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—Original—

Activation with Ethanol Improves Embryo Development of ICSI-Derived Oocytes by Regulation of Kinetics of MPF Activity

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Abstract. Developmental potential of bovine embryos that are not artificially activated after intracytoplasmic sperm injection (ICSI) is generally very low. In this study, we investigated effects of artificial activation with ethanol on kinetics of maturation promoting factor (MPF) activity ($p34^{cdc2}$ kinase activity) and development of bovine oocytes following ICSI. Treatment of oocytes with ethanol at 4 h after ICSI improved their first cleavage and further preimplantation development (51% vs. 13%, 14% vs. 4%: treatment with vs. without ethanol, respectively). MPF activity of oocytes was lowered until at least 2 h after ICSI. In oocytes without activation after ICSI, MPF activity temporarily elevated at 6 h after ICSI, whereas this phenomena was not observed in the oocytes treated with ethanol. Furthermore, MPF activity was elevated 20 h after ICSI in oocytes activated with ethanol, whereas this elevation of MPF activity was not shown in oocytes without activation. These results indicate that the stimulus of sperm was sufficient to lower MPF activity of oocytes following ICSI, and moreover the activation treatment of bovine oocytes with ethanol after ICSI served to maintain the low levels of MPF activity until the next cell cycle started.

Key words: Intracytoplasmic sperm injection (ICSI), Activation, Bovine embryo, Development, MPF activity

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The first intracytoplasmic sperm injection (ICSI) in mammals was reported by Uehara and Yanagimachi [1]. They injected hamster or human spermatozoa into mature hamster oocytes, and observed the transformation of the sperm head into a male pronucleus. Since then, in mammalian species such as rabbit [2, 3], cattle [4, 5], mouse [6], monkey [7], human [8, 9], hamster [10], live offspring have been obtained from oocytes fertilized by ICSI.

Pronuclear formation in sperm-injected bovine oocytes was reported by Westhusin *et al.* [11]. Since that report, many investigators have tried to improve the efficiency of production of bovine ICSI embryos. However, the cleavage and developmental rate of ICSI-derived embryos to the blastocyst stage remain very low. Artificial activation of bovine oocytes after ICSI has been required for embryos to progress beyond the pronuclear stage and continue further development. While mechanical stimulus by an injection pipette can provide an activating stimulus for matured oocytes in some species, fewer than 5 % of bovine oocytes have been activated by sham-injection [12, 13]. Parthenogenetic activation of bovine oocytes can be induced by various stimuli, such as exposure to ethanol [14, 15], calcium

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ionophore A23187 [16], and electric stimuli [17, 18]. In addition, artificial activation after ICSI can be induced by calcium ionophore A23187 [19], ionomycin [12], ethanol [20–22] and electric stimuli [23].

Ethanol is widely used for activation of mammalian oocytes such as those of mouse [24], cow [15], porcine [25], rabbit [26] and marmoset [27]. Horiuchi *et al.* [20, 21] reported that bovine oocytes fertilized by ICSI showed a high cleavage rate and high developmental rate to the blastocyst stage after activation treatment with ethanol at 4 h after ICSI. Also, they reported that ICSI using a piezo-micromanipulator improved the embryonic development of ICSI-derived embryos. In addition, Katayose *et al.* [28, 29] showed that the rate of male pronuclear formation of bovine oocytes fertilized by ICSI using a piezo-micromanipulator was significantly higher than that by conventional ICSI.

Oocyte activation depends on inactivation of maturation promoting factor (MPF), which is composed of $p34^{cdc2}$ kinase and cyclin B. MPF activity elevates following oocytes meiotic division and reaches its peak in the metaphase. Inactivation of MPF induces mitotic division of fertilized oocytes in mouse, rabbit and bovine [30, 31]. Intracellular Ca^{2+} concentration evoked by fertilization and artificial activation reduces MPF activity, thus causing a resumption of meiotic division [32]. After completion of fertilization, an elevation of MPF activity is required for mitotic progression beyond the pronuclear stage. The kinetics of MPF activity may affect preimplantation development of ICSI-derived embryos. To improve early embryonic development, we need to consider these kinetics. To the best of our knowledge, MPF activity of bovine oocytes fertilized by ICSI has not been clarified. Thus, in the present study, we investigated the relationship between MPF activity and embryonic development of bovine ICSI embryos with or without ethanol treatment following ICSI.

Materials and Methods

Collection and in vitro maturation of oocytes

Bovine ovaries were obtained from an abattoir and transported to the laboratory in 0.85% physiological saline within 8 h after slaughter. Follicular oocytes were recovered by aspirating the follicles of 2- to 8-mm diameter with a 21-gauge needle. Only oocytes surrounded by compact, dense cumulus cell layers were selected. Cumulus-oocyte complexes were matured at 39 C for 21 h under 5% CO_2 in air under mineral oil (Sigma-Aldrich) in 25 mM HEPES-buffered TCM 199 (Earle's Salt; Gibco BRL, Grand Island, NY, USA) supplemented with 1 mg/ml polyvinylalchol (Sigma-Aldrich, St. Louis, MO, USA), 0.2 mM sodium pyruvate (Sigma-Aldrich), 0.02 AU/ml FSH (Antrin, Denka Pharmaceutical, Kanagawa, Japan), 1 μg/ml estradiol-17β (Sigma-Aldrich), 75 μg/ml potassium penicillin G (Nacalai tesque. Inc., Kyoto, Japan) and 50 μg/ml streptomycin sulfate (Nacalai tesque).

ICSI, oocyte activation and oocyte culture

After 21 h *in vitro* maturation, cumulus cells were removed from oocytes by vortexing for 4 min in 1 mg/ml hyaluronidase (Nacalai tesque) dissolved in HEPES-buffered TCM 199 (Hanks' Salt; Gibco). The denuded oocytes were washed in TCM 199 (Earle's Salt), transferred to TCM 199 supplemented with 10% fetal bovine serum (FBS, Gibco), and cultured at 39 C under 5% CO_2 in air until ICSI.

Frozen-thawed bovine spermatozoa were used for ICSI in this experiment. One straw of frozen semen was thawed in water at 37 C, and spermatozoa were washed twice with TCM 199. Then, resuspended spermatozoa were layered over 1 ml TCM 199 (Earle's Salt) supplemented with 10 mM pentoxifylline (Sigma-Aldrich) and swum up for 30 min at 39 C under 5% CO_2 in air.

ICSI was carried out according to the method reported by Horiuchi *et al.* [21]. We performed ICSI using a piezomicropipette-driving unit (PMAS-CT150, Prime Tech, Tsukuba, Japan). Briefly, a motile spermatozoon was selected and was immobilized by applying a piezo-pulse to the sperm tail. The holding pipette held a denuded oocyte with a polar body in either the 6 or 12 o'clock positions. The tip of the injection pipette containing the aspirated spermatozoon approached the oocyte from the 3 o'clock position. The zona and the cytoplasmic membrane of the oocyte were broken by several piezo-pulses. Then the spermatozoon was injected into the oocyte.

Injected oocytes were cultured in CR1aa supplemented with 3 mg/ml BSA (Fatty-acid-free, Sigma-Aldrich) at 39 C under 5% CO_2, 5% O_2, 90% N_2. At 4 h after ICSI, oocytes were activated with

7% ethanol in TCM 199 containing 1 mg/ml PVP (PVP-40, Sigma-Aldrich) for 5 min. Then, some injected oocytes were washed and cultured in CR1aa with 3 mg/ml BSA for the first 3 days, and then cultured further in CR1aa with 5% calf serum (CS, Gibco) for the following 5 days at 39 C under 5% CO_2, 5% O_2, 90% N_2. After 18 h of culture, some of the injected oocytes were mounted whole between a slide and a coverslip, and fixed with acetic alcohol (1:3, glacial acetic acid:ethanol) overnight. The fixed oocytes were stained with 1% aceto-orcein, and the presence of male and female pronuclei were examined under a phase contrast microscope. The other injected oocytes were used to measure MPF activity of oocytes. *In vitro*-fertilized (IVF) oocytes were used as a control. IVF was performed according to the method reported by Saeki *et al.* [33].

Oocyte extract preparation for MPF activity assay

Oocytes at 2, 4, 6, 8, 12, 16, 20 and 24 h after ICSI were used as samples. As a control, MPF activity of IVF oocytes fertilized *in vitro* was examined at 6, 10, 14, 18, 22 and 26 h after insemination.

The oocytes were washed several times in PBS and put into plastic tubes containing 5 μl cell lysis buffer (Cell Signaling Technology, USA) with 1 mM PMSF (Sigma-Aldrich). After the oocytes were suspended, the samples were frozen in liquid nitrogen, thawed and sonicated using an ultrasonic disruptor (UD-200; Tomy, Tokyo, Japan) three times for several seconds at 1 C. Cell extracts were frozen and stored at –80 C until just before use.

In vitro p34^{cdc2} kinase assay

The p34^{cdc2} kinase assay was performed using a Mesacup cdc2 kinase assay kit (code no. 5234; MBL, Nagoya, Japan), according to the method described by Shojo *et al.* [30] and Shimada *et al.* [31].

Briefly, 5 μl of oocyte extract (containing 10 oocytes) was mixed with 45 μl of kinase assay buffer B composed of 25 mM HEPES buffer, 10 mM $MgCl_2$, 10% (v/v) MV peptide solution (SlLYSSPGGAYC), and 0.1 mM ATP (Sigma-Aldrich), and the mixture was incubated for 30 minutes at 30 C. The reaction was terminated using 200 μl PBS containing 50 mM EGTA. Phosphorylation of MV peptides was detected using ELISA analysis. Values were expressed as the fold strength of p34^{cdc2} kinase activity in MII oocytes just after *in vitro* maturation.

Statistical analysis

All data were analyzed with Fisher's exact test.

Results

Activation with ethanol improves development of ICSI-derived embryos

We examined the effects of activation treatment on bovine oocytes fertilized by ICSI. The results of the developmental rate to the pronuclear stage after ICSI with or without ethanol treatment are shown in Table 1, and the cleavage rate and the developmental rate to the blastocyst stage after ICSI with or without ethanol are both shown in Table 2. The rate of pronuclear formation (91% *vs.* 71%), the cleavage rate (51% *vs.* 13%) and the developmental rate to blastocyst stage (14% *vs.* 4%) were significantly higher ($p<0.05$) in oocytes activated with ethanol after ICSI than those in oocytes without activation after ICSI. There was no difference between the rate of pronuclear formation in oocytes activated with ethanol after ICSI and that in oocytes fertilized *in vitro* (91% *vs.* 91%).

ICSI oocytes activated with ethanol exhibit different kinetics of MPF activity from those without activation

We examined the effects of ethanol treatment on

Table 1. Effect of ethanol treatment on pronuclear formation in bovine oocytes fertilized by ICSI

	Ethanol treatment	No. of replicates	No. of oocytes examined*	No. (%) of oocytes with two pronuclei
Control (IVF)	–	3	45	41(91)[a]
ICSI	–	3	85	60(71)[b]
ICSI	+	3	67	61(91)[a]

*: 18 h after ICSI or IVF.

[a-b] : Values with different superscripts are significantly different ($p<0.05$).

Table 2. Effects of ethanol treatment on cleavage and subsequent development in bovine oocytes fertilized by ICSI

	Ethanol treatment	No. of replicates	No. of eggs cultured	No. (%) of embryos cleaved	No. (%) of blastocysts
Control (IVF)	–	4	109	78(72)[a]	26(24)[a]
ICSI	–	4	84	11(13)[b]	3(4)[b]
ICSI	+	4	98	50(51)[c]	14(14)[c]

[a-c]: Values with different superscripts within the same column are significantly different ($p<0.05$).

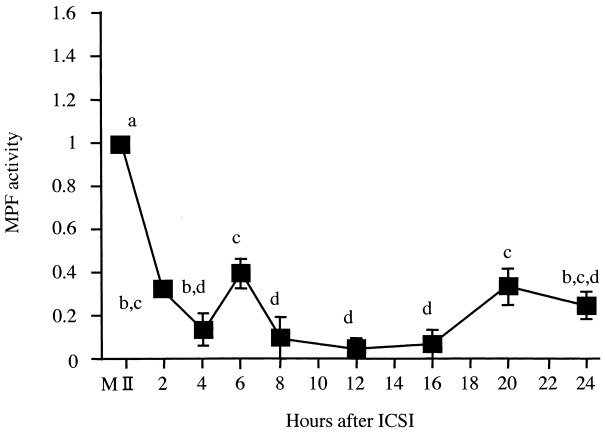

Fig. 1. MPF activity kinetics of oocytes without activation after ICSI. MPF activity of oocytes without activation after ICSI was lowered to 30% of the value of metaphase II oocytes at 2 h. It was lowered further at 4 h. At 6 h, it was temporarily raised, but it was lowered again at 8 h. MPF activity of oocytes was elevated again at 20 h, however its level was about 40 % of the value of metaphase II oocytes. Data are presented as the mean ± SEM (n=3). Different alphabetical letters show significant differences between groups ($p<0.05$).

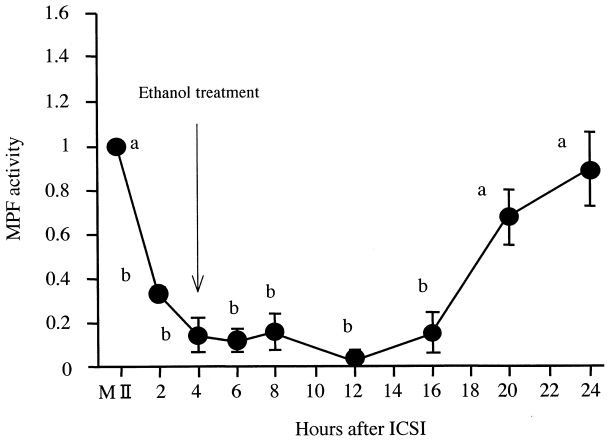

Fig. 2. MPF activity kinetics of oocytes activated with ethanol after ICSI. MPF activity of oocytes activated with ethanol after ICSI was lowered to 30% of the value of metaphase II oocytes at 2 h, and was lowered further at 4 h. MPF activity of oocytes was maintained at this low level until 16 h, when it began elevating, equaling the level of metaphase II oocytes at 20 h. Data are presented as the mean ± SEM (n=3). Different alphabetical letters show significant differences between groups ($p<0.05$).

the MPF activity in bovine oocytes fertilized by ICSI. The results of MPF activity kinetics of oocytes activated with or without ethanol after ICSI are shown in Figs. 1 and 2. Each MPF activity is shown as a value relative to the MPF activity of metaphase II oocytes that was defined as one. In ICSI, MPF activity of the oocytes fertilized by ICSI was reduced to 33% of the value of metaphase II oocytes at 2 h after ICSI. At 4 h after ICSI, it was reduced further. MPF activity of oocytes fertilized by ICSI was significantly lowered before activation with ethanol. The MPF activity of oocytes without activation after ICSI was temporarily elevated at 6 h after ICSI, whereas this temporary elevation was not observed in oocytes activated with ethanol. Then, the MPF activity of oocytes activated with or without ethanol was maintained at low levels until 16 h after ICSI. At 20 h after ICSI, the MPF activity of oocytes activated with ethanol had elevated to equal level of metaphase II oocytes. On the other hand, the oocytes without activation after ICSI did not show such an elevation.

MPF activity kinetics of oocytes fertilized *in vitro* is shown in Fig. 3. The MPF activity of oocytes fertilized *in vitro* was lowered to 50% of the value of metaphase II oocytes at 6 h after insemination, and was significantly lowered to 27% of the value of metaphase II oocytes at 10 h after insemination. MPF activity of oocytes was maintained at low levels until 22 h after insemination, followed by elevation of MPF activity at 26 h after insemination.

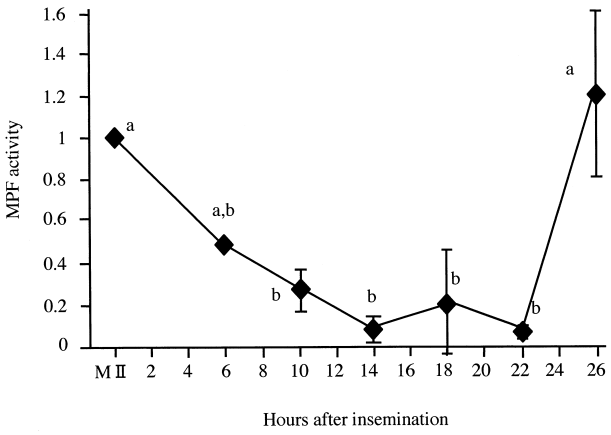

Fig. 3. MPF activity kinetics of oocytes fertilized *in vitro*. MPF activity of oocytes fertilized in vitro was lowered to 50% of the value of metaphase II oocytes at 6 h, and it was significantly lowered to 27% of the value of metaphase II oocytes at 10 h after insemination. MPF activity of oocytes was maintained at a low level until 22 h after insemination, when it began elevating to equal the level of metaphase II oocytes at 26 h. Data are presented as the mean ± SEM (n=3). Different alphabetical letters show significant differences between groups ($p<0.05$).

Discussion

Activation treatment with calcium ionophore (A23187) following bovine ICSI slightly promoted the developmental rate of embryos [34]. In the present study, ethanol was used for artificial activation following ICSI, and rates of cleavage and of development to the blastocyst stage higher than those of ICSI-embryos without activation were shown. Horiuchi *et al.* [20, 21] reported that activation treatment with ethanol performed at 4 h after ICSI was effective for the improvement of the subsequent development of bovine embryos. Thus, ethanol treatment following ICSI appears to be one of the most effective treatments for successful development of embryos fertilized by ICSI.

In normal fertilization, sperm penetration causes an increase in the intracellular Ca^{2+} level in the ooplasm. Subsequently, Ca^{2+} calmodulin dependent kinase II (CaMK II) is raised, and then the MPF activity of oocyte is lowered by degradation of cyclin B. As shown in this study, MPF activity of oocytes fertilized by ICSI was significantly lowered compared with that of metaphase II oocytes before activation treatment with ethanol. MPF activity of oocytes fertilized *in vitro* was also significantly lowered to 27% of the value of metaphase II oocytes at 10 h after insemination. We consider that in the case of ICSI, injected sperm caused a transient rise of Ca^{2+} level in the ooplasm, thereby lowering the MPF activity of oocytes at 2 h after ICSI. When we examined effects of mechanical stimulus due to injection on MPF activity of oocytes by carrying out a sham-injection (manipulation without sperm injection), MPF activity of the oocytes at 2, 4 and 6 h after sham-injection was gradually lowered every 2 h, but not as much as that of metaphase II oocytes (data not shown). Thus, we concluded that such mechanical stimulus alone was insufficient to cause a reduction in the MPF activity of oocytes after ICSI.

A spermatozoon is capable of acquiring the ability to fertilize an oocyte through the acrosome reaction: the removal of outer acrosomal membrane and exposure of inner acrosomal membrane. After the spermatozoon passes through the zona, the nuclear membrane of the sperm head fuses with the membrane of the oocyte and disappears into the ooplasm. Following this sperm and egg fusion, transient Ca^{2+} oscillation waves happen repeatedly. The Ca^{2+} oscillations in bovine oocytes after fertilization last for 17–18 h [35]. In the case of ICSI, a spermatozoon is directly injected into the ooplasm, therefore the sperm membrane is intact, because the acrosome reaction has not been induced. The ICSI procedure bypasses the normal process of zona penetration and fusion of sperm and egg membrane, thus the initial steps involved in destabilizing the sperm nucleus may also be bypassed. In addition, bovine spermatozoa have been shown to be more stably packaged than human, mouse, and hamster sperm cells [36]. It was reported that, treating bull spermatozoa with dithiothreitol (DTT: an agent that specifically reduces disulfide bonds), before sperm injection into bovine oocytes, caused enlargement of the sperm nuclei and oocyte activation was promoted [12]. This suggests that the initial process of fertilization by ICSI may be affected by sperm condition.

Unlike human and mouse oocytes, bovine oocytes require additional stimuli for embryonic development after ICSI. There have been many reports that, when sperm are not treated, sperm-injected bovine oocytes remain inactivated or the sperm nuclei remain condensed for many hours

after ICSI [12, 13, 20, 23]. In normal fertilization, the membrane fusion between sperm and oocytes is essential for incorporation of sperm into the ooplasm. Such incorporation of the sperm head into the ooplasm results in release from meiotic arrest of the oocyte. In the case of ICSI, the soluble sperm factors are the only oocyte activation factors, except for the physical stimulation of the injection procedure [37].

Our results indicate that the MPF activity of oocytes after ICSI without activation was lowered at 2 h, then temporarily elevated at 6 h after ICSI. In bovine, temporary Ca^{2+} elevation induces inactivation of MPF, however it is quickly restored and activated oocytes re-enter a new metaphase arrest [38]. Thus, we speculate that the transient rise of Ca^{2+} in the ooplasm was induced only by the presence of the injected sperm. Accordingly, the MPF activity of oocytes without activation after ICSI was temporarily raised but could not be maintained at low levels.

MPF activity is usually lowered by degradation of cyclin B and is elevated again before starting the next cell cycle by production of new cyclin B. Following the elevation of Ca^{2+} concentration, cyclin B is degraded and the MPF activity of oocytes is lowered. The MPF activity of oocytes without activation after ICSI was temporarily elevated at 6 h and lowered again at 8 h after ICSI. There is a possibility that this temporary elevation of MPF activity was caused by a quick and temporary renewal of cyclin B in bovine oocytes after ICSI. We presume that cyclin B was consumed by the temporary rise of MPF activity of oocytes at 6 h after ICSI, and a shortage of cyclin B in the ooplasm was induced by the temporary elevation. Therefore, the MPF activity of oocytes without activation after ICSI was elevated to about only 40% of the value of metaphase II oocytes at 20 h after ICSI.

On the other hand, MPF activity of oocytes activated with ethanol after ICSI was lowered at 2 h and this low level was maintained until 16 h, when it elevated again at 20 h after ICSI. In oocytes activated with ethanol after ICSI, the temporary elevation of MPF activity in oocytes at 6 h after ICSI was apparently inhibited. Therefore, the elevated MPF activity of oocytes activated with ethanol at 20 h after ICSI was probably due to a sufficient amount of cyclin B promoting progression toward the first cleavage. This suggests that ethanol treatment after ICSI inhibits the temporary rise of the MPF activity at 6 h after ICSI, and thus promotes subsequent embryonic development.

In conclusion, the stimulus due to the injected sperm alone was sufficient to lower the MPF activity of oocytes after ICSI, however, it was not maintained. Activation treatment with ethanol after ICSI influenced the MPF activity of oocytes fertilized by ICSI, maintaining low levels. Thus, the difference in MPF activity kinetics of oocytes with or without ethanol treatment after ICSI are likely to be the cause of the difference in the subsequent embryonic development.

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