Effects of EDTA saturated with Ca^{2+} (Ca–EDTA) on pig, bovine and mouse oocytes at the germinal vesicle stage during maturation culture and the involvement of chelation of Zn^{2+} in pronuclear formation induction by Ca–EDTA

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EDTA saturated with Ca^{2+} , Fe^{3+} or Cu^{2+} can induce parthenogenetic activation of pig oocytes at the germinal vesicle stage, whereas EDTA saturated with Zn^{2+} , which is unable to chelate Zn^{2+} , does not, indicating that chelation of Zn^{2+} with EDTA saturated with Ca^{2+} (Ca–EDTA) in maturing pig oocytes plays a pivotal role in the induction of parthenogenetic activation of oocytes. In the present study, the involvement of Zn^{2+} chelation in the induction of parthenogenetic activation of pig oocytes at the germinal vesicle stage was confirmed first by examining the effects of concomitant addition of Zn^{2+} , Cu^{2+} or Ni^{2+} at various concentrations together with 1 mmol Ca–EDTA l^{-1} to the maturation medium. The titration experiments revealed that the pronuclear formation induced by 1 mmol Ca–EDTA l^{-1} was completely inhibited by the addition of $> 30 \mu\text{mol Zn}^{2+} \text{l}^{-1}$ to the medium, but not by the addition of Cu^{2+} and Ni^{2+} at any concentration examined. Second, bovine and mouse oocytes at the germinal vesicle stage were cultured in medium with or without 1 mmol Ca–EDTA l^{-1}

for 48 h to examine the effects of Ca–EDTA treatment on these oocytes during maturation culture. Most (70–86%) of the bovine oocytes that underwent germinal vesicle breakdown matured to the MII stage via the MI phase, regardless of whether Ca–EDTA was present for the first 24 h of culture. However, 61% of oocytes that had been cultured with Ca–EDTA for 48 h formed a pronucleus without a second polar body, whereas oocytes cultured in the absence of Ca–EDTA were not observed to form a pronucleus at any time during culture. However, even when mouse oocytes at the germinal vesicle stage were cultured for up to 48 h in maturation medium containing Ca–EDTA, pronuclear formation was not observed. Finally, when bovine oocytes that had been cultured with 1 mmol Ca–EDTA l^{-1} for 48 h from the germinal vesicle stage were cultured further in medium without Ca–EDTA that was supplemented with 5% fetal calf serum, only 26% of the oocytes developed to the cleaved stage, and none could develop further.

Introduction

Preovulatory mammalian oocytes at the germinal vesicle (GV) stage, when removed from follicles, can spontaneously undergo meiotic maturation in culture, resulting in secondary oocytes arrested at metaphase of the second meiotic division (MII). The duration of the period required for the meiotic maturation of mammalian oocytes in culture varies depending on the species: in mice, cattle and pigs it is about 12, 24 and 48 h, respectively. During the meiotic maturation period, there are two distinct successive metaphases: metaphase I (MI) and metaphase II (MII). The interval between the two meiotic divisions, known as interkinesis, is very short and is not accompanied by an S phase (or DNA replication).

Meiotic progression is regulated by the activity of metaphase-promoting factor (MPF), a heterodimer composed of a catalytic p34^{cdc2} kinase and a cyclin B-regulatory

subunit. Entry into metaphase requires an accumulation of MPF activity (reviewed by Nigg, 1993). When the MPF activity of oocytes at the GV stage is inhibited during meiosis I by moderate ectopic expression of Wee1 kinases (Nakajo *et al.*, 2000), or by failure to accumulate cyclin B due to injection of antisense *c-mos* oligonucleotide (O'Keefe *et al.*, 1989, 1991), although the oocytes undergo germinal vesicle breakdown (GVBD), interphase nuclear formation can be induced just after meiosis I.

It has been reported that treatment of pig oocytes at the GV stage with a cell membrane-impermeable metal ion chelator, EDTA or EDTA saturated with Ca^{2+} (Ca–EDTA) at 0.1–2.0 mmol l^{-1} , induced formation of a pronucleus after 48 h of maturation culture. Moreover, time-course experiments on the nuclear status of oocytes treated with EDTA during the maturation period revealed that the pronuclear formation resulted from the lack of meiotic maturation before the completion of meiosis I (Azuma *et al.*, 2001). Notably, the oocytes with a pronucleus that formed precociously as a result of EDTA treatment could develop parthenogenetically

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to the blastocyst stage during additional embryonic culture (Azuma *et al.*, 2001). Unfortunately, it is still unknown how EDTA induces precocious formation of the pronucleus during meiosis I, leading to parthenogenetic development to the blastocyst stage. However, although Fe^{3+} - or Cu^{2+} -saturated EDTA, like EDTA and Ca-EDTA, could activate the oocytes, Zn^{2+} -saturated EDTA (Zn-EDTA) and a Ca^{2+} -specific chelator (EGTA) could not induce precocious pronuclear formation of the oocytes during the maturation period. However, most of the oocytes treated with Zn-EDTA and EGTA reached the MII stage in the maturation cultures (Azuma *et al.*, 2001). The results of these experiments indicate that extracellular chelation of Zn^{2+} by EDTA results in parthenogenetic activation of maturing oocytes, which induces precocious formation of a pronucleus followed by the development of blastocysts.

The aim of the present study was to test the hypothesis that the effects of EDTA might be due to the extracellular chelation of Zn^{2+} , by examining the effects of concomitant addition of Zn^{2+} , Cu^{2+} or Ni^{2+} at various concentrations with 1 mmol Ca-EDTA l^{-1} to maturation medium on the induction of pronuclear formation in pig oocytes at the GV stage. Second, the effects of Ca-EDTA on pig oocytes were compared with the effects on bovine and mouse oocytes at the GV stage, the maturation periods of which are approximately 50% and 25% of the value for pig oocytes (48 h), respectively. Therefore, the effects of Ca-EDTA treatment of bovine and mouse oocytes at the GV stage during various periods throughout *in vitro* maturation on the nuclear status of the oocytes were examined.

Materials and Methods

Collection of oocytes

Pig and bovine ovaries were collected at an abattoir and transported in saline (9 g NaCl l^{-1}) at 35–38°C within 3 h to the laboratory. Oocytes surrounded by compact cumulus cells and with evenly granulated cytoplasm were aspirated from small antral follicles (2–6 mm in diameter) with a 21-gauge needle attached to a 10 ml disposable syringe, and then the cumulus-enclosed oocytes were washed with Hepes-buffered Tyrode's solution with 0.1% (w/v) polyvinylalcohol (PVA; Sigma, St Louis, MO) before they were used for experiments. Cumulus-enclosed mouse oocytes were collected from isolated ovaries by needle puncture from 6–8-week-old ICR strain females 44–48 h after i.p. injection of 5 iu equine chorionic gonadotrophin (eCG; Sankyo Inc., Tokyo). The collection medium used was Dulbecco's PBS supplemented with 0.2 mmol 3-isobutyl-1-methyl xanthine l^{-1} (Sigma) to inhibit resumption of meiosis. The female mice were anaesthetized with ether and killed by cervical dislocation. All procedures involving animals were approved by the Kyoto University Animal Care and Use Committee.

Cytological examination

The nuclear morphology of pig, bovine and mouse oocytes after culture was examined by stripping surrounding

cumulus cells by repeated pipetting. The denuded oocytes were mounted on slides, fixed in 25% (v/v) acetic alcohol and stained with 1% (w/v) orcein as described by Azuma *et al.* (2001) and Ikeda *et al.* (2000). The stained oocytes were examined under a phase-contrast microscope (Nikon, Tokyo).

Experimental design

In Expt 1, the effects of concomitant addition of divalent cationic metal ions, Zn^{2+} , Cu^{2+} or Ni^{2+} , at various concentrations with 1 mmol Ca-EDTA l^{-1} to the maturation medium on Ca-EDTA-induced pronuclear formation of pig oocytes at the GV stage during the maturation culture period were examined. The basal medium used for the maturation culture referred to as maturation medium was TCM199 with Earle's balanced salt solution (Nissui, Tokyo) supplemented with 18 mmol NaHCO_3 l^{-1} , 3 mmol glucose l^{-1} , 1 mmol sodium pyruvate l^{-1} , 10 iu hCG (Sankyo Inc.), 10 iu eCG and 0.1% (v/v) PVA. Cumulus-enclosed oocytes were cultured in maturation medium with 1 mmol Ca-EDTA l^{-1} (Wako Pure Chemical Inc., Osaka) supplemented with ZnCl_2 , CuCl_2 or NiCl_2 at the various concentrations indicated below for 48 h at 38.5°C under 5% CO_2 in air. The nuclear morphology of oocytes after culture was observed as described above.

In Expt 2, the effects of Ca-EDTA treatment during maturation culture on the nuclear status of bovine and mouse oocytes at the GV stage were examined. The basal medium used for bovine maturation cultures was the same as that used for pig oocytes, whereas Eagle's minimum essential medium (Nissui) supplemented with 3 mg crystallized BSA ml^{-1} (Sigma) was used for mouse maturation cultures. Bovine and mouse cumulus-enclosed oocytes were cultured in their respective maturation media with or without 1 mmol Ca-EDTA l^{-1} for 0, 12, 24, 36 and 48 h under 5% CO_2 in air at 38.5 or 37.0°C, respectively. At the end of the culture period, the nuclear morphology of the oocytes was examined according to the method described above.

In Expt 3, the *in vitro* developmental competence during the preimplantation periods of bovine and mouse oocytes that had formed pronuclei in response to Ca-EDTA treatment was examined according to the methods described.

Statistical analysis

Experiments were repeated three times. Data were analysed by ANOVA and Fisher's protected least significant difference test. Percentage data were subjected to arcsine transformation before statistical analysis. A value of $P < 0.05$ was considered to be an indication of statistical significance.

Results

The first experiment examined the effects of concomitant addition of various concentrations of divalent cationic

Table 1. Effects of concomitant addition of divalent cation metal ions to maturation medium with Ca-EDTA on pronucleus formation of germinal vesicle (GV) oocytes^a

Metal ion ($\mu\text{mol l}^{-1}$)	Ca-EDTA (mmol l^{-1})	Number of oocytes examined	Percentage of oocytes at each stage				
			GV	Maturing (GVBD– Ana/Telo)	MII	PN	Degenerated
–	0	30	14	23	63	0	0
–	1	33	0	14	0	77	9
Cu²⁺							
30	1	34	3	15	0	58	24
300	1	31	7	10	3	67	13
Ni²⁺							
30	1	31	4	6	0	84	6
300	1	33	6	9	3	81	0
Zn²⁺							
5	1	36	3	5	17	56	19
30	1	41	5	27	65	3	0
300	1	30	13	7	67	0	13
1000	1	26	0	0	62	0	38

^aCumulus-enclosed oocytes at the GV stage were cultured for 48 h in maturation medium with or without 1 mmol Ca-EDTA l^{-1} , in the presence or absence of each metal ion at the concentrations indicated.

Ca-EDTA: Ca²⁺-saturated EDTA; GVBD: germinal vesicle breakdown; Ana/Telo: anaphase I and telophase I; MII: metaphase II; PN: pronucleus.

metal ions (Zn²⁺, Cu²⁺ or Ni²⁺, with 1 mmol Ca-EDTA l^{-1}) to the maturation medium on pronuclear formation in pig oocytes at the GV stage during 48 h of culture (Table 1). Although 63% of oocytes cultured for 48 h in maturation medium without addition of divalent cationic metal ions (as a negative control) reached the MII stage, 77% of oocytes cultured for 48 h in medium with 1 mmol Ca-EDTA l^{-1} (as a positive control) escaped from meiotic maturation to form a pronucleus. Morphological observations of the nuclei of the oocytes activated with Ca-EDTA showed that > 95% of the oocytes formed one pronucleus without any polar bodies, in agreement with findings reported by Azuma *et al.* (2001). Culture of GV oocytes in maturation medium with 1 mmol Ca-EDTA l^{-1} supplemented with the metal ions at various concentrations (Zn²⁺ at 5, 30, 300 and 1000 $\mu\text{mol l}^{-1}$, and Cu²⁺ or Ni²⁺ at 30 and 300 $\mu\text{mol l}^{-1}$) for 48 h revealed that the addition of Zn²⁺ at concentrations of $\geq 30 \mu\text{mol l}^{-1}$ almost completely inhibited the induction of pronuclear formation by Ca-EDTA treatment, and 62–67% of the oocytes completed maturation to the MII stage. In contrast, the addition of Cu²⁺ or Ni²⁺ at 30 or 300 $\mu\text{mol l}^{-1}$ to the medium in the presence of Ca-EDTA resulted in rates (58 and 67% or 84 and 81%, respectively) of pronuclear formation in oocytes at the GV stage similar to that (77%) of the positive control. These results are consistent with previous findings that the formation of the pronucleus in oocytes at the GV stage treated with Ca-EDTA during

the maturation period is due to extraembryonic chelation of Zn²⁺.

The second experiment examined whether treatment of bovine and mouse oocytes at the GV stage with 1 mmol Ca-EDTA l^{-1} induced pronuclear formation during maturation culture. The duration required for cultured bovine and mouse oocytes at the GV stage to reach the MII stage (24 and 12 h, respectively) is shorter than that (48 h) of pig oocytes. When bovine oocytes at the GV stage were cultured in maturation medium with or without 1 mmol Ca-EDTA l^{-1} for 24 h, the oocytes that underwent germinal vesicle breakdown (GVBD) subsequently matured to the MII stage via the MI phase, regardless of whether Ca-EDTA was present or absent (86 and 70%, respectively) (Table 2). However, further culture of oocytes in medium with or without Ca-EDTA for 24 h revealed that in the presence of Ca-EDTA, pronuclear formation occurred in 14% of the oocytes at 36 h after commencement of culture, and then the proportion of oocytes that formed a pronucleus increased to 61% at 48 h of culture. However, in the absence of Ca-EDTA, oocytes did not form pronuclei at any time during the culture period, and 77% of oocytes remained at the MII stage until 48 h of culture. All the activated oocytes formed one pronucleus but did not extrude the second polar body. In contrast, when mouse oocytes at the GV stage were cultured for 48 h in medium with 1 mmol Ca-EDTA l^{-1} (Table 3), they did not form a

Table 2. Effects of Ca-EDTA on nuclear status of bovine oocytes during maturation in culture^a

Culture period (h)	Ca-EDTA (mmol l ⁻¹)	Number of oocytes examined	Percentage of oocytes at each stage					
			GV	Maturing (GVBD–Ana/Telo)	MII	CC	PN	Degenerated
24	0	30	0	23	70	0	0	7
	1	21	0	10	86	0	0	4
36	0	30	0	23	77	0	0	0
	1	29	0	20	66	0	14	0
48	0	34	0	23	77	0	0	0
	1	31	0	13	11	6	61	9

^aCumulus-enclosed oocytes at the GV stage were cultured for 48 h in maturation medium with or without 1 mmol Ca-EDTA l⁻¹.

Ca-EDTA: Ca²⁺-saturated EDTA; GV: germinal vesicle; GVBD: germinal vesicle breakdown; Ana/Telo: anaphase I and telophase I; MII: metaphase II; CC: condensed chromatin; PN: pronucleus.

Table 3. Effects of Ca-EDTA on nuclear status of mouse oocytes during maturation in culture^a

Culture period (h)	Ca-EDTA (mmol l ⁻¹)	Number of oocytes examined	Percentage of oocytes at each stage					
			GV	Maturing (GVBD–Ana/Telo)	MII	CC	PN	Degenerated
12	0	30	3	9	88	0	0	0
	1	31	6	10	84	0	0	0
24	0	29	4	3	93	0	0	0
	1	31	0	3	97	0	0	0
36	0	31	6	3	91	0	0	0
	1	32	6	3	69	16	0	6
48	0	31	0	3	97	0	0	0
	1	30	7	7	50	10	0	27

^aCumulus-enclosed oocytes at the GV stage were cultured for 48 h in maturation medium with or without 1 mmol Ca-EDTA l⁻¹.

Ca-EDTA: Ca²⁺-saturated EDTA; GV: germinal vesicle; GVBD: germinal vesicle breakdown; Ana/Telo: anaphase I and telophase I; MII: metaphase II; CC: condensed chromatin; PN: pronucleus.

pronucleus. Instead, most (84%) of the oocytes reached the MII stage at 12 h of culture, and the nuclear status of the oocytes remained at the MII phase until 24 h of culture. Thereafter, in 10–16% of the oocytes, the nuclear contents changed from metaphase chromosomes to condensed chromatin, with formation of a single chromatin cluster 36 and 48 h after the commencement of culture, although 50–69% of the oocytes were still in the MII phase. In control oocytes cultured without Ca-EDTA, most (88–97%) of the oocytes examined reached the MII phase by 12 h of culture, and remained at this phase until 48 h of culture (Table 3).

In Expt 3, the developmental ability of bovine oocytes that had formed a pronucleus in response to Ca-EDTA treatment during the preimplantation period was examined. After bovine cumulus-enclosed oocytes at the GV stage had

been cultured with or without 1 mmol Ca-EDTA l⁻¹ for 48 h, oocytes freed from surrounding cumulus cells were cultured further in modified synthetic oviduct fluid (mSOF) with 5% heat inactivated fetal calf serum for 48 or 192 h at 38.5°C under 5% CO₂, 90% N₂ and 5% O₂. The oocytes treated with Ca-EDTA could develop to the cleaved stage, and the cleavage rates were significantly higher in the Ca-EDTA-treated oocytes (26%) than in control untreated oocytes (1%) (Table 4). However, none of the cleaved stage oocytes could develop further.

Discussion

Pig oocytes at the GV stage can be parthenogenetically activated during the maturation stage of culture by treatment

Table 4. Developmental ability of bovine oocytes after treatment with Ca-EDTA*

Ca-EDTA (mmol l ⁻¹)	Number of oocytes examined	Percentage of oocytes	
		Cleaved (48 h) [†]	Developed to blastocyst (192 h) [†]
0	157	1 ^a	0
1	199	26 ^b	0

Ca-EDTA: Ca²⁺-saturated EDTA.*Cumulus-enclosed oocytes at the germinal vesicle stage were cultured with 1 mmol Ca-EDTA l⁻¹ for 48 h.[†]*In vitro* culture periods after treatment with Ca-EDTA.^{ab}Within the same column, values with different superscripts are significantly different ($P < 0.05$).

with a cell membrane-impermeable chelator, Ca-EDTA, at 1 mmol l⁻¹, leading to pronuclear formation followed by mitotic cell divisions (Azuma *et al.*, 2001). Moreover, although Cu²⁺- or Fe³⁺-saturated EDTA, like Ca-EDTA, can activate the oocytes, Zn²⁺-saturated EDTA could not induce pronuclear formation, indicating that extracellular chelation of Zn²⁺ by Ca-EDTA is an important regulatory event in pronuclear formation and parthenogenetic activation of pig oocytes at the GV stage (Azuma *et al.*, 2001).

The results of the present study support the involvement of chelation of Zn²⁺ by Ca-EDTA in the parthenogenetic activation of pig oocytes at the GV stage. In these experiments, the effects of addition of divalent cationic metal ions, Zn²⁺, Cu²⁺ or Ni²⁺, at various concentrations to maturation medium with or without 1 mmol Ca-EDTA l⁻¹ were examined. When GV oocytes were cultured in medium with 1 mmol Ca-EDTA l⁻¹ supplemented with Zn²⁺ at $\geq 30 \mu\text{mol l}^{-1}$ for 48 h, the induction of pronuclear formation by Ca-EDTA treatment was almost completely inhibited, and most of the oocytes matured to the MII stage. However, the titration of 1 mmol Ca-EDTA l⁻¹ with Cu²⁺ or Ni²⁺ at 30 or 300 $\mu\text{mol l}^{-1}$ did not affect the ability of Ca-EDTA to induce pronuclear formation. These results are consistent with our previous finding that the precocious formation of a pronucleus in pig oocytes at the GV stage treated with Ca-EDTA is due to extracellular chelation of Zn²⁺. However, the reason why Ca-EDTA failed to induce pronuclear formation even when the molar ratio of EDTA:Zn²⁺ decreased below 1:1 remains unknown. This finding is consistent with a report that the addition of an excess of chelator for Zn²⁺ (Ca-EDTA) to the culture medium was required for complete prevention of Zn²⁺-induced oxidative neuronal injury in mouse cortical cultures (Noh *et al.*, 1999). A possible explanation for the requirement for an excess of Ca-EDTA relative to Zn²⁺ may be that the chelating efficiency of Zn²⁺ by Ca-EDTA is affected by the culture conditions.

With regard to the potential function of Zn²⁺, it has been found that a substantial amount of Zn²⁺ per unit mass of

protein is localized in the plasma membranes of cells, and that Zn²⁺ in the membrane has a stabilizing or protective effect on the structure and function of the membrane (Bettger and O'Dell, 1981). Interestingly, it was reported that Zn²⁺ affects the motility and capacitation of sperm cells in many species (Bettger and O'Dell, 1981; Rizzo *et al.*, 1992; Andrews *et al.*, 1994). Moreover, treatment of ejaculated spermatozoa with EDTA elicits decondensation of sperm nuclear chromatin, and this effect is reversibly inhibited by Zn²⁺ supplementation (Kvist and Bjorndahl, 1985). These findings indicate that alteration of Zn²⁺-mediated structures of oocytes, which are likely to be induced by removal of Zn²⁺ by Ca-EDTA, may play a pivotal role in the effects of Ca-EDTA on pig oocytes.

The second study examined whether Ca-EDTA treatment could induce pronucleus formation during maturation culture of bovine and mouse oocytes at the GV stage. Time-course experiments using bovine oocytes revealed that although GV oocytes cultured in medium with Ca-EDTA underwent the normal maturation process until the MII stage during the first 24 h of culture, when the oocytes that had reached the MII stage were cultured in the presence of Ca-EDTA for an additional 24 h, pronuclear formation was observed 36–48 h after the commencement of maturation culture. However, in the absence of Ca-EDTA, none of the oocytes cultured for 48 h formed a pronucleus. Surprisingly, it was found that the culture period (36–48 h) required for pronuclear formation induced by Ca-EDTA treatment in bovine oocytes at the GV stage was very similar to that in pig oocytes at the GV stage. In the case of pig oocytes, the oocytes at the GV stage reached the MI stage at about 24 h after the commencement of culture and then entered interphase, followed by the formation of the pronucleus. These comparative findings in bovine and pig oocytes imply that GV-stage oocytes from both species treated with Ca-EDTA become disposed to exit meiosis and enter interphase 24 h after the initiation of the treatment with Ca-EDTA. Furthermore, it was found that treatment of bovine oocytes at the GV stage with Zn-EDTA at 1 mmol l⁻¹ for 48 h could not induce pronuclear formation (data not shown). Therefore, findings from the present study in both pig and bovine oocytes contribute similar insights into the regulatory mechanisms that are required for the Ca-EDTA-induced transition from meiosis to interphase in oocytes at the GV stage. In contrast, even when mouse oocytes at the GV stage were cultured in maturation medium in the presence of 1 mmol Ca-EDTA l⁻¹ for 48 h, pronuclear formation was not observed, and most of the oocytes proceeded to the MII stage. Although 10–16% of the oocytes that had attained the MII stage at 24 h of culture appeared to undergo a change of nuclear morphology from metaphase chromosomes to condensed chromatin and to form a single chromatin cluster, the nuclear status of these oocytes did not proceed to the stage of nuclear decondensation and deposition of the nuclear envelope associated with formation of the pronucleus.

When bovine oocytes that had formed a pronucleus

during culture in medium with Ca-EDTA for 48 h were cultured further in medium without Ca-EDTA, 26% of the oocytes developed to the cleavage stage but none developed beyond this stage. However, our previous experiments indicated that pig oocytes that had formed a pronucleus in response to Ca-EDTA treatment during their maturation culture period could undergo further preimplantation development to the blastocyst stage. The lower developmental ability of Ca-EDTA-treated bovine oocytes compared with that of Ca-EDTA-treated pig oocytes might be related to their ageing characteristics, because although the timing of pronucleus formation induced in pig oocytes by Ca-EDTA treatment was almost the same as that of the activation of MII oocytes by fertilization, the timing of pronucleus formation in Ca-EDTA-treated bovine oocytes was about 16 h later than that of the activation of MII oocytes by fertilization.

In summary, the results of the present study support the proposal that chelation of Zn^{2+} by Ca-EDTA treatment induces formation of the pronucleus in pig oocytes at the GV stage during the maturation culture period, leading to parthenogenetic development to the blastocyst stage. Furthermore, it was found that addition of 1 mmol Ca-EDTA l^{-1} could induce pronuclear formation in bovine oocytes at the GV stage during maturation culture at 36–48 h after the commencement of Ca-EDTA treatment, whereas mouse oocytes at the GV stage were not activated by Ca-EDTA treatment during their maturation culture.

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