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Parthenogenetic development of porcine oocytes treated by ethanol, cycloheximide, cytochalasin B and 6-dimethylaminopurine

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Abstract

This study was carried out to investigate the various concentrations and exposure times of ethanol, one of many intracellular calcium elevating agents, and a sequential combination of ethanol (8%), cycloheximide (CHX, 10 μg/ml), cytochalasin B (CCB, 7.5 μg/ml) and 6-dimethylaminopurine (6-DMAP, 2 mM) to improve parthenogenetic activation and development of in vitro matured porcine oocytes. Cumulus-oocyte complexes (COCs) were matured in tissue culture medium (TCM) 199 for 44 h at 38.5 °C, 5% CO_2 in air. Cumulus-free oocytes showing first polar body were activated by concentrations of 0, 5, 6, 7, 8, 9 and 10% ethanol for 10 min and exposure times of 0, 5, 8, 10, 12 and 15 min with 8% ethanol in HEPES buffered (25 mM) NCSU-23 medium. Also, oocytes were activated with the NCSU-23 medium containing 8% ethanol for 10 min. After that, oocytes were incubated in the NCSU-23 medium supplemented with CHX, CCB, 6-DMAP, CHX + CCB, CHX + 6-DMAP, CCB + 6-DMAP and CHX + CCB + 6-DMAP for 3 h, respectively. Following activation, oocytes were transferred into the NCSU-23 medium containing 0.4% BSA for further culture of 20 and 144 h at 38.5 °C, 5% CO_2 in air. The activation rates of oocytes were higher in 6, 7 and 8% ethanol concentrations compared with 0, 5, 9 and 10% ethanol concentrations. Significantly, more oocytes (29.3–33.7%) were activated in the exposure for 8, 10, 12 and 15 min than those in the exposure for 0 and 5 min, but there was no difference due to exposure to 8% ethanol for 8–15 min. Oocytes treated by chemical agents (40.5–70.5%) after exposure to ethanol significantly improved the rate of oocyte activation compared with ethanol alone (31.2%). The percentage of cleaved oocytes was higher in the ethanol + CHX + CCB + 6-DMAP treatment (66.4%) than in other treatments (24.9–57.6%). Also,

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the rate of blastocyst formation was higher in the ethanol + CHX + CCB + 6-DMAP treatment (25.0%) than in other treatments (0.0–19.3%). In conclusion, the optimal activation treatment of ethanol exposure alone for the in vitro matured porcine oocytes was 8% ethanol for 8–15 min. Oocytes activated by 8% ethanol for 10 min and incubated in the NCSU-23 medium supplemented with CHX, CCB and 6-DMAP for 3 h were more efficient for parthenogenetic development of in vitro matured porcine oocytes.

Keywords: Porcine oocyte; Parthenogenesis; Ethanol; Activation

1. Introduction

Activation of oocytes matured in vitro is essential for the success of animal cloning by nuclear transfer. Artificial stimuli elevating the cytoplasmic levels of calcium ions can induce activation of the oocytes even without penetration of the sperm into the oocyte. Chemical activating stimuli such as ethanol (Nagai, 1987), calcium ionophore and ionomycin (Jones et al., 1995; Tesarik and Sousa, 1995) are used to induce the artificial activation of mammalian oocytes. Ethanol was shown to induce oocyte activation in the mouse (Cuthberston et al., 1981; Cuthberston, 1983) and cattle (Nagai, 1987, 1992; Yang et al., 1994). Yang et al. (1994) reported that the combined treatment with 7% ethanol for 10 min and cycloheximide (CHX), the protein synthesis inhibitor, improved the rate of activation of the young oocytes of cattle. The histone kinase inhibitor, 6-dimethylaminopurine (6-DMAP), has been reported to accelerate and enhance the formation of pronuclei in non-age metaphase II oocytes from mice and cattle (Leal and Liu, 1998). Also, cytochalasin B (CCB) prevents the release of the second polar body after activation of the oocytes, which would result in diploid development (Fukui et al., 1992), and may also help prevent fragmentation of embryos (Collas and Robl, 1990; Yang et al., 1992). Beux et al. (2003) reported that 6-DMAP, cycloheximide, roscovitine and butyrolactone I could be used to block meiotic resumption in porcine oocytes in NCSU-23 culture medium. Although, many reports on porcine oocyte activation have been described, limited information is available on directly comparing the activation efficiency of various chemical agents mentioned above.

The purpose of this study was to investigate the effects of the different concentrations and exposure times of ethanol, and a sequential combination of ethanol, cycloheximide, cytochalasin B and 6-DMAP to improve parthenogenetic activation and development of in vitro matured porcine oocytes.

2. Materials and methods

2.1. Oocyte collection and in vitro maturation

Ovaries were collected from prepubertal gilts at a local slaughter house, and transported to the laboratory in 0.9% NaCl solution containing 75 μg/ml potassium penicillin G, 50 μg/ml streptomycin sulfate at 30–35 °C. Cumulus-oocyte complexes (COCs) were aspirated from

antral follicles (3–6 mm in diameter) using a 18-gauge needle fixed to a 10 ml disposable syringe. COCs were washed three times in Tyrode-lactate-pyruvate-polyvinylalcohol (mTLP-PVA) as described by Bavister (1989), and were washed twice with a maturation medium. Thirty–fourty COCs were transferred to 500 μl of the same medium that had been covered with mineral oil in a 4-well multidish (Nunc, Roskilde, Denmark) and equilibrated at 38.5 °C, 5% CO_2 in air. The medium used for oocyte maturation was tissue culture medium (TCM) 199 supplemented with 26.19 mM sodium bicarbonate, 0.9 mM sodium pyruvate, 10 μg/ml insulin, 2 μg/ml vitamin B_{12}, 25 mM HEPES, 10 μg/ml bovine apotransferrin, 500 μM cysteamine, 10 IU/ml PMSG (Folligon, Intervet International B.V. Boxmeer, Holland), 10 IU/ml hCG (Chorulon, Intervet International B.V. Boxmeer, Holland), 10 ng/ml EGF (Sigma E-4127, USA), 0.4% BSA (Fraction V, Sigma A-8022, USA), 75 μg/ml sodium penicillin G, 50 μg/ml streptomycin sulfate and 10% pFF. After about 22 h of culture, oocytes were cultured without cysteamine and hormones for 22 h at 38.5 °C, 5% CO_2 in air.

2.2. Activation and culture of oocytes

After the completion of culture of oocytes for IVM, cumulus cells were removed with 0.1% hyaluronidase in mTLP–PVA, and washed three times with HEPES buffered (25 mM) NCSU-23 medium. Cumulus-free oocytes showing first polar body were selected and activated by various concentrations and exposure times of ethanol in the NCSU-23 medium.

Also, oocytes were activated with the NCSU-23 medium containing 8% ethanol for 10 min. After that, oocytes were incubated in the NCSU-23 medium supplemented with 10 μg/ml cycloheximide, 7.5 μg/ml cytochalasin B and 2 mM 6-dimethylaminopurine, with a single treatment or combination treatments for 3 h, respectively. Following activation, oocytes were washed three times with the NCSU-23 culture medium containing 0.4% BSA, and were transferred into 500 μl of the same medium for further culture of 20 and 144 h at 38.5 °C, 5% CO_2 in air.

2.3. Examination of oocytes

At 20 h after activation, oocytes were fixed for 48 h in 25% acetic acid (v:v) in ethanol at room temperature, and stained with 1% (w:v) orcein in 45% (v:v) acetic acid to examine activated oocytes under a phase-contrast microscope at ×400 magnification. Blastocysts at 144 h were stained with Hoechst 33342, and nuclei were counted under fluorescent microscope (Olympus, Japan).

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.4. Experimental design

Experiment 1 was conducted to investigate activation of in vitro matured oocytes after exposure to ethanol for 10 min on the concentrations of 0, 5, 6, 7, 8, 9 and 10% ethanol, respectively.

Experiment 2 investigated activation of in vitro matured oocytes after 8% ethanol treatment for varying of 0, 5, 8, 10, 12 and 15 min exposure time, respectively.

Experiment 3 was carried out to investigate the effects of chemical activation agents. All oocytes were activated with the NCSU-23 medium containing 8% ethanol for 10 min. After that, oocytes were incubated in the NCSU-23 medium supplemented with CHX, CCB, 6-DMAP, CHX + CCB, CHX + 6-DMAP, CCB + 6-DMAP and CHX + CCB + 6-DMAP for 3 h, respectively.

2.5. Statistical analysis

Analyses of variance (ANOVA) were carried out using the SAS software package (1996). Duncan's multiple range test was used to compare mean value of individual treatments, when the *F*-value was significant ($P < 0.05$).

3. Results

3.1. Effects of ethanol concentrations on activation of porcine oocytes

As shown in Table 1, seven different concentrations of ethanol were used to compare activation of porcine oocytes matured in vitro. The activation rates of oocytes were higher in 6, 7 and 8% ethanol concentrations compared with 0, 5, 9 and 10% ethanol concentrations. However, more oocytes were activated in the 8% ethanol treatment than in the other treatments. This concentration was therefore chosen for the successive experiments.

3.2. Effects of 8% ethanol exposure times on activation of porcine oocytes

As shown in Table 2, significantly more oocytes (29.3–33.7%) were activated in the exposure for 8, 10, 12 and 15 min than those in the exposure for 0 and 5 min, but there was no difference due to exposure to 8% ethanol for 8–15 min.

Table 1
Effects of ethanol concentrations on activation of porcine oocytes matured in vitro[a]

Concentration of ethanol (%)	No. of oocytes examined[b]	No. and percentage activated
0	119	7 (5.6 ± 3.6^{c})
5	111	29 (25.8 ± 2.8^{b})
6	116	34 (29.7 ± 1.8^{ab})
7	121	37 (30.7 ± 0.9^{ab})
8	118	41 (34.9 ± 1.6^{a})
9	120	32 (26.4 ± 1.6^{b})
10	121	14 (11.3 ± 3.1^{c})

[a] Oocytes were exposed to ethanol for 10 min after 44 h maturation in tissue culture medium (TCM) 199.
[b] Experiments were repeated four times.
[abc] Means ± S.E. in the same column with different superscripts differ significantly ($P < 0.05$).

Table 2
Effect of activation of porcine oocytes matured in vitro according to length of exposure by 8% ethanol

Length of exposure (min)	No. of oocytes examined[a]	No. and percentage activated
0	106	9 (8.7 ± 3.3[c])
5	100	24 (24.0 ± 1.7[b])
8	102	30 (29.3 ± 2.0[ab])
10	103	35 (33.7 ± 2.1[a])
12	102	32 (31.5 ± 1.9[ab])
15	107	33 (30.1 ± 2.0[ab])

[a] Experiments were repeated four times.
[abc] Means ± S.E. in the same column with different superscripts differ significantly ($P < 0.05$).

Table 3
Effects on activation of porcine oocytes matured in vitro treated by ethanol or ethanol plus various chemical agents

Treatment	No. of oocytes examined[a]	No. and percentage activated
Ethanol	103	36 (31.2 ± 3.3[e])
	Ethanol plus	
CHX	109	52 (46.6 ± 2.7[cd])
CCB	107	55 (50.5 ± 2.4[c])
6-DMAP	108	46 (40.5 ± 3.8[d])
CHX + CCB	108	69 (62.7 ± 3.0[ab])
CHX + 6-DMAP	108	52 (47.8 ± 3.1[cd])
CCB + 6-DMAP	107	59 (54.3 ± 3.3[bc])
CHX + CCB + 6-DMAP	105	75 (70.5 ± 3.3[a])

CHX: 10 μg/ml Cycloheximide, CCB: 7.5 μg/ml Cytochalasin B, 6-DMAP: 2 mM 6-dimethylaminopurine.
[a] Experiments were repeated five times.
[abcde] Means ± S.E. in the same column with different superscripts differ significantly ($P < 0.05$).

3.3. Effect of chemical agents for parthenogenetic activation and development

As shown in Table 3, the chemical agent treatments followed by exposure to ethanol significantly improved ($P < 0.05$) the rate of oocyte activation compared with that of the ethanol alone. The ethanol + CHX + CCB + 6-DMAP treatment significantly improved the rate of oocyte activation compared with that of the other seven treatments.

As shown in Table 4, the percentages of cleaved oocytes and blastocyst formation were higher with the ethanol + CHX + CCB + 6-DMAP treatment than in the other seven treatments. The chemical agent treatments improved ($P < 0.05$) the cell numbers per blastocyst compared with those of ethanol alone, but there were no differences among the chemical agent treatments.

4. Discussion

Oocyte activation was induced by ethanol in cattle (Nagai, 1987, 1992; Yang et al., 1994). Liu et al. (1998) reported that both sequential combination activation procedures of

Table 4
Effect on development of porcine oocytes matured in vitro treated by ethanol or ethanol plus various chemical agents

Treatment	No. of oocytes examined[a]	% of cleaved oocytes	% of blatocyst from cleaved oocytes	Cell no. per blastocyst
Ethanol	100	24.9 ± 4.3^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{b}
Ethanol plus				
CHX	98	43.8 ± 3.4^{de}	8.5 ± 2.3^{de}	21.4 ± 0.8^{a}
CCB	98	48.0 ± 2.6^{cd}	12.2 ± 1.1^{cd}	23.3 ± 0.9^{a}
6-DMAP	97	39.3 ± 1.0^{e}	4.4 ± 1.9^{ef}	21.3 ± 1.3^{a}
CHX + CCB	107	57.6 ± 1.5^{b}	19.3 ± 1.5^{b}	24.6 ± 1.2^{a}
CHX + 6-DMAP	106	45.7 ± 2.5^{cde}	10.0 ± 2.6^{cd}	21.5 ± 0.8^{a}
CCB + 6-DMAP	104	52.6 ± 1.4^{bc}	14.5 ± 0.7^{bc}	24.5 ± 1.2^{a}
CHX + CCB + 6-DMAP	108	66.4 ± 2.0^{a}	25.0 ± 2.3^{a}	25.6 ± 1.3^{a}

CHX: 10 μg/ml Cycloheximide, CCB: 7.5 μg/ml Cytochalasin B, 6-DMAP: 2 mM 6-dimethylaminopurine.
[a] Experiments were repeated five times.
[abcdef] Means ± S.E. in the same column with different superscripts differ significantly ($P < 0.05$).

calcium ionophore A23187 (CaA) + 6-dimethylaminopurine, and CaA + cycloheximide + cytochalasin D were effective for the activation and development of young bovine oocytes. In this study, we were interested in testing the effects of the optimal concentration and length of exposure to ethanol for activating porcine oocytes matured in vitro. The synergistic effect of ethanol with CHX, CCB and 6-DMAP treatments was also investigated. Our results indicated that exposure to 8% ethanol for 10 min activated 33.7–34.9% of the porcine oocytes matured in vitro. This rate of activation was similar to other reports on activation of bovine oocytes with ethanol (Nagai, 1987; Yang et al., 1994). It is known that ethanol can induce a single intracellular free-calcium increase in oocytes which can cause activation of some oocytes (Cuthberston et al., 1981). Liu et al. (1998) reported that optimal parthenogenetic development of newly matured bovine oocytes can be obtained by calcium ionophore treatment by incubation with either 6-DMAP or cycloheximide + cytochalasin D. This study confirmed that treatment with ethanol alone resulted in low activation, cleavage and development rates compared with the combined treatments of ethanol plus various chemical agents.

A protein synthesis inhibitor, cycloheximide has been used to induce pronuclear development in matured mouse oocytes (Siracusa et al., 1978; Clarke and Masui, 1983) and in cattle (Sirard et al., 1989). Presicce and Yang (1994a,b) reported that activation rates achieved with the combined ethanol and cycloheximide treatment were high for both young and aging oocytes. Ethanol-induced Ca^{2+} elevation would inactivate the existing cytostatic factor (CSF), and the subsequent cycloheximide exposure would prevent renewal of CSF synthesis in the oocyte. A protein serine/threonine kinase (or phosphorylation) inhibitor, 6-dimethylaminopurine has been shown to enhance the activation stimulus and to accelerate pronuclear formation and parthenogenetic development in the young mouse and bovine oocytes (Moses and Masui, 1994; Moses et al., 1995; Takahashi et al., 1996). After the combined treatments with ethanol plus various chemical agents, we also observed an increased rate of parthenogenetic development in activated oocytes compared with the treatment with

ethanol alone. However, the activation, cleavage and blastocyst formation rates of oocytes with the treatments with ethanol + CHX + CCB, ethanol + CCB + 6-DMAP and ethanol + CHX + CCB + 6-DMAP were significantly increased compared with the treatments with ethanol + CHX, ethanol + CCB, ethanol + 6-DMAP and ethanol + CHX + 6-DMAP. Cytochalasin was reported to inhibit extrusion of the second polar body; in the presence of cytochalasin, segregation of the chromosomes occurred, but cytokinesis did not take place. This resulted in diploid zygotes with two pronuclei (Presicce and Yang, 1994a,b; Liu et al., 1998). The protein kinase inhibitor 6-DMAP, on the other hand, is known to cause the second meiotic spindle to disintegrate and the oocytes to pass directly into interphase (Navara et al., 1994). In our experiment, the activation, cleavage and blastocyst formation rates were more effective following incubation in the treatments with cytochalasin B. In conclusion, the optimal activation treatment for ethanol exposure alone for the porcine oocytes matured in vitro was 8% ethanol for 8–15 min. Also, we can conclude that the combined treatment of in vitro matured porcine oocytes with ethanol + CHX + CCB + 6-DMAP significantly increased the activation, cleavage and blastocyst formation rates. Further comparative investigations will be needed to show what effects each inhibitor has on oocyte quality and further developmental competence.

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References

Bavister, B.D., 1989. A consistently successful procedure for in vitro fertilization of golden hamster eggs. Gamete Res. 23, 139–158.

Beux, G.L., Richard, F.J., Sirard, M.A., 2003. Effect of cycloheximide, 6-DMAP, roscovitine and butylolactone I on resumption of meiosis in porcine oocytes. Theriogenology 60 (6), 1049–1058.

Clarke, H.J., Masui, Y., 1983. The induction of reversible and irreversible chromosome decondensation by protein synthesis inhibition during meiotic maturation of mouse oocytes. Dev. Biol. 97, 291–301.

Collas, P., Robl, J.M., 1990. Factors affecting the efficiency of nuclear transplantation in the rabbit embryo. Biol. Reprod. 43, 877–884.

Cuthberston, K.S.R., Whittingham, D.G., Cobbold, P.H., 1981. Free calcium increase in exponential phases during mouse oocyte activation. Nature 294, 754–757.

Cuthberston, K.S.R., 1983. Parthenogenetic activation of mouse oocytes in vitro with ethanol and benzyl alcohol. J. Exp. Zool. 226, 311–314.

Fukui, Y., Sawai, K., Furudate, M., Sato, N., Iwazumi, Y., Ohsaki, K., 1992. Parthenogenetic development of bovine oocytes treated with ethanol and cytochalasin B after in vitro maturation. Mol. Reprod. Dev. 33, 357–362.

Jones, K.T., Carroll, J., Whittingham, D.G., 1995. Ionomycin, thapsigargin, ryanodine and sperm induced Ca^{2+} release increase during meiotic maturation of mouse oocytes. J. Biol. Chem. 270, 6671–6677.

Leal, C.L., Liu, L., 1998. Differential effects of kinase inhibitor and electrical stimulus on activation and histone H kinase activity in pig oocytes. Anim. Reprod. Sci. 52 (1), 51–61.

Liu, L., Ju, J.C., Yang, X.Z., 1998. Differential inactivation of maturation-promoting factor and mitogen-activated protein kinase following parthenogenetic activation of bovine oocytes. Biol. Reprod. 59, 537–545.

Moses, R.M., Masui, Y., 1994. Enhancement of mouse egg activation by the kinase inhibitor, 6-dimethylaminopurine (6-DMAP). J. Exp. Zool. 270, 211–218.

Moses, R.M., Kline, D., Masui, Y., 1995. Maintenance of metaphase in colcemid-treated mouse eggs by distinct calcium- and 6-dimethlyaminopurine (6-DMAP)-sensitive mechanisms. Dev. Biol. 167, 829–837.

Nagai, T., 1987. Parthenogenetic activation of cattle follicular oocytes in vitro with ethanol. Gam. Res. 16, 243–249.

Nagai, T., 1992. Development of bovine in vitro-matured follicular oocytes activated with ethanol. Theriogenology 37, 869–875.

Navara, C.S., First, N.L., Schatten, G., 1994. Microtubule organization in the cow during fertilization, polyspermy, parthenogenesis and nuclear transfer: the role of the sperm aster. Dev. Biol. 162, 29–40.

Presicce, G.A., Yang, X., 1994a. Nuclear dynamics of parthenogenesis of bovine oocytes matured in vitro for 20 and 40 h and activated with combined ethanol and cycloheximide treatment. Mol. Reprod. Dev. 37, 61–68.

Presicce, G.A., Yang, X., 1994b. Parthenogenetic development of bovine oocytes matured in vitro for 24 h and activated by ethanol and cycloheximide. Mol. Reprod. Dev. 38 (4), 380–385.

SAS Institute Inc., 1996. The SAS system for Windows. Release 6.12, Cary, NC.

Siracusa, G., Whittingham, D.G., Molinaro, M., Vivarelli, E., 1978. Parthenogenetic activation of mouse oocytes induced by inhibitors of protein synthesis. J. Embryol. Exp. Morphol. 43, 157–166.

Sirard, M.A., Florman, H.M., Leibfried-Rutledge, M.L., Barnes, F.L., First, N.L., 1989. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biol. Reprod. 40, 1257–1263.

Takahashi, S., Kubota, C., Ogata, Y., Tokunaga, T., Imai, H., 1996. Parthenogenetic activation and development of bovine oocytes treated with protein synthesis or protein phosphorylation inhibitors. Theriogenology (Suppl. 1) 45 (1), 156.

Tesarik, J., Sousa, M., 1995. More than 90% fertilization rates after intracytoplasmic sperm injection and artificial induction of oocyte activation with calcium ionophore. Fertil. Steril. 63, 343–349.

Yang, X., Jiang, S., Shi, Z., 1992. Improved activation by combined cycloheximide and electrical pulse treatment of bovine follicular oocytes matured in vitro for 23–24 h. Biol. Reprod. (Suppl.) 46, 117 (abstract).

Yang, X., Presicce, G.A., Moraghan, L., Jiang, S.E., Foote, R.H., 1994. Synergistic effect of ethanol and cycloheximide on activation of freshly matured bovine oocytes. Theriogenology 41, 395–403.