FULL PAPER *Theriogenology*

The optimal period of Ca-EDTA treatment for parthenogenetic activation of porcine oocytes during maturation culture

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ABSTRACT. The changes triggered by sperm-induced activation of oocytes, which are required for normal oocyte development, can be mediated by other agents, thereby inducing the parthenogenesis. In this study, we exposed porcine oocytes to 1 mM Ca-EDTA, a metal-ion chelator, at various intervals during 48 hr of *in vitro* maturation to determine the optimum period of Ca-EDTA treatment for parthenogenetic activation. When the oocytes were cultured with or without Ca-EDTA from 36 hr (post-12), 24 hr (post-24), 12 hr (post-36) and 0 hr (post-48) after the start of maturation culture, the blastocyst formation rates were significantly higher ($P<0.05$) in the post-24, post-36 and post-48 groups (3.3%, 4.0% and 2.6%, respectively) than those in the control group without treatment (0%). Furthermore, when the oocytes were cultured with Ca-EDTA for 0 hr (control), 12 hr (pre-12), 24 hr (pre-24), 36 hr (pre-36) and 48 hr (pre-48) from the start of maturation culture, the oocytes formed blastocysts only in the pre-36 and pre-48 groups (0.4% or 0.8%, respectively). Pronuclei (<66.7%) were observed only when the periods of Ca-EDTA treatment were more than 12 hr during maturation culture. In the control group, no pronuclei were detected. Our findings demonstrate that porcine immature oocytes can be parthenogenetically activated by Ca-EDTA treatment for at least 24 hr to 36 hr during maturation culture, leading to pronucleus formation followed by the formation of blastocysts.
KEY WORDS: blastocyst, Ca-EDTA, nuclear status, parthenogenesis, porcine oocyte

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Mammalian oocytes are arrested at metaphase II (MII) of meiosis and are only released from this stage when an activation signal is naturally provided by the entry of sperm [18, 26]. Sperm-induced egg activation triggers a series of events, such as induction of intracellular Ca^{2+} oscillations that reduce the activity of the maturation promoting factor (MPF) and enable resumption of meiosis [7]; this is followed by the formation of male and female pronuclei (PN) [1]. These events are indispensable for normal oocyte development [8].

Artificially, oocyte activation can be induced by stimuli, such as ethanol [16, 17], calcium ionophore A23187 [25], ionomycin [5], electroporation [6], $CaCl_2$ [14], protein kinase inhibitors [15], G protein [14], cycloheximide [19], and strontium and barium ions [20], mimicking normal fertilization [21]. These reagents replace the natural activation signal of sperm [22] and induce the process of parthenogenesis, which is essential for the success of nuclear cloning and reprogramming of the donor nucleus. In pigs, parthenogenotes are also used for embryo transfer to promote pregnancy and piglet production. It has been demonstrated that a normal piglet was delivered by the co-transfer of *in vitro* produced embryos with parthenogenotes produced by electrical activation [9]. Parthenogenotes usually die by the 30th day of pregnancy, therefore useful to establish and maintain pregnancy in place of normal embryos.

Ethylenediamine tetraacetic acid (EDTA) saturated with Na^+ (Na-EDTA) has been successfully used for parthenogenetic activation of porcine immature oocytes at the germinal vesicle (GV) stage, thereby enabling blastocyst formation from immature oocytes. In this case, PN formation starts directly from interphase, between the germinal vesicle breakdown (GVBD) and anaphase I/telophase I (AT) stages, and not via the MII stage. [3, 4]. Ca-EDTA at a concentration of 1 mM has also been shown to induce PN formation from porcine and bovine GV-stage oocytes after 48 hr of maturation culture; however, mouse oocytes were not activated by this treatment [4]. Moreover, PN formation in bovine oocytes required Ca-EDTA treatment for 36-48 hr during the culture period. These studies indicate that EDTA treatment during maturation culture can induce the precocious formation of a pronucleus followed by the development of blastocysts by passing over the second meiotic maturation period. However, at present, little information is available regarding the parthenogenetic activation conditions required during Ca-EDTA treatment for the development of porcine oocytes.

To establish a new method of chemical activation, we examined the optimal period of Ca-EDTA treatment during maturation culture for enabling the parthenogenetic development of porcine oocytes to the blastocyst stage.

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MATERIALS AND METHODS

Collection and in vitro maturation (IVM) of oocytes: Porcine ovaries were obtained from prepubertal cross-bred gilts (Landrace, Large White and Duroc breeds) at a slaughterhouse and transported to the laboratory in 0.9% physiological saline at 35°C within 3 hr of slaughter. Cumulus-oocyte complexes (COCs) were aspirated from follicles measuring 3 to 6 mm in diameter by using an 18-gauge needle connected to a 5-m*l* disposable syringe and collected in modified phosphate-buffered saline (m-PBS; Nihonzenyaku, Fukushima, Japan) supplemented with 100 IU/m*l* penicillin G potassium (Meiji, Tokyo, Japan) and 0.1 mg/m*l* streptomycin sulphate (Meiji). Only COCs with a uniform ooplasm and a compact cumulus cell mass were used in this study. COCs were matured in maturation medium (IVM medium), as described by Azuma *et al*. [3] with minor modifications. Briefly, IVM medium consisted of tissue culture medium (TCM) 199 (Earle's salts) with 25 mM HEPES buffer (TCM 199; Life Technologies, Carlsbad, CA, U.S.A.) supplemented with 0.1% polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MO, U.S.A.), 3.05 mM D-glucose (Wako, Osaka, Japan), 0.91 mM sodium pyruvate (Life Technologies), 10 IU/m*l* equine chorionic gonadotropin (Kawasaki-Mitaka K.K., Kawasaki, Japan), 10 IU/m*l* human chorionic gonadotropin (Kawasaki-Mitaka) and 50 μg/m*l* gentamicin (Sigma-Aldrich). COCs were treated with 1 mM Ca-EDTA (Wako) according to the treatment schedules shown in Fig. 1. Oocytes were matured totally for 48 hr in IVM medium including term incubated in IVM medium with 1 mM Ca-EDTA. Approximately 10 COCs were cultured in each 100-μ*l* drop of IVM medium covered with a layer of mineral oil (Sigma-Aldrich) in a 35 × 10 mm petri dish (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). All cultures were performed at 38.5°C in a humidified incubator containing 5% CO_2.

Embryo culture: After IVM culture, all oocytes were mechanically denuded of cumulus cells in TCM 199 supplemented with 0.1% (w/v) hyaluronidase (Sigma-Aldrich) and cultured in North Carolina State University (NCSU) 23 medium [23] supplemented with 4 mg/m*l* bovine serum albumin (BSA; Sigma-Aldrich) and 50 μg/m*l* gentamicin in an atmosphere containing 5% CO_2 at 38.5°C. After 72 hr, the embryos were transferred to fresh NCSU 23 medium supplemented with 4 mg/m*l* BSA (Sigma-Aldrich), 5.55 mM d-glucose and 50 μg/m*l* gentamicin, and cultured for additional 5 days. The rates of cleavage and blastocyst formation were examined on day 3 and day 8 after *in vitro* culture, respectively.

Assessment of nuclear status: To evaluate the nuclear status of porcine oocytes after 48 hr of IVM culture, oocytes were denuded of cumulus cells by repeated pipetting, mounted on slides, fixed in acetic acid : ethanol (1:3) for 2 days, stained with 1% (w/v) orcein (Sigma-Aldrich) in 45% (v/v) acetic acid (Wako) and examined under a phase contrast microscope.

Assessment of embryo development: On day 8 of culture, all embryos were fixed and permeabilized in PBS containing 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich) at room temperature for 15 min and incubated in PBS containing 0.3% (w/v) polyvinylpyrrolidone at room temperature for another 15 min. Embryos were placed in a drop of mounting medium consisting of 90% (v/v) glycerol containing 1.9 μM Hoechst 33342 (Sigma-Aldrich) on a slide, overlaid with a cover slip supported by four droplets of vaseline/paraffin, incubated overnight at 4°C and examined under a fluorescence microscope with a 355-nm wavelength excitation filter. Embryos with a clear blastocoele were defined as blastocysts. The numbers of cleaved embryos and blastocysts were recorded.

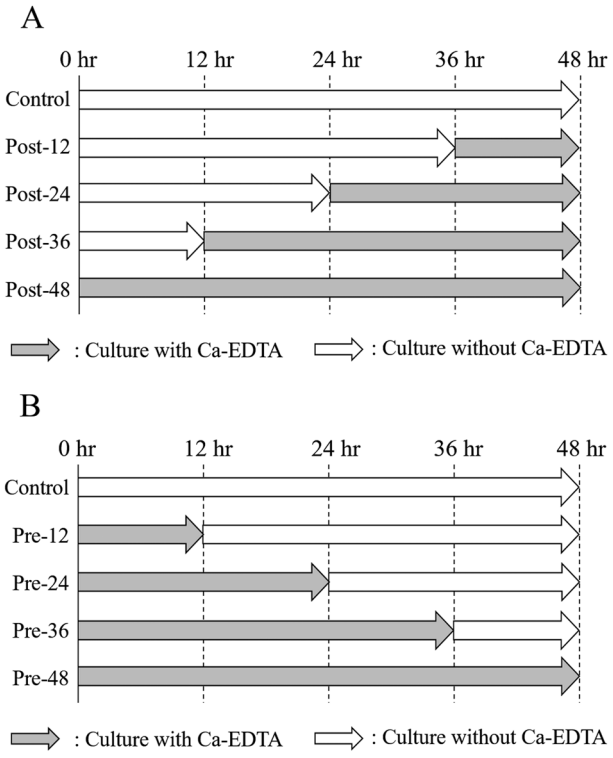

Fig. 1. Experimental design representing the schedules of Ca-EDTA exposure to porcine oocytes during maturation culture. A: treatment groups for pre-treatment experiment (Experiment 1); B: treatment groups for post-treatment experiment (Experiment 2). Filled and open arrows indicate culture with and without Ca-EDTA, respectively.

Experimental design: In experiment 1, the induction of parthenogenetic development of porcine oocytes by post-treatment with Ca-EDTA during maturation culture was examined. COCs were randomly allocated to one of five treatment groups (Fig. 1A), cultured in IVM medium without Ca-EDTA for 48 hr, 36 hr, 24 hr, 12 hr and 0 hr and subsequently incubated in IVM medium supplemented with 1 mM Ca-EDTA for 0 hr (control), 12 hr (post-12), 24 hr (post-24), 36 hr (post-36) and 48 hr (post-48). After 48 hr of IVM culture, some oocytes were fixed and stained to examine the nuclear status of oocytes treated with Ca-EDTA. Remaining oocytes were cultured in NCSU 23 medium for 8 days and examined the embryo development as described

above.

In experiment 2, the development of porcine oocytes by pre-treatment with Ca-EDTA during maturation culture was examined. COCs were incubated in IVM medium supplemented with 1 mM Ca-EDTA for 0 hr (control), 12 hr (pre-12), 24 hr (pre-24), 36 hr (pre-36) and 48 hr (pre-48), and subsequently cultured in IVM medium without Ca-EDTA (Fig. 1B). After 48 hr of IVM culture, some oocytes were fixed and stained to examine the nuclear status of oocytes treated with Ca-EDTA. Remaining oocytes were cultured in NCSU 23 medium for 8 days.

Statistical analysis: Experiments were repeated four times per treatment. The percentages of cleaved embryos and embryos developed to the blastocyst stage were subjected to arcsine transformation before analysis of variance (ANOVA). The transformed data were tested by ANOVA, followed by protected Fisher's least significant difference (LSD) test, using the StatView software (Abacus Concepts, Berkeley, CA, U.S.A.). Differences with a probability value of $P<0.05$ were considered statistically significant.

RESULTS

Experiment 1: As shown in Table 1, when COCs were treated with Ca-EDTA after at least 24 hr during maturation culture (post-24, post-36 and post-48 groups), more oocytes cleaved parthenogenetically and developed to the blastocyst stage than those in the control and post-12 groups. The cleavage rate of oocytes in the post-36 group was significantly higher ($P<0.05$) than that of oocytes in the post-24 and post-48 groups; however, there were no significant differences in the rates of blastocyst formation and number of cell per blastocyst among the three groups. In the control and post-12 groups, no oocytes developed to the blastocyst stage.

Experiment 2: When COCs were treated with Ca-EDTA for 36 hr and 48 hr from the start of maturation culture (pre-36 and pre-48 groups), the cleavage rate of oocytes gradually increased, and some oocytes developed to the blastocyst stage (Table 2). However, the rates of blastocyst formation were very low to verify significant differences among the groups, irrespective of the Ca-EDTA treatment. In the control, pre-12 and pre-24 groups, no oocytes developed to the blastocyst stage.

As shown in Fig. 2, the PN formation of Ca-EDTA treated oocytes was detected when porcine oocytes were post-incubated with Ca-EDTA for at least 12 hr (post-12, post-24, post-36 and post-48). The percentages increased gradually with longer treatment periods, and the rates (34.2% - 66.7%) of PN formation in the post-24, post-36 and post-48 groups were significantly higher ($P<0.05$) than those (0%) in the control group (Fig. 2 A). Furthermore, when COCs were pre-incubated with Ca-EDTA for at least 12 hr (pre-12, pre-24, pre-36 and pre-48), the oocytes formed PN and the rates (13.4%–59.2%) of PN formation were significantly higher ($P<0.05$) than those (0%) of the control group (Fig. 2B).

DISCUSSION

In this study, Ca-EDTA treatment during maturation culture not only activated porcine immature oocytes but also induced parthenogenetic development to blastocyst stage. It had been shown that MII-stage oocytes can be parthenogenetically activated by artificial stimuli and that activated oocytes can cleave and develop to the blastocyst stage [12, 13]. Historically, Azuma *et al*. [3] were the first to report that porcine GV-stage oocytes can be parthenogenetically activated by treatment with Na-EDTA during maturation culture, inducing PN formation, followed by blastocyst formation. Moreover, they demonstrated that Ca-EDTA treatment could also activate porcine oocytes and induce precocious PN formation during oocyte meiosis [3, 4]. They suggested that the treatment of porcine oocytes with Ca-EDTA leads to a deficiency of extracellular Zn^{2+}, alters the composition of the membrane and modifies intracellular conditions, eventually resulting in the promotion of parthenogenetic development. Furthermore, they also suggested that Ca-EDTA can promote PN formation not via the MII stage. However, the optimal period for Ca-EDTA treatment during maturation culture to activate oocytes and successful blastocyst formation from GV-stage oocytes remained unclear.

This study demonstrated that when oocytes were treated with Ca-EDTA from 24 hr or 36 hr after the start of maturation culture to the end of the culture period or during the entire IVM (post-24, post-36 and post-48 groups, respectively) and for more than 36 hr from the start of maturation culture (pre-36 and pre-48 groups), oocytes could cleave and develop to the blastocyst stage. These results indicate that Ca-EDTA treatment of at least 24 hr to 36 hr during a maturation culture period of 48 hr is effective in activating porcine immature oocytes to exit meiosis, enter interphase and develop into blastocysts. Also, the oocytes matured for 24 hr to 36 hr from the start of maturation culture are sensitive to Ca-EDTA activation. In this study, when we performed Ca-EDTA treatment from the start of maturation culture, incubating oocytes with Ca-EDTA for 36 hr was necessary to produce blastocysts. Furthermore, the result of experiment 1 showed that treating oocytes with Ca-EDTA after 24 hr from the start of maturation culture was effective on PN and blastocysts formation. These results indicate that 24 hr to 36 hr from the start of IVM is important period for parthenogenesis of oocytes. In pigs, GVBD typically occurs and metaphase I (MI)-stage oocytes appear between 24 hr and 26 hr after the start of cultivation, and oocytes reach the MII stage after 40 hr to 44 hr of cultivation [2, 24]. Kikuchi *et al*. [10] reported that *in vitro* matured porcine oocytes reach to MII stage after 30 hr from the start of maturation culture. We also examined the nuclear status of oocyte matured without Ca-EDTA, and MII-stage oocytes appeared between after 24 hr to 36 hr from the start of IVM (data not shown). On the other hand, Kim *et al*. [11] reported the importance of Zn^{2+} to exit meiosis in mice. Zn^{2+} content is significantly higher in MII stage oocytes than GV stage oocytes. They also showed that the deficiency of Zn^{2+} during IVM makes the oocyte retain the telophase I spindle, resulting in the failure to reach

Table 1. Effects of post-treatment with Ca-EDTA during *in vitro* maturation culture on the parthenogenetic development of porcine oocytes*

Treatment group**	No. of oocytes cultured	No. (%) of embryos		No. of cells per blastocyst
		cleaved	developed to blastocyst	
Control	365	45 (12.5 ± 2.7)[a)]	0 (0.0)[a)]	-
Post-12	298	23 (7.7 ± 1.6)[a)]	0 (0.0)[a)]	-
Post-24	300	83 (27.7 ± 2.4)[b)]	10 (3.3 ± 0.7)[b)]	31.5 ± 3.7
Post-36	285	105 (37.0 ± 5.0)[c)]	11 (4.0 ± 1.2)[b)]	29.4 ± 2.5
Post-48	300	81 (27.2 ± 2.6)[b)]	9 (2.6 ± 0.8)[b)]	34.7 ± 3.1

*All of the experiments were repeated 4 times. Data are expressed as the mean ± SEM. **Porcine oocytes were cultured without Ca-EDTA from the start of maturation culture and incubated with 1 mM Ca-EDTA for the designated times until 48 hr of total culture period (Fig. 1). As a control, oocytes were cultured in maturation medium without Ca-EDTA. a–c) Values with different superscript letters in the same column are significantly different (P<0.05).

Table 2. Effects of pre-treatment with Ca-EDTA during *in vitro* maturation culture on the parthenogenetic development of porcine oocytes*

Treatment group**	No. of oocytes cultured	No. (%) of embryos		No. of cells per blastocyst
		cleaved	developed to blastocyst	
Control	265	21 (8.1 ± 2.9)[a)]	0 (0.0)	–
Pre-12	260	24 (9.2 ± 1.3)[a)]	0 (0.0)	–
Pre-24	264	28 (10.5 ± 2.9)[a)]	0 (0.0)	–
Pre-36	281	49 (17.8 ± 3.9)[a, b)]	1 (0.4 ± 0.4)	34
Pre-48	270	62 (22.7 ± 4.4)[b)]	2 (0.8 ± 0.8)	24.5

*All of the experiments were repeated 4 times. Data are expressed as the mean ± SEM. **Porcine oocytes were incubated with 1 mM Ca-EDTA for the designated times from the start of maturation culture and cultured without Ca-EDTA until 48 hr of total culture period (Fig. 1). As a control, oocytes were cultured in maturation medium without Ca-EDTA. a, b) Values with different superscript letters in the same column are significantly different (P<0.05).

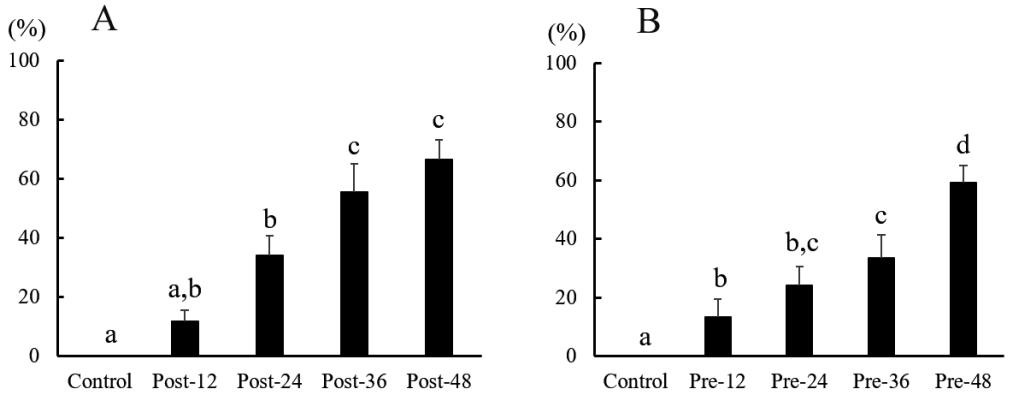

Fig. 2. Effects of post-treatment (A) and pre-treatment (B) with Ca-EDTA during *in vitro* maturation culture on the pronucleus formation of porcine oocytes after 48 hr of culture (mean ± SEM). (A) Porcine oocytes were cultured with Ca-EDTA for 0 hr (control), 12 hr (post-12), 24 hr (post-24), 36 hr (post-36) and 48 hr (post-48) after 48 hr, 36 hr, 24 hr, 12 hr and 0 hr from the start of incubation during maturation culture, respectively. (B) Oocytes were cultured with Ca-EDTA for 0 hr (control), 12 hr (pre-12), 24 hr (pre-24), 36 hr (pre-36) and 48 hr (pre-48) from the start of incubation during maturation culture. Four replicates per treatment group were performed and analyzed. [a–d)] Bars with different letters differ significantly (P<0.05).

the MII stage. It is possible that the deficiency of Zn^{2+} exerts an effect on the release of the polar body (PB) and the PN formed not via the MII stage. Considering these results, the deficiency of Zn^{2+} by Ca-EDTA treatment during 24 to 36 hr after the start of maturation culture can be especially effective to oocytes parthenogenesis, which is the period for the transition from the MI to the MII stage. Azuma *et al*. [4] reported that 77% of oocytes activated with 1 mM Ca-EDTA for 48 hr formed a PN and >95% of the oocytes with PN did not have any PB. In our study, similar rate (67%) of PN formation was observed in the oocytes treated with Ca-EDTA for 48 hr (post-48 group), but 23% of the oocytes with

PN had the PB (data not shown). The difference on the rates of oocytes with the release of PB is unclear, but our results indicate that some oocytes formed PN after completion of MII. Therefore, further research is necessary to reveal the exact mechanism of the parthenogenetic activation by Ca-EDTA during maturation culture.

In conclusion, porcine immature oocytes can be parthenogenetically activated by Ca-EDTA treatment for at least 24 hr to 36 hr during maturation culture, leading to PN formation followed by the formation of blastocysts. Furthermore, oocytes are most sensitive to such treatment at 24 hr to 36 hr after the start of maturation culture. Our study determined the optimal period of Ca-EDTA treatment required for the parthenogenetic activation of porcine oocytes; however, the underlying mechanism remained unclear. In future studies, we intend to investigate other effectors of porcine parthenogenetic activation to investigate mechanism of chemical activation.

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