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Activation of Bovine Oocytes by Combined Treatment with Ionomycin and cdc2 Kinase Inhibitor

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SUMMARY

The success of nuclear transplantation with mammalian oocytes depends critically on the potential of oocytes activation, which mainly caused to prevent the re-accumulation of maturation promoting factor (MPF). This study was conducted to compare the effect of combined treatment of Ionomycin with a H1-histone kinase inhibitor (dimethylaminopurine, DMAP) or cdc2 kinase inhibitor (sodium pyrophosphate, SPP) on activation of bovine oocytes.

In vitro matured bovine oocytes with the first polar body (PB) and dense cytoplasm were assigned to 3 experimental groups. For activation treatment, oocytes were exposed to 5 μM Ionomycin for 5 min (Group 1), and followed by 1.9 mM dimethylaminopurine (DMAP) for 3 h (Group 2) or followed by 2 mM sodium pyrophosphate (SPP) for 3 h (Group 3). The activation effects in the three treatments and the control group (untreated) were judged by the extrusion of the second PB and formation of a pronucleus (PN). Differences among groups were analyzed using one-way ANOVA after arc-sine transformation of proportional data.

All three treatments led to high activation rates (90% to 95%), with significant difference from the control. However, the extrusion of the second PB and the rate of PN formation differed remarkably among treatments. In Group 1 and 3, about 95% of the oocytes had extruded the second polar body, but one PN had formed in a higher proportion of oocytes in Group 3 than in Group 1 (90% vs. 5%). In experiment 2, the rates of cleavage and development into blastocysts in Group 1 were significantly lower than those of Group 2 and 3 (8.9% and 0% vs. 50.5% and 11.6%, and 44.6% and 7.2%, respectively, $P<0.05$). In experiment 3, ~80% of parthenotes in Group 1 were developed with haploid chromosomal sets. However, when ionomycin was followed immediately by DMAP (Group 2), only 20% of parthenotes were haploid. In Group 3, combined treatment with ionomycin and SPP, the appearance of abnormal chromosomal sets was significantly ($P<0.05$) reduced and the proportion of haploid parthenotes was increased to 85% (17/20) than in Group 2.

These results demonstrate that SPP acted as a cdc2 kinase inhibitor and formed the haploidy in oocyte activation. Thus, the present study suggests that cdc2 kinase inhibitor, such as sodium pyrophosphate, may have an effective role in oocyte activation for the production of cloned embryos/animals by nuclear transplantation.

(Key words: cdc2 kinase inhibitor, ploidy, oocyte activation, bovine oocytes)

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INTRODUCTION

It has been suggested that activation of recipient oocytes is one of the critical components of the current nuclear transplantation regimen (Stice and Robl, 1990; Yang et al., 1994). However, the absence of a paternal gamete in mammalian parthenotes appeared to limit their embryonic development. Successful embryo biotechnology procedures, including nuclear cloning and transgenesis, require an understanding as well as *in vitro* control of this activation phenomenon.

The artificial activation of bovine oocytes has been induced by several agents, including Ca^{2+} ionophore (Ware et al., 1989; Liu et al., 1998ab; Bodo et al., 1998; Ernst et al., 1999), ethanol (Nagai, 1987; Moraghan et al., 1992; Presicce et al., 1994ab; Kuznietsova et al., 2000), electrical stimulation (Aoyagi et al., 1992; Moraghan et al., 1992; Bodo et al., 1998), and inhibitors of protein synthesis (First et al., 1992; Presicce et al., 1994ab; Bodo et al., 1998; Ernst et al., 1999, Ledda et al., 1996; Liu et al.,1998ab). Basically this depends on the influx of extracellular Ca^{2+} into the eggs, and followed by Ca^{2+} transients, to reload intracellular stores. Ca^{2+} ionophore has been widely used for this purpose. The Ca^{2+} influx into eggs results in stimulating the release of cortical granule, extruding the second polar body, and followed by forming of both gametes pronuclei in various invertebrate and vertebrate (Steinhardt et al., 1974; Ducibella et al., 1990; Marcus 1990; Funahashi et al., 1994). The treatment with ethanol resulted in the activation of metaphase II oocytes. Cultured the eggs in the medium containing 7~8% ethanol for 5~7 min induces pronuclear formation and development into later stage embryo, causing by promoting a rapid potentiation of IP_3-mediated Ca^{2+} release through stimulation of inositol- triphosphate (IP-3) formation at the plasma membrane (Ilyin and Parker, 1992). However, the efficiencies of those chemicals for oocytes activation were low, often varied with the age of the oocytes, and usually result in development limited to 1 or 2 cleavages. The reasons for such limited development of parthenotes remain unclear, but may be related to their ploidy, or absence of a paternal genome (De La Fuente and King, 1998).

Thus, the combined treatment of calcium release or influx chemicals with inhibitors of protein synthesis or phosphorylation, or histone kinase to prevent the re-accumulation of MPF has been proved to be effective methods of inducing parthenogenesis. Parthenogenesis has been induced in bovine oocytes by exposure to a Ca^{2+} ionophore followed by cycloheximide or 6-dimethylaminopurine (DMAP), resulting in the resumption of embryonic cell cycles and a high percentage of blastocysts formation (Susko-Parrish et al., 1994). The effects of DMAP on chromatin and microtubule configurations during meiosis are mediated by the inhibition of protein kinases and consequently protein phosphorylation (Szollosi et al., 1993; Rime et al., 1989). Treatment with 6-DAMP after bovine oocytes activation induces pronuclear formation and drives the parthenote into interphase of the first mitotic cell cycle, presumably as a uniform diploid (Susko-Parrish et al., 1994). However, the examination of chromosomal complements in bovine parthenotes occurring spontaneously (King et al., 1988) or produced by activation protocols (Winger et al., 1997) suggests the presence of heteroploidy in a high number of embryos. The oocytes activated with ionomycin and a protein phosphorylation inhibitor displayed some alterations of karyokinesis during the first cell cycle (De La Fuente and King, 1998). Similarly, cycloheximide not only induced a depletion of proteins maintaining MPF activity, but also prohibited the translation of cytoplasmic proteins controlling the initiation of DNA replication (Soloy et al., 1997). Therefore, sodium

pyrophosphate, a potent fully reversible cdc2 kinase inhibitor, was used for the production of haploidy-activated oocytes.

This study evaluated whether ionomycin alone or combination with sodium pyrophosphate or DMAP can affect on pronuclear formation, *in vitro* development, and incidence of chromosomal abnormality in parthenogenetically activated bovine oocytes.

MATERIALS AND METHODS

1. Media

Chemicals and media were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise specified. The medium used for oocyte maturation was M199 containing 10% (v/v) steer serum (Cansera Inc., Rexdale, ON, Canada), 2.5 mM Na pyruvate, 1 mM L-glutamine, 1.0% penicillin-streptomycin (10,000 IU and 10,000 μg/ml respectively; Pen-Strep; GIBCO). The medium used for *in-vitro* culture (IVC) of embryos was M199 containing 10% FCS, 2.5 mM Na pyruvate, 1 mM L-glutamine, and 0.5% Pen-Strep. Tyrode's medium with albumin, lactate, and pyruvate (TALP) was used for preparing 5 μM solutions of ionomycin; TALP supplemented with 10 mM HEPES (HEPES-TALP) was used for sperm preparation, and TALP supplemented with 0.02 mg/ml heparin (IVF-TALP) was used for *in vitro* fertilization (IVF). The IVC medium was used for preparing the 1.9 mM solution of 6-dimethylaminopurine (DMAP) and the 2 mM solution of sodium pyrophosphate (SPP). For both the IVM and IVC media, the pH was adjusted to 7.4 and the osmolality to 280 mOsm/Kg.

2. Oocyte Preparation

Cumulus-oocyte-complexes (COCs) collected from ovaries harvested at a local abattoir were matured in 50 μl droplets of IVM medium under paraffin oil (Yakuri, Japan) at 39C in a humidified atmosphere of 5% CO_2 in air. After 24 h culture, the expanded cumulus cells were removed by vortexing for 2 min in 3% Na-citrate solution. Oocytes suitable for activation (with the first polar body and dense cytoplasm) were selected under an inverted microscope ($\times$200 magnification).

3. Oocyte Activation and Culture

Oocytes were selected and assigned to 3 experimental groups. Group 1: Oocytes were exposed to 5 μM ionomycin for 5 min. Group 2: Oocytes were exposed to the ionomycin for 5 min, and then exposed to 1.9 mM DMAP for 3 h. Group 3: Oocytes were exposed to ionomycin for 5 min, and then exposed to 2 mM SPP for 3 h.

All oocytes were then co-cultured with bovine oviductal epithelial cells (BOEC) in sets of 15 in 50 μl drops of M199 as described by Rho et al. (1998). These co-cultures were maintained for 192 h post-activation. At 48h and 120h post-activation, the cultures were "fed" by adding 25 μl of IVC medium to each drop. Embryonic development was assessed with a stereomicroscope at 24h intervals.

4. *In Vitro* Fertilization

For comparison with parthenotes, embryos were produced by IVF. For the sperm preparation, frozen semen were thawed and isolated intact sperm by Percoll-density gradient method as described by Rosenkrans et al. (1993). After 22 h of culture the oocytes were removed partially its cumulus cells by vortexing them for 10 sec in HEPES-TALP medium. Sets of 15 oocytes were then inseminated with sperm which had been prepared as mentioned above at a final concentration of 2×10^6 sperm/ml in the 50 μl drops of IVF-TALP media. At 16 h post-insemination, sets of 15 presumptive zygotes were co-cultured with BOEC using the same method as for the parthenotes. The rates of cleavage and blastocyst development were exami-

Table 1. Nuclear observations of bovine oocytes at 18 h after various activation procedures

Groups (treatment)	Oocytes used	Nuclear configuration (%)				
		MII	AII-TII	1PN	2PN	>3PN
1. Ionomycin	64	10(16)	40(63)[a]	14(22)[a]	0	0
2. Ionomycin +DMAP	72	3(4)	7(10)[b]	40(56)[b]	12(17)[a]	10(14)[a]
3. Ionomycin + SPP	68	3(4)	5(7)[b]	58(85)[c]	1(2)[b]	1(2)[b]

* Percentages with different superscripts within columns indicate significant differences ($P<0.05$).
DMAP: dimethylaminopurine, SPP: sodium pyrophosphate.
MII: metaphase II, AII: anaphase II, TII: telophase II, PN: pronuclues

ned at 48 h and 192 h post-activation/ insemination, respectively.

5. Cytological Procedures

At 18 h after activation, oocytes were fixed overnight in methanol:acetic acid (3:1, v/v) and stained with 1% aceto-lacmoid to reveal the presence of pronuclei (PN) formation and nuclear status at ×400 magnification.

At 96 h post-activation, parthenotes were prepared and examined for their cytogenetic composition, as described by King et al. (1979). The stained chromosome spreads and nuclei were counted under a compound microscope at ×200 magnification. The chromosomes were evaluated at ×1,000 with oil-immersion optics. Embryos were classified as being haploid, diploid, polyploid or mixoploid.

6. Experimental Design

This study comprised 3 experiments. In Experiment 1, the nucleus status and PN formation of parthenotes were assessed by three different activation regimens. In total, 204 oocytes were used in 5 replicates. In Experiment 2, parthenotes produced by the three different activation regimens and IVF controls were compared for the rates of cleavage by 48 h post-activation/insemination and development to the blastocyst stage by 192 h post-activation/insemination. Finally, in Experiment 3, parthenotes produced at 96 h post-activation by three different activation regimens were compared for their ploidy. A total of 50 8-cell stage parthenotes were analyzed.

7. Statistical Analysis

Differences among treatments were analyzed using one-way analysis of variance (ANOVA) after arcsine transformation of the proportional data of PN formation, cleavage, development, and ploidy. Differences were considered significant ($P<0.05$).

RESULTS

1. Pronuclear Formation of Oocytes following Different Activation Treatments

At 18 h post-activation, the activation rates were determined based on the formation of pronucleus. Table 1 shows the results of Experiment 1. In Group 2 and 3, most oocytes (~88%) were developed into PN stage, but the formation of a pronucleus had ensued in a higher proportion of oocytes in Group 3 than in Group 2 (85% vs. 55%, $P<0.05$). In Group 2, the formations of 2PN and 3PN were significantly ($P<0.05$) higher than in Group 3 (17% and 14% vs. 2% and 2%, respectively). In Group 1, more than 85% oocytes were activated by the presence of the second polar body, but 21% of them formed a PN.

Table 2. The rates of cleavage and development of oocytes after various activation procedures

Groups (treatment)	Oocytes used	Development to(%)	
		Cleavage	Blastocyst
IVF control	75	42(56)[a]	17(23)[a]
1. Ionomycin	67	6(9)[b]	0
2. Ionomycin + DMAP	69	35(51)[a]	8(12)[b]
3. Ionomycin + SPP	66	29(45)[a]	5(7)[b]

* Percentages with different superscripts within columns indicate significant differences (P<0.05).
DMAP: dimethylaminopurine, SPP: sodium pyrophosphate.

2. Cleavage and Development of Parthenotes following Different Activation Treatments

The results of Experiment 2 are summarized in Table 2, which shows that the rates of cleavage and development varied among treatment groups. In Group 1, 9% of oocytes cleaved, but none of them developed to the blastocyst stage. Cleavage rates were greatly increased to ~50% in the use of combination of ionomycin and DMAP/SPP in Groups 2 and 3. However, development of parthenotes to the blastocyst stage remained low to 12% in Group 2 and 7% in Group 3, respectively. Although there was no significant difference in cleavage among the oocytes in Groups 2, 3 and IVF control, but development into blastocysts stage was significantly (P<0.05) higher in IVF control than in Groups 2 and 3.

3. Ploidy of Parthenotes following Different Activation Treatments

The results of Experiment 3 are shown in Table 3. Of the 50 parthenotes analyzed for their chromosomal composition at 96 h post-activation. The chromosomal composition differed significantly (P<0.05) among treatments. The ionomycin treatment alone produced 80% parthenotes with haploid chromosomal sets. However, when ionomycin was followed immediately by DMAP (Group 2) only 20% of parthenotes were haploid and the others were diploid (10%), polyploid (60%) or mixoploid (10%). In Group 3, combined treatment with ionomycin and SPP, the appearance of abnormal chromosome sets was reduced and the proportion of haploid parthenotes was increased to 90% (18/20), significantly higher than in Group 2.

DISCUSSION

For oocyte activation, numerous stimuli, including calcium, electric currency, ethanol and enzyme

Table 3. Ploidy in the parthenotes produced by various activation procedures

Groups (treatment)	Embryos used	Chromosomal status (%)			
		Haploid	Diploid	Polyploid	Mixoploid
1. Ionomycin	10	8(80)[b]	0(0)	2(20)[a]	0(0)
2. Ionomycin + DMAP	20	4(20)[a]	2(10)	12(60)[b]	2(10)
3. Ionomycin + SPP	20	18(90)[b]	1(5)	1(5)[a]	0(0)

* Percentages with different superscripts within columns indicate significant differences (P<0.05).
DMAP: dimethylaminopurine, SPP: sodium pyrophosphate.

have been used with or without combination of protein/H1-histone kinase inhibitor (Soloy et al., 1997; Liu et al., 1998a), resulting in development to blastocysts (Liu et al., 1998a) and establishment of a 35-day pregnancy in cattle (Fukui et al., 1992; Susko-Parrish et al., 1994). Better results on *in vitro* development of parthenotes were achieved in the use of chemicals being increased intracellular calcium with the combination of protein synthesis inhibitor (Hagemann et al., 1995; Bos-Mikich et al., 1995) or protein phosphorylation inhibitor (Szollosi et al., 1993). However, cycloheximide and DMAP non-specifically affect several metabolic pathways in oocytes and consequently may impair further embryonic development. Oocytes activated with ionomycin and a protein phosphorylation inhibitor displayed some alterations in the DNA content reflecting an abnormal pattern of karyokinesis during the first cell cycle (De La Fuente and King, 1998).

The present study shown in Experiment 1 has demonstrated that oocytes activated with ionomycin and followed by DMAP or sodium pyrophosphate treatment enhance the efficiency of PN formation than ionomycin alone, suggesting that being arrested the MPF level of the activated oocytes with calcium to be minimal amount plays more important role. However, between two compounds of DMAP and sodium pyrophosphate, the numbers of PN from the oocytes differ significantly. In DMAP treated, ~88% of the oocytes were developed into PN stage, but the formation of a pronucleus had ensued in a higher proportion of oocytes treated with sodium pyrophosphate. The high incidences of ≤ 2 PN formation in DMAP treated oocytes may cause to inhibition of the second polar body extrusion. Another reason why have been high in PN number is re-entering of the nucleus to S-phase to the cell cycle without having passed through metaphase (De La Fuente and King, 1998). While high activation rates have been achieved after the use of DMAP, chromosomal abnormalities and premature DNA synthesis have been observed in the produced parthenotes (De La Fuente and king, 1998; Rho et al., 1998). Protein phosphorylation inhibitors could prevent reactivation of MPF indirectly, via destruction of c-mos, a Ser/Thr kinase and component of CSF (Susko-Parrish et al., 1994), that has been shown to be destroyed after oocyte activation treatment (Masui, 1991; Wu et al 1997). DMAP-activated bovine oocytes show a premature PN formation with some of the pronuclear structures resembling those observed in c-mos deficient mice. This suggests that inhibition of protein phosphorylation by DMAP inactivates c-mos and MAP kinase (De La Fuente and King, 1998). Neither PN formation of mouse oocytes (Moses and Masui, 1994) nor cleavage of bovine oocytes was registered after the incubation with DMAP alone (Liu et al., 1998b).

When the oocytes were activated with ionomycin alone, ~9% of oocytes cleaved, but none of them developed to the blastocyst stage. Cleavage rates were greatly increased to ~50% in the use of combination of ionomycin and DMAP/sodium pyrophosphate. However, development of parthenotes to the blastocyst stage remained low to 7 ~ 12% than of IVF embryos (~20%). This result similar to others that reported protein phosphorylation inhibitor, DMAP, enhances the activation stimulus and accelerates PN formation and partherogenetic development in the bovine oocytes (Moses and Masui, 1994; Moses et al., 1995; Susko-Parrish et al., 1994; Szollosi et al., 1993; Takahashi et al., 1996).

The results shown in Experiment 3 have indicated that DMAP treatment to the oocytes induces high incidence of chromosomal abnormality as assessed by mixoploidy and polyploidy. As described in the formation of PN, the occurrence of the abnormality may be due to failure of the second polar body extrusion, with

some nuclei presumably re-entering to S-phase of the cell cycle without having passed through metaphase. In contrast to this, sodium pyrophosphate leads to high rates of haploid parthenotes. Previous studies using butyrolactone I (BLI), an inhibitor of cyclin-dependent kinases (CDKI), indicate that the specific inhibition of cyclin-dependent kinases (CDKs) in metaphase II oocytes induces a decrease of cdc2 kinase activity, followed by MAP kinase deposphorylation and subsequent formation of nuclear envelope (Motlik et al., 1998). And the efficiency of the use of specific CDKI, such as a bohemine and inhibitors from the same family for oocytes activation after ICSI or nuclear transfer is useful, and in vitro matured bovine oocytes can be successfully activated by a synthetic inhibitor of CDKs (Alberio et al., 2000). As the result of this study, sodium pyrophosphate is a specific CDK inhibitor, which may allow study of the role of MPF inhibition in the parthenogenetic activation of bovine oocytes. Therefore, DMAP can be probably explained by the fact that it did not inactivate MPF directly as sodium pyrophosphate does, but inhibits phosphorylation of cdc25, an essential kinase for the activation of MPF.

In summary, the present data indicate that DMAP treatment to the oocytes results in high incidence of abnormality on PN formation and chromosomal ploidy. However, the treatment with cdc2 kinase inhibitor (sodium pyrophosphate) greatly reduces those of abnormality with reasonable development rate into blastocyst stage of parthenotes. It is also suggested that oocyte activation by the combined treatment with ionomycin and sodium pyrophosphate is beneficial for the procedures of nuclear transfer and intracytoplasmic sperm injection.

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