Chemical, Osaka, Japan), 0.57 mM cysteine hydrochloride hydrate (Sigma), 10 ng/ml epidermal growth factor (Sigma), 10 IU/ml eCG (Teikoku-Zoki, Tokyo, Japan), 10 IU/ml hCG (Teikoku-Zoki), 100 $\mu\text{g/ml}$ amikacin sulfate (Meiji Seika, Tokyo, Japan), 0.1% (w:v) PVA and 10% (v:v) pig follicular fluid. After 38–42 h of culture, cumulus cells were removed by pipetting with 0.1% (w:v) hyaluronidase (Sigma). Oocytes with a polar body were selected for the experiments.

Induction of cytoplasmic protrusions

HEPES-buffered TCM-199 (Gibco) supplemented with 0.4% (w:v) bovine serum albumin (HEPES-TCM-BSA) was used as the basic medium. *In vitro*-matured and denuded oocytes were cultured in 100 μl of HEPES-TCM-BSA supplemented with demecolcine (Sigma) and sucrose at various concentrations for various times under 5% CO_2 , 5% O_2 and 90% N_2 at 38.5 °C. For the last 5 min of each culture, 5 $\mu\text{g/ml}$ Hoechst 33342 (Sigma) was added to the medium. After culture, the oocytes were examined for the presence of a cytoplasmic protrusion under an inverted microscope. Oocytes with a cytoplasmic protrusion were examined to determine the location of the chromosomes under ultraviolet light.

Experimental designs

In experiment 1, the effects of the demecolcine concentration on the incidence of cytoplasmic protrusions were examined by culturing oocytes in HEPES-TCM-BSA supplemented with 20 mM sucrose [20] and 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 1.5 or 2.0 $\mu\text{g/ml}$ demecolcine for 60 min. The osmolarity of each medium was 314 mOsm when measured using a vapor pressure osmometer (Wescor, Logan, UT, USA).

In experiment 2, the effects of the treatment times of demecolcine and sucrose on the incidence of cytoplasmic protrusions were

examined. Oocytes were cultured in HEPES-TCM-BSA supplemented with 0.2 $\mu\text{g/ml}$ demecolcine and 20 mM sucrose for 15, 30, 60 or 90 min. The measured osmolarity of the medium was 314 mOsm.

In experiment 3, to examine the effects of the sucrose concentration on the incidence of cytoplasmic protrusions, oocytes were cultured in HEPES-TCM-BSA supplemented with 0.2 $\mu\text{g/ml}$ demecolcine and 0, 10, 20, 40, 80 or 160 mM sucrose for 30 min. The measured osmolarities of the medium containing 0, 10, 20, 40, 80 and 160 mM sucrose were 292, 301, 314, 336, 372 and 454 mOsm, respectively.

In experiment 4, the effects of the presence or absence of demecolcine with or without sucrose on the incidence of cytoplasmic protrusions were examined by culturing oocytes in HEPES-TCM-BSA supplemented with 0.2 $\mu\text{g/ml}$ demecolcine and/or 20 mM sucrose for 30 min. Regardless of the presence or absence of demecolcine, the measured osmolarities of the medium with and without sucrose were 314 and 292 mOsm, respectively.

Statistical analysis

All percentage data were subjected to an arcsin transformation in each replicate. The transformed values were analyzed by one-way (experiments 1, 2 and 3) or two-way (experiment 4) ANOVA followed by Fisher's protected least significant difference test. A probability of $P < 0.05$ was considered statistically significant.

Results

As shown in Table 1, the rates of oocytes with a cytoplasmic protrusion (Fig. 1a) at 0.2–1.0 $\mu\text{g/ml}$ demecolcine (62.7–68.0%) were significantly ($P < 0.05$) higher than those at 0.01–0.05 $\mu\text{g/ml}$ demecolcine (34.4–42.2%), although these rates were not different from those at 0.1 and 1.5–2.0 $\mu\text{g/ml}$ demecolcine (50.6–55.8%). In 96.8–100% of the oocytes, the cytoplasmic protrusions contained chromosomes (Fig. 1b), with no significant differences among the different concentrations of demecolcine.

When oocytes were cultured for 15 min with demecolcine and sucrose, 35.1% of them extruded a cytoplasmic protrusion; this rate was significantly ($P < 0.05$) lower than those (55.6–63.9%) of

Fig. 1. A pig oocyte treated with $0.2 \mu\text{g/ml}$ demecolcine and 20 mM sucrose for 60 min under normal (a) and ultraviolet (b) light. The oocyte extruded a cytoplasmic protrusion (arrow) containing chromosomes (arrowhead). Scale bar= $50 \mu\text{m}$.

oocytes cultured for 30 , 60 or 90 min (Table 2). The cytoplasmic protrusions of all of the oocytes (100%) contained chromosomes, regardless of the different treatment times.

As shown in Table 3, significantly ($P<0.05$) fewer oocytes extruded a cytoplasmic protrusion after culture with 160 mM sucrose (16.1%) than with 0 – 80 mM sucrose (61.0 – 73.4%). There were no significant differences in the rates (61.0 – 73.4%) among the oocytes cultured with 0 – 80 mM sucrose. In the absence of sucrose, however, cytoplasmic protrusions were difficult to observe because of the small perivitelline spaces of the oocytes (Fig. 2). In 88.9 – 100% of the oocytes, the cytoplasmic protrusions contained chromosomes, with no significant differences among the different concentrations of sucrose.

Significantly ($P<0.01$) more oocytes extruded a cytoplasmic protrusion after culture with demecolcine (50.0 – 54.7%) than without it (3.3 – 6.7%), regardless of the presence or absence of sucrose (Table 4). The cytoplasmic protrusions contained chromosomes in all of the oocytes (100%), regardless of the presence or absence of demecolcine with or without sucrose.

Discussion

The results of the present study show that the cytoplasmic protrusion containing chromosomes in the pig oocyte is attributable to demecolcine and that sucrose does not affect its formation.

The mechanisms involved in formation of a cytoplasmic protrusion in oocytes treated with demecolcine and/or sucrose are unclear. The cytoplasmic protrusion containing chromosomes in the mouse oocyte is considered to be induced by hypertonicity, the

Table 2. The incidence of cytoplasmic protrusions in pig oocytes treated with demecolcine and sucrose for different periods of time^a

Treatment time (min)	No. of trials	No. of oocytes examined	No. (%) ^b of oocytes with a cytoplasmic protrusion	No. (%) ^c of oocytes with a cytoplasmic protrusion containing chromosomes
15	8	168	59 (35.1) ^d	59 (100)
30	8	160	89 (55.6) ^e	89 (100)
60	8	155	99 (63.9) ^e	99 (100)
90	7	103	59 (57.3) ^e	59 (100)

^aOocytes were cultured in the presence of $0.2 \mu\text{g/ml}$ demecolcine and 20 mM sucrose. The osmolarity of the medium was 314 mOsm . ^bPercentage per oocytes examined. ^cPercentage per oocytes with a cytoplasmic protrusion. ^{d,e}Values with different superscripts are significantly different ($P<0.05$).

Table 3. The incidence of cytoplasmic protrusions in pig oocytes treated with different concentrations of sucrose^a

Concentration of sucrose (mM)	Osmolarity (mOsm)	No. of trials	No. of oocytes examined	No. (%) ^b of oocytes with a cytoplasmic protrusion	No. (%) ^c of oocytes with a cytoplasmic protrusion containing chromosomes
0	292	3	60	39 (65.0) ^d	39 (100)
10	301	3	60	40 (66.7) ^d	40 (100)
20	314	4	94	69 (73.4) ^d	69 (100)
40	336	3	59	36 (61.0) ^d	36 (100)
80	372	4	78	48 (61.5) ^d	78 (100)
160	454	3	56	9 (16.1) ^e	8 (88.9)

^aOocytes were cultured for 30 min in the presence of $0.2 \mu\text{g/ml}$ demecolcine. ^bPercentage per oocytes examined. ^cPercentage per oocytes with a cytoplasmic protrusion. ^{d,e}Values with different superscripts are significantly different ($P<0.05$).

Fig. 2. A pig oocyte extruded a cytoplasmic protrusion (arrow) after culture for 30 min with 0.2 $\mu\text{g/ml}$ demecolcine. Scale bar=50 μm .

common role for sucrose in cryopreservation [24–27], because hypertonic solutions containing high concentrations of other sugars, such as maltose, trehalose, sorbitol, mannitol and glucose, or NaCl can also induce the same phenomenon [22]. Sucrose, which is unable to permeate through the plasma membrane, induces water molecules to move out of oocytes and, thus, effectively causes them to shrink. Liu *et al.* [22] estimated that shrinkage of mouse oocytes induced by hypertonicity of more than 470 mOsm brings the spindle-chromosome complex, where both poles of the spindle are anchored to the cortex by microtubule and non-microtubule elements, closer to the plasma membrane. The chromosomes are then attached to the plasma membrane, and cytoplasmic protrusions are formed due to bulging of the rigid spindle-chromosome complex through the plasma membrane. The same protocol could be applied to bovine oocytes, although hypertonicity of more than 671 mOsm, adjusted by adding more than 300 mM sucrose, is needed to induce a cytoplasmic protrusion containing chromosomes in them [23]. In contrast, this protocol might not be useful for pig oocytes; although 60.0% of the oocytes extruded a cytoplasmic protrusion after culture in HEPES-TCM-BSA with osmolarity adjusted to 608 mOsm by addition of 300 mM sucrose, the protrusions contained chromosomes in only 33.3% of the oocytes in our preliminary experiment (unpublished data).

On the other hand, cytoplasmic protrusions containing chromosomes could be induced in pig oocytes in medium with the osmolarity adjusted to 337 mOsm by addition of 50 mM sucrose

only if demecolcine was present [12, 19]. The mechanisms of this phenomenon have been explained as follows [19]. The meiotic spindle of oocytes treated with demecolcine is disorganized, and the chromosomes become condensed. The condensed chromosomes become closer, but are not attached to the plasma membrane due to the low concentration of sucrose. However, the condensed chromosomes anchored to the actin-rich domain might provide a more rigid structure for the oocyte cortex. Although we agree with this hypothesis, the movement of condensed chromosomes to the plasma membrane might not be attributable to the shrinkage of oocytes induced by sucrose because cytoplasmic protrusions containing chromosomes were observed even when pig oocytes were treated with demecolcine in isotonic medium without sucrose (292 mOsm). The condensation of maternal chromosomes by microtubule disrupters, such as demecolcine, would be related to their movement to the plasma membrane. In the absence of demecolcine, a high concentration of sucrose is essential for movement of maternal chromosomes to the plasma membrane because the movement is only attributable to shrinkage of the oocytes. In the presence of demecolcine, however, chromosomes might spontaneously move to the plasma membrane after condensation, and in this case, addition of sucrose might not be needed. In contrast to the observations of Liu *et al.* [23] showing that cytoplasmic protrusions containing chromosomes were observed in bovine oocytes in medium with the osmolarity adjusted to 671–1587 mOsm by addition of 300–900 mM sucrose, Tani *et al.* [28] reported that the same phenomenon can be induced in isotonic medium containing demecolcine. Furthermore, shrinkage of oocytes induced by sucrose might prevent movement of chromosomes condensed by demecolcine because in the presence of demecolcine, the rate of oocytes with a cytoplasmic protrusion in medium with the osmolarity adjusted to 454 mOsm by addition of 160 mM sucrose is significantly lower than that in isotonic medium without sucrose.

It has been reported that the basic medium is not involved in induction of a cytoplasmic protrusion containing chromosomes in mouse oocytes using sucrose [22]. However, the effects of the basic medium on the phenomenon induced by demecolcine or in other species are unclear. Under our experimental conditions, the adequate concentration and treatment time of demecolcine for inducing a cytoplasmic protrusion containing chromosomes in pig oocytes were 0.2–1.0 $\mu\text{g/ml}$ and 30–90 min, respectively. These results are in agreement with those of a previous report [19] in which high rates of pig oocytes with a cytoplasmic protrusion con-

Table 4. The incidence of cytoplasmic protrusions in pig oocytes treated with demecolcine and/or sucrose^a

Supplement		Osmolarity (mOsm)	No. of oocytes examined	No. (%) ^b of oocytes with a cytoplasmic protrusion	No. (%) ^c of oocytes with a cytoplasmic protrusion containing chromosomes
Demecolcine	Sucrose				
–	–	292	60	4 (6.7) ^d	4 (100)
+	–	292	56	28(50.0) ^e	28 (100)
–	+	314	60	2 (3.3) ^d	2 (100)
+	+	314	86	47(54.7) ^e	47 (100)

^aOocytes were cultured for 30 min in the presence of 0.2 $\mu\text{g/ml}$ demecolcine and/or 20 mM sucrose. Experiments were repeated three times. ^bPercentage per oocytes examined. ^cPercentage per oocytes with a cytoplasmic protrusion. ^{d,e}Values with different superscripts are significantly different ($P<0.01$).

taining chromosomes were obtained after culture for 30–60 min with 0.1–0.4 $\mu\text{g/ml}$ demecolcine. HEPES-TCM-BSA and NCSU 37 [29] were used as the basic medium for induction of cytoplasmic protrusions in the present study and the previous report, respectively. Therefore, the basic medium is probably not an important factor for induction of a cytoplasmic protrusion containing chromosomes in pig oocytes using demecolcine.

The results of the present study indicated clearly that sucrose is not needed to induce a cytoplasmic protrusion containing chromosomes in pig oocytes in the presence of demecolcine. However, cytoplasmic protrusions are easier to observe with the addition of sucrose because it enlarges the perivitelline space in oocytes [12, 19]. Therefore, the presence or absence of sucrose would affect the enucleation efficiency of oocytes after treatment with demecolcine. On the other hand, it is possible that sucrose treatment has detrimental effects on the developmental potential of cloned embryos, although normal cloned pigs and miniature pigs have been produced that were derived from oocytes whose maternal chromosomes were removed after treatment with demecolcine and sucrose [12, 19, 20]. Therefore, further studies are required to determine whether sucrose is needed for chemically assisted enucleation of pig oocytes.

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