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Parthenogenetic Activation of Mouse Oocytes by Strontium

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Abstract: *The purposes of this study were to determine the optimal conditions for inducing parthenogenetic activation by strontium and to evaluate the developmental ability of the activated oocytes in mice. MII oocytes collected from B6CBF1 and CD-1 mice were cultured for 2–60 min in medium containing 1.7 mM strontium. The proportion of oocytes activated increased in a time-dependent manner, with an apparent difference between the B6CBF1 and CD-1 strains in sensitivity to strontium. In oocytes derived from B6CBF1 mice more than 90% of oocytes were activated by the treatment with strontium for 30 min, whereas more than 80% of the oocytes from CD-1 mice were activated by the treatment for 5 min. The majority of oocytes formed the second polar body and a single pronucleus in both cases. Of the parthenogenetically activated 1-cell embryos with the haploid genome, around 39% developed to the blastocyst stage in the B6CBF1, and in contrast the developmental ability was low (11%) in the CD-1 strain. The ability to develop to blastocysts was significantly improved in diploid parthenogenetic embryos of both the strains; 93% and 58% were developed to the blastocyst stage, respectively. After transfer of the diploid parthenogenetic embryos to recipient mice, 11% of the embryos developed to day 10 of gestation. The record of intracytoplasmic Ca^{2+} concentrations showed that the exposure of oocytes to medium containing 1.7 mM strontium induced repetitive intracellular Ca^{2+} transients. These data clearly showed that strontium is a potent stimulus for inducing parthenogenetic activation and supporting in vitro and in vivo development in mouse oocytes.*

Key words: *Mouse oocyte, Parthenogenetic activation, Development, Strontium, Calcium transients.*

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Oocytes are ovulated at the metaphase of the second meiosis and arrested at this stage of the cell cycle until fertilization. This process is regulated by the cytoplasmic maturation promoting factor (MPF), a complex of cyclin and $p34^{cdc2}$, which is stabilized by cytostatic factor (CSF) [1–4]. Intracellular Ca^{2+} oscillations at fertilization, which are seen every 10–20 min and last for 3–4 h, are necessary for resumption of the second meiosis of the oocytes [4, 5]. Artificial stimuli which cause intracytoplasmic Ca^{2+} increases can also induce oocyte activation. These artificial stimuli, such as exposure to ethanol, the Ca^{2+} ionophore A 23187, or direct electric pulses, cause a single Ca^{2+} increase caused by the influx of exogenous Ca^{2+} [6], but strontium (Sr^{2+}) treatment mimics the repetitive Ca^{2+} oscillations seen at fertilization [7]. How Sr^{2+} causes the repetitive intracellular Ca^{2+} oscillations and meiotic resumption is unclear, but it is thought to provide a more efficient procedure for the induction of parthenogenetic activation in mouse oocytes, compared to the other stimuli which induce a single intracellular Ca^{2+} increase [5–9]. In addition, the difference between fertilization and parthenogenesis in cytoplasmic Ca^{2+} mobility may reflect the ability of the cytoplasm to support further development of embryos.

Although no precise experiment has been conducted, it is known that a wide range of concentrations of Sr^{2+}, 1.7–10 mM, are effective in inducing intracellular Ca^{2+} oscillations [7–9] and oocyte activation in mice [8–10]. Incubation with medium containing 10 mM Sr^{2+} for 2 to 24 h was found to be quite efficient for oocyte activation; more than 90% of the oocytes formed the second polar body and pronucleus. The parthenogenetic 2-cell embryos, which had been diplodized by inhibition of the second polar body extrusion, developed to the blastocyst stage in the range of 73–87% *in vitro*, but few fetuses were obtained after transfer to recipients [9]. A

possibility is that over stimulation by incubation with 10 mM Sr^{2+} medium for several hours has a detrimental effect on embryo development. Studies so far show that Sr^{2+} is certainly efficient in the induction of parthenogenetic activation in mouse oocytes, but optimal conditions for its use have not yet been established. We therefore examined the effects of duration of incubation with Sr^{2+} medium on the activation rate and the ability of the oocytes to develop to blastocysts and fetuses by using oocytes from B6CBF1 and CD-1 mice.

Material and Methods

Oocyte collection

B6CBF1 (C57BL/6N × CBA) and CD-1 mice were used as oocyte donors. They were superovulated with injections of 5 iu of equine chorionic gonadotrophin (eCG; Peamex, Sankyo Ltd., Tokyo, Japan) and 5 iu of human chorionic gonadotrophin (hCG; Puberogen, Sankyo Ltd.) given 48 h apart. Oocytes at metaphase II were released from the oviducts 15 h after the hCG injection, and the cumulus cells were removed by digestion with 300 units/ml hyaluronidase in M2 medium [10]. After being washed with M2 medium several times, normal MII oocytes were selected and used for the experiments.

Artificial activation

To induce oocyte activation, 1.7 mM $SrCl_2$ (Sr^{2+}) was prepared in Ca^{2+}-free M16 medium [12] supplemented with 0.4% polyvinyl alcohol (PVA) and adjusted to 260 mOsm. Oocytes were cultured in a 50 μl drop of Sr^{2+} medium for 2–60 min in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37°C. The oocyte activation was evaluated by extrusion of the second polar body and formation of the pronuclei at 6 h after the treatment. Activated oocytes were classified into the following 4 types according to Webb *et al.* (1986) [13]: 1) oocytes which formed a second polar body and single pronucleus (1PN + 1PB), 2) oocytes which formed two pronuclei without the second polar body (2PN), 3) oocytes immediately cleaved (IC), 4) oocytes with a single pronucleus without the second polar body (1PN), 5) oocytes which formed a second polar body and metaphase III chromosomes (MIII), and 6) others including degraded oocytes. The experiments were repeated 5 to 8 times.

In vitro culture and embryo transfer

After culture with Sr^{2+} medium for 1 h, the oocytes were washed with M2 medium several times and cultured in a drop of M16 medium supplemented with 0.4% bovine serum albumin (BSA) and 100 μM EDTA in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37°C. When diploid parthenogenetic embryos were produced, following the incubation with Sr^{2+} medium the oocytes were cultured in M16 medium containing 5 μg/ml cytochalasin B (CB) to inhibit an extra-polar body extrusion and to induce the formation of two pronuclei.

The blastocysts derived from B6CBF1 diploid parthenogenetic embryos were transferred to the uterine horns of CD-1 females on day 3 of pseudopregnancy (2.5 days post coitum).

Intracellular Ca^{2+} measurements

To monitor intracellular Ca^{2+}, oocytes were incubated for 15–20 min in 2 μM fura 2 AM (Sigma) in M2 containing 0.002% pluronic. The zona pellucida was removed by a brief exposure to acidified Tyrode's medium, and the oocyte was transferred to a heated chamber (37°C) on a Zeiss inverted fluorescent microscope. Fura-2 was excited at 340 and 380 nm and emitted light >520 nm was collected. Images were acquired using a cooled CCD camera and were processed using Ionvision software (Coventry, UK). Fluorescence records are presented as the ratio of fluorescence intensities acquired at 340 and 380 nm.

Evaluation of meiotic resumption

To assess meiotic resumption after parthenogenetic activation, whole mount preparations of the oocytes were prepared at 0, 15, 30, 60, 90, 120, 180, 240 and 360 min after the beginning of the treatment. After being mounted on slide glass, oocytes were fixed with methanol 3: acetic acid 1 solution for 2 days and stained with 1% lacmoid.

Stastical analysis

Data were analyzed by χ^2-test.

Results

Proportion of activated oocytes

In the first experiment, oocytes were incubated with 1.7 mM Sr^{2+} medium for 2–60 min to assess the optimal time for the induction of parthenogenetic activation. The activated oocytes were classified into 4 types (Table 1). The proportion of activated B6CBF1 mouse oocytes, which was estimated by extrusion of the second polar body, was increased with the time of incubation, and 93% of the oocytes were activated when incubated for 30 and 60 min. The majority of the activated oocytes extruded the second polar body and formed a single pronucleus. Of the oocytes incubated for 30 min, 15%

were arrested at MIII, where the oocytes extruded the second polar body but failed to form a pronucleus. In the 60 min cultures, the proportion of MIII oocytes was significantly ($p<0.05$) decreased to 4%, and the oocytes that formed two pronuclei without the second polar body extrusion was significantly ($p<0.05$) increased to 12%. The oocytes from CD-1 mice were much more sensitive to Sr^{2+}. When these oocytes were incubated with 1.7 mM Sr^{2+} medium for only 5 min, 81% of the oocytes were activated (Table 2). Prolongation of the incubation time did not increase the proportion of oocytes activated, of which 69–73% formed the second polar body and a single pronucleus.

Development of parthenogenetic embryos

To assess the ability to develop to blastocysts, the parthenogenetic 1PN + 1PB 1-cell embryos were cultured for 4 days *in vitro*. The developmental ability of the embryos from B6CBF1 oocytes was improved with a prolongation of incubation time with Sr^{2+} medium. The highest proportion of the embryos which developed to blastocyst stage was 39% in the 60 min incubation. In contrast in the CD-1, 11% of blastocysts were obtained by 15 min incubation, but the proportion tended to be decreased by prolonged duration. The developmental ability of the parthenogenetic embryos was significantly enhanced when the second polar body extrusion was inhibited with cytochalasin B. In the diploid parthenogenetic embryos, 93% (B6CBF1) and 58% (CD-1) developed to blastocysts. These rate of developmental are similar to those of fertilized embryos studied in our laboratory.

It is known that parthenogenetic embryos produced by other artificial stimuli and gynogenetic embryos pro-

Table 1. Parthenogentic activation of mouse oocytes by strontium

Exposure time (min)	No. of oocytes examined	No. of oocytes activated (%)	Types of activated oocytes (%)#					Abnomal
			1PN + 1PB	2PN	IC	1PN	M III	
B6CBF1								
0	109	4 (4)	2 (18)	1	0	0	1	1
5	103	45 (44)****	28 (27)****	0	0	0	16****	1
15	103	70 (68)*	59 (57)***	1	0	0	9	3
30	102	95 (93)	76 (75)	3	0	1	15	1
60	107	99 (93)	83 (78)	12**	0	0	4**	2
CD-1								
0	101	33 (33)	9 (9)	0	1	0	21	1
2	100	30 (30)****	20 (20)*	0	0	0	9**	5
5	115	94 (81)****	85 (74)****	0	1	0	8	1***
15	142	114 (80)	99 (70)	4*	4	1	5	8**
30	129	101 (78)	89(69)	4	5	0	1	6
60	132	110 (83)	97 (73)	7	4	0	1	13

#See text for the abbreviations. Values with asterisks are significantly different from the preceding value; *p<0.1, **p<0.05, ***p<0.01, ****p<0.001.

Table 2. *In vitro* development of parthenogentic mouse embryos activated by strontium

Exposure time (min)	No. of activated oocytes developed to blastocysts/examined (%)	
	B6CBF1	CD-1
Haploid parthenotes		
5	2/28 (7)	3/85 (4)
15	11/59 (19)	11/99 (11)*
30	28/76 (37)*	10/89 (9)
60	32/83 (39)	6/97 (6)
Diploid parthenotes#		
60	102/110 (93)****	72/124 (58)****

#Diploid parthenogentic embryos were produced by inhibition of the second polar body extrusion. Values with asterisks are significantly different from the preceding value in each strain; *p<0.1, ****p<0.001.

duced by pronuclear transfer are able to develop by day 10 of gestation, 20- to 25-somite fetuses. To reveal this ability in the parthenogenetic embryos activated by Sr^{2+} medium, a total of 104 diploid blastocysts derived from the B6CBF1 oocytes were transferred into 10 pseudopregnant females. Of the transferred embryos, 11% developed to day 10 of gestation showing the various stages of development; and they had 8 to 30 somites (Table 3, Fig. 1).

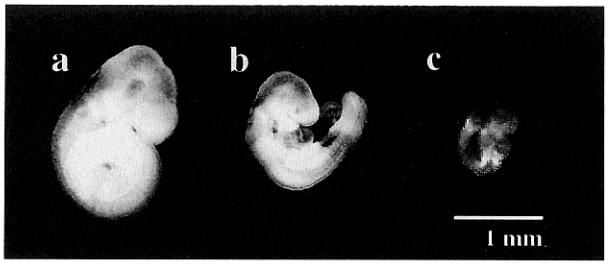

Fig. 1. Day 9.5 mouse fetuses, about 30 somites (a), 20 somites (b) and 10 somites (c), derived from oocytes activated parthenogenetically by strontium.

Cytokinetic changes in activated oocytes

The progress of meiosis after the parthenogenetic activation was examined in B6CBF1 oocytes incubated with 1.7 mM Sr^{2+} medium for 60 min (Table 4). The transit from metaphase to anaphase of the second meiotic division started 15 min after the beginning of culture with 1.7 mM Sr^{2+} medium. At 30 min after the start of incubation, 86% of the oocytes were at the telophase of the second meiosis. The oocytes started to extrude the second polar body at 90 min after the treatment, when chromosomes were at the telophase stage, and within 120 min after the activation, most of the oocytes com-

Table 3. Postimplanation development of parthenogenetic mouse embryos activated by strontium

No. recipients	No. of embryos transferred	No. of embryos implanted	No. of fetuses	
1	12	10	0	
2	16	8	1	10 somites; 1
3	14	8	0	
4	11	11	0	
5	20	16	0	
6	13	10	3	25 somites; 1, 10–15 somites; 1, 10 somites; 1
7	8	7	5	20–25 somites; 1, 10–15 somites; 2, 8–10 somites; 2
8	13	12	1	10 somites; 1
9	14	13	1	23 somites; 1
10	11	9	4	25–30 somites; 1, 25 somites; 1, 8–10 somites; 2
Total 10	132	104	15	
		78.8%	11.4%	

Table 4. Meiotic progress in mouse oocytes activated by strontium

Time after# activation (min)	No. of oocytes examined	Meiotic stages					
		MII	AnaII	TeloII	Telo/Retic[1]	Reticular[2]	Pronuclear
0	15	15	0	0	0	0	0
15	25	23	2	0	0	0	0
30	29	1	3	25	0	0	0
60	29	1	0	28	0	0	0
90	21	0	0	13	8	0	0
120	24	0	0	4	20	0	0
180	21	0	0	0	0	21	0
240	20	0	0	0	0	0	20
360	21	0	0	0	0	0	21

Oocytes were incubated with medium containing 1.7 mM Sr^{2+} for 60 min. 1. Telo/Retic; oocytes that have the extruded second polar body, but the chromosomes are still condensed. 2. Reticular; oocytes that contain reticular stage nucleus.

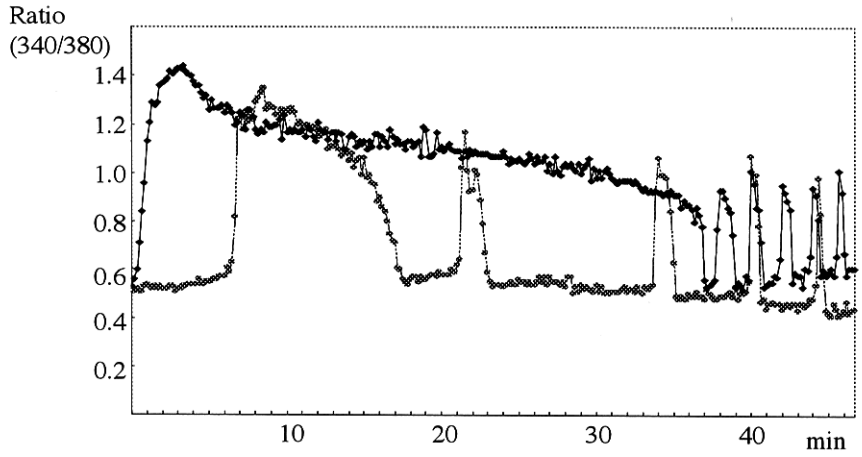

Fig. 2. Records of intracellular Ca^{2+} oscillations in the high- (◆-◆) and low-sensitive (●-●) mouse oocytes exposed to the strontium medium.

pleted the second polar body extrusion. Nuclear decondensation was seen from 180 min after activation, in which the nucleus was in the reticular stage, and pronuclei with nucleoli were observed at 240 min after activation.

Intracellular Ca^{2+} oscillations

To confirm whether parthenogenetic oocyte activation with Sr^{2+} medium is induced by an increase in intracellular Ca^{2+}, intracellular Ca^{2+} levels were recorded. Intracellular Ca^{2+} oscillations were seen in all cases examined (n=6). Figure 2 shows two records which represent high-sensitive and low-sensitive oocytes. The oocytes that sensitively responded to Sr^{2+} had a long first intracellular Ca^{2+} transient which lasted for 30 min, starting 1 min after exposure to Sr^{2+} medium. In these cases the first transient was followed by repeatad Ca^{2+} transients at 5–10 min intervals. The low-sensitive oocytes showed the first Ca^{2+} transient from 7 min after the exposure; it lasted for 10 min and was followed by repetitive Ca^{2+} transients at 10–15 min intervals.

Discussion

The present study has confirmed that Sr^{2+} medium can be used to induce parthenogenetic activation and intracellular Ca^{2+} oscillations in mouse oocytes, as reported previously [7, 8, 14]. The analysis of cytokinesis in oocytes activated by Sr^{2+} showed that the majority of oocytes formed a single haploid nucleus after the extrusion of the second polar body [8–10]. The present study showed that the sequential events after incubation with Sr^{2+} medium occurred in similarly to those seen in the fertilized oocytes, in which the second polar body extrusion occurred 90–120 min after the beginning of incubation in Sr^{2+} medium and formed a pronucleus 2.5–3 h later. Nevertheless, the sensitivity of oocytes to Sr^{2+} is apparently different in the two mouse strains, B6CBF1 and CD-1. A 30-min incubation in Sr^{2+} medium was required to induce parthenogenetic activation in the oocytes from B6CBF1 mice. In contrast, the oocytes from CD-1 mice sensitively responded to Sr^{2+} medium and were activated by 5-min incubation. The reason for the difference between the two strains in sensitivity to Sr^{2+} medium is unclear, but it has been reported that resistance to electric stimuli of the two strains also differs [15]. There is no evidence that Sr^{2+} is a potent stimulus for oocyte activation in other species; at least bovine oocytes do not respond to Sr^{2+} medium (unpublished data).

The ability of Sr^{2+} to activate mouse oocytes was first reported by Fraser [16], who showed that oocytes incubated in Sr^{2+} medium formed a second polar body and pronuclei. Kline and Kline [7] demonstrated that Sr^{2+} is able to mimic the repetitive intracellular Ca^{2+} transients seen at fertilization. Moreover, it is known that the proportion of aneuploidy in embryos activated with Sr^{2+} medium is significantly lower than in embryos activated by brief exposure to ethanol medium [10]. In view of these reports, Sr^{2+} is thought to be an efficient stimulus for inducing parthenogenetic activation in mouse oocytes, but the developmental ability of the oocytes has not been closely studied. A recent report by Bos-Mikich *et al.* [9] showed that oocytes incubated in Sr^{2+}

medium for 2–24 h developed to the blastocyst stage *in vitro* and the number of inner cell mass cells of blastocysts was significantly increased when oocytes were activated by the prolonged incubation in Sr^{2+} medium. This suggests that the intracellular Ca^{2+} oscillations play a role not only in the resumption of meiosis but also in the subsequent embryo development. But these embryos failed to develop to fetuses after transfer to recipient females except in a few cases. The present study on B6CBF1 oocytes clearly showed that oocytes activated with Sr^{2+} have the ability to develop *in vitro* and *in vivo*. The proportion of the activated oocytes developed to blastocysts was affected by the duration of incubation in Sr^{2+} medium. The developmental ability of oocytes incubated for short periods was poor, being areested until the 8-cell stage. As reported previously, the developmental ability of parthenogenetic 1-cell embryos was apparently enhanced by diplodization: 93% and 58% developed to blastocysts in B6CBF1 and CD-1 embryos, respectively.

The development of parthenogenetic embryos after implantation is generally limited in mammals [17, 18]. In mice, parthenogenetic and gynogenetic embryos are unable to develop beyond day 10 of gestation [19, 20]. The proportion of embryos which developed to fetuses has varied among the reports and among the mouse species used. Gynogenetic mouse embryos which are produced by pronuclear exchange and contain only the maternal genome exhibit a slightly better ability to develop *in vitro*, but the post implantation development is not improved; only 10% of embryos develop to day 9.5 fetuses after transfer to recipient females [21]. In the reported by Bos-Mikich *et al.* [9], 60–70% of the embryos transferred became successfully implanted but only 2% of the embryos were developed to day 10 of gestation. In the present study, regarding the developmental ability of the parthenogenetic embryos, 11% of the embryos transferred developed to day 10 of gestation. Our results revealed that diploid parthenogenetic embryos that are activated by Sr^{2+} medium are able to develop to day 10 of gestation, as can the gynogenetic embryos. These results suggest that insufficient stimulation with Sr^{2+} results in poor development of the parthenogenetic embryos *in vitro*, but over stimulation may have a detrimental effect on development, especially after implantation.

In conclusion, Sr^{2+} is a potent stimulus for inducing parthenogenetic activation in mouse oocytes, but the duration of incubation in Sr^{2+} medium affects activation of the oocytes and the subsequent development *in vitro* and *in vivo*.

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