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Effect of artificial oocyte activation by calcium ionophore on one-day-old unfertilized oocytes after ICSI

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Abstract: At our clinic, a successfully fertilized human oocyte is defined as one with two polar bodies and two pronuclei (PN) 18 h after ICSI, but when some oocytes do not form PN. Recently, a successful pregnancy and live birth following artificial oocyte activation (AOA; using Ca^{2+} ionophore) of ICSI-treated one-day-old unfertilized oocytes was reported. In this study, we performed AOA on PN-lacking unfertilized oocytes, 18 h after ICSI (defined as Day 1-AOA, since ICSI=Day 0). The abilities of these oocytes to form PN and develop were compared with those of oocytes fertilized by ICSI (defined as No-AOA). Piezo-ICSI was performed on 168 oocytes, 50 of which failed to fertilize (0PN) but were subsequently artificially activated. 2PN formation rate was significantly lower in Day 1-AOA, and the rates of 1PN and ≥4PN were significantly higher than in No-AOA. In Day 1-AOA, 8 oocytes formed 2PN, 6 zygotes underwent cleavage and 2 of them developed to the morula stage, but none formed blastocysts. The rates of cleavage, morula, and blastocyst formation were significantly lower in Day 1-AOA than in No-AOA. These results suggest that the AOA protocol has room for improvement in the activation of unfertilized oocytes 18 h after ICSI.

Key words: ICSI, Pronuclear formation, Ca^{2+} ionophore, Artificial oocyte activation

Introduction

In the intracytoplasmic sperm injection (ICSI) procedure, one sperm is directly injected into the cytoplasm of an oocyte under a microscope. ICSI is the most efficient and widely accepted treatment in assisted reproduction technology, and it is used to treat patients with severe oligozoospermia and patients who have previously experienced fertilization failure using conventional *in vitro* fertilization (IVF). Normal fertilization involves pronuclei (PN) formation, in which both the male and female PN are formed within 16 h after ICSI [1]. However, not all ICSI-treated oocytes progress to further development, and 70–80% of oocytes that fail to fertilize following ICSI remain at the metaphase II (MII) stage [2, 3]. At our clinic, the formation of two pronuclei (2PN) and extrusion of the second polar body are checked for at 18 h after ICSI, but PN formation is not observed in some oocytes. Recently, we demonstrated that in approximately 90% of oocytes that do not have PN at both 18 and 24 h after ICSI, premature chromosome condensation (PCC) of the sperm head is observed [4]. This PCC process does not require activation of the oocyte and the presence of condensing factors (e.g., M-phase/maturation promoting factor: MPF) in the cytoplasm, preventing the transformation of sperm nuclei into male PN [5]. Taken together, these results suggest that failure of oocyte activation is the principal cause of fertilization failure in ICSI.

Oocytes that are not fertilized by ICSI can be subsequently activated using chemical substances or electroporation, a process called artificial oocyte activation (AOA), and these activated oocytes can successfully form PN [6, 7]. This combination of ICSI with AOA has resulted in pregnancies and the birth of healthy babies by patients with repeated fertilization failure [8, 9]. In these cases, the AOA processes were applied in the cycle immediately following a repeated fertilization failure and performed within 2 h after ICSI [10–12]. However, at this

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time point, it is difficult to forecast the failure of fertilization after ICSI, and the AOA treatment may disturb normal fertilization because the incidence of chromosome aberrations increases with time between AOA and ICSI in the mouse [13].

Lu et al. [14] reported that activation using the Ca^{2+} ionophore A23187 (A23187) on one-day-old unfertilized oocytes after ICSI led to a successful pregnancy and full-term delivery for a couple with oligoasthenoteratozoospermia, who had repeatedly experienced extremely low fertilization rates. They performed AOA at 18 h after ICSI, and three of nine (33.3%) unfertilized oocytes were activated with 2PN formation. The zygotes developed to four-cell, five-cell, and several-cell embryos in culture over three days. The two embryos with minimal fragmentation were transferred on the fourth day after oocyte retrieval and a healthy baby was delivered [14]. Importantly, AOA can only be performed on oocytes with fertilization failure in which PN are not observed. Therefore, AOA at 18 h after ICSI may be a useful procedure for rescuing oocytes with fertilization failure.

In this study, we performed AOA with A23187 on one-day-old unfertilized oocytes lacking PN formation at 18 h after ICSI, and analyzed whether it can actually induce oocyte activation. Furthermore, we compared the developmental competence of normally fertilized oocytes in the same ICSI cycle with those of oocytes with 2PN formation after one-day-old AOA.

Materials and Methods

Patients and ethics

This study investigated only ICSI cycles that generated unfertilized oocytes between June and October 2014 with the informed consent of patients. A total of 310 oocytes were retrieved in 41 cycles (41 cases). The average age of the women was 39.3 ± 4.5 (mean ± SD) years, ranging from 29 to 48 years. Ovarian stimulation was performed using letrozole (26 cycles), clomifene (13 cycles), or chemical-stimulation-free (2 cycles) treatment. This study was approved by the Ethical and Scientific Committee of Ochi Yume Clinic Nagoya, Japan.

Sperm preparation

Routine semen analysis was carried out according to the criteria specified by the World Health Organization. Motile sperm were separated by a density gradient centrifugation method (90%: 68%: 45%) using ISolate (Irvine Scientific, CA, USA). After suspension in a sperm washing medium (Irvine Scientific), the sperm were stored at room temperature until ICSI. The final concentration of sperm was less than 2.0×10^7/ml.

Oocyte collection and ICSI

Cumulus-oocyte complexes (COCs) were retrieved transvaginally with a 20–23 G needle (Kitazato Medical, Japan) under ultrasound guidance. The maturity of the oocytes was determined under an inverted microscope (Olympus, Japan) and oocytes at the MII stage were stripped of their cumulus cells using 60 IU/ml hyaluronidase (Sigma). ICSI was performed using a Piezo Micro Manipulator (Prime Tech Ltd, Japan) (piezo-ICSI) within 3–6 h after oocyte retrieval (ICSI=Day 0). The injected oocytes were cultured in Sydney IVF Fertilization Medium (Cook Medical, IN, USA) or Human Tubal Fluid medium (Origio, Denmark) in a humid incubator (K-Systems Kivex Biotec Ltd, Denmark). All culture procedures were performed at 37 °C in an atmosphere containing 6% CO_2, 5% O_2, and 89% N_2, and all media were covered with mineral oil (Nakamedical, Japan).

Artificial oocyte activation on Day 1 (Day 1-AOA)

We prepared a 10 mM stock solution of A23187 (Sigma) with dimethyl sulfoxide (Sigma) and diluted it with fertilization medium to a final concentration of 10 μM (activation medium) [15]. PN formation was observed under inverted microscopes, and oocytes without PN at 18 h after ICSI (Day 1) were defined as unfertilized. The unfertilized oocytes were transferred to the activation medium in darkness and incubated for 5 min at 37 °C. After three washes, the oocytes were cultured for an additional 24 h in Continuous Single Culture medium (Irvine Scientific), Sydney IVF Cleavage Medium (Cook Medical), or Quinn's Advantage Protein Plus Cleavage Medium (Origio) in an EmbryoScope time-lapse system (Unisense FertiliTech, Denmark). On the morning of Day 2, PN formation was observed for a second time. The time course of this study is shown in Fig. 1.

Evaluation of AOA

On Day 2, oocytes showing 2PN were continuously cultured for four days. Oocytes with a single pronucleus (1PN), three pronuclei (3PN), or more than four pronuclei (≥4PN) were recorded. The time of PN formation after AOA was analyzed using the image data of EmbryoScope.

Statistical analyses

Fisher's PLSD test was performed to assess the significance of differences in the rates of PN formation and embryo development. The time of PN appearance was analyzed using one-way ANOVA. A probability of $P <$

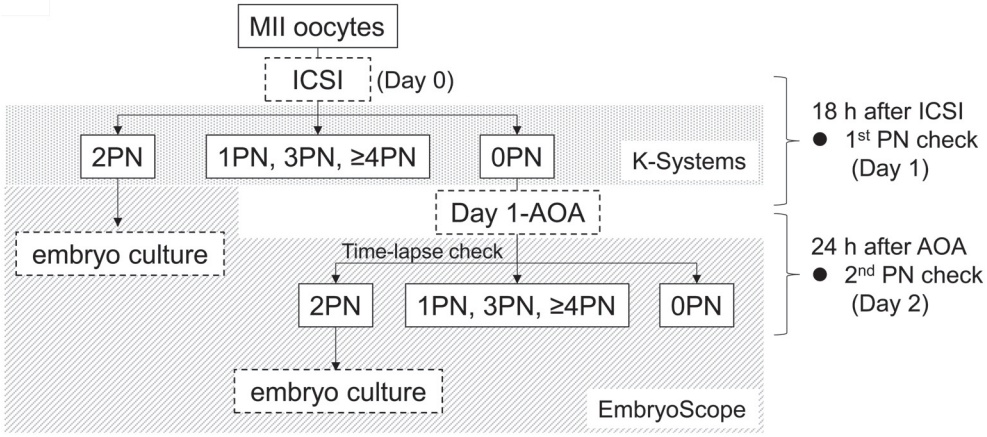

Fig. 1. Time course of this study. All ICSI-treated oocytes were cultured in K-Systems incubator (Day 0). At 18 h after ICSI (Day 1), PN formation was checked (1st PN check; No-AOA in Table 1) and 2PN zygotes were cultured in an EmbryoScope time-lapse system until Day 5 (No-AOA in Table 3). AOA with A23187 was performed on oocytes without PN (0PN) followed immediately by culture in the EmbryoScope (Day 1-AOA). At 24 h after AOA (Day 2), PN formation was checked for the second time (2nd PN check; Day 1-AOA in Table 1 and Table 2), and 2PN zygotes were continuously cultured in the EmbryoScope until Day 6 (Day 1-AOA in Table 3).

Table 1. Pronuclear formation after AOA of one-day-old unfertilized oocytes following ICSI

	No-AOA (Control)	Day 1- AOA	*P*-value	
No. of oocytes undergoing ICSI	168		–	
No. of oocytes without PN (%)	50 (29.8)	–	–	
No. of oocytes undergoing AOA	–	50	–	
No. of oocytes without PN (%)	–	19 (38.0)	–	
Total no. of oocytes with PN (%)	110 (65.5)	28 (56.0)	P=0.51	ns
1PN (%)	3 (1.3)	9 (18.0)	$P \leq 0.05$	
2PN (%)	101 (60.1)	8 (16.0)	$P \leq 0.05$	
3PN (%)	4 (2.3)	3 (6.0)	P=0.16	ns
≥4PN (%)	2 (1.2)	8 (16.0)	$P \leq 0.05$	
No. of oocytes degenerated or fragmented (%)	8 (4.7)	3 (6.0)	P=0.70	ns

0.05 was considered to indicate statistical significance.

Results

In the present study, a total of 168 oocytes at the MII stage were retrieved in 41 cycles and inseminated by piezo-ICSI. The time course and results following ICSI are described in Fig. 1 and Table 1, respectively. The majority of oocytes (60.1%: 101/168) formed 2PN within 18 h after ICSI (No-AOA). However, 29.8% (50/168) of oocytes failed to fertilize and did not show PN formation (Table 1).

We treated the 50 oocytes that failed to fertilize after ICSI with A23187 at 18 h after ICSI (Day 1-AOA). On Day 1-AOA, 56.0% (28/50) of these oocytes were activated and formed PN (Table 1); 16.0% (8/50) were activated and formed 2PN. However, the rate of 2PN formation in the group of Day 1-AOA oocytes was significantly lower than in No-AOA oocytes (60.1%). The rates of 1PN and ≥4PN formation were significantly higher in Day 1-AOA (1PN: 18.0%; ≥4PN: 16.0%) than in No-AOA oocytes (1PN: 1.3%; ≥4PN: 1.2%).

Table 2 shows the time of PN appearance in Day 1-AOA oocytes. Oocytes were treated with A23187 and immediately transferred to an EmbryoScope (=0 h). 1PN, 2PN, 3PN, and ≥4PN appeared at 7.0 ± 4.4, 8.2 ± 2.4, 6.0 ± 0.8, and 13.2 ± 9.1 h after AOA, respectively, and there were no significant differences among the number of PN.

Table 3 shows the development of zygotes with 2PN formation in the No-AOA and Day 1-AOA groups. The

Table 2. Time of PN appearance after Day 1-AOA

	1PN	2PN	3PN	≥4PN
No. of oocytes	9	8	3	8
Time (h)*	7.0 ± 4.4	8.2 ± 2.4	6.0 ± 0.8	13.2 ± 9.1

*Oocytes treated with A23187 were imediately cultured in an EmbryoScope time-lapse system (=0 h). Times are mean ±SD.

Table 3. Development of zygotes with 2PN formation after Day 1-AOA

	No-AOA* (Control)	Day 1-AOA	*P*-value
No. of 2PN zygotes cultured	16	8	–
Cleaved (%)	16 (100.0)	6 (75.0)	$P \leq 0.05$
Morulae (%)	14 (87.5)	2 (25.0)	$P \leq 0.05$
Blastocysts (%)	9 (56.3)	0 (0.0)	$P \leq 0.05$

*Zygotes in the same cycles in which 2PN was formed after ICSI without artificial activation were used as a control.

majority of zygotes (75.0%: 6/8) in the Day 1-AOA group cleaved; however, the rate was lower than in the No-AOA group (100.0%). Among the cleaved zygotes, two embryos (25.0%) developed to the morula stage, but they did not develop to blastocysts.

Discussion

At our clinic, on average, more than 80% of oocytes that have been subjected to piezo-ICSI form 2PN [16, 17]. When zygotes with 2PN formation are continuously cultured to blastocysts in an EmbryoScope, the unfertilized oocytes are also cultured simultaneously to confirm that no PN forms after 18 h. The present study analyzed only the ICSI cycles that generated unfertilized oocytes, and their rate of 2PN formation was lower than is typically observed, with only 16.0% of the unfertilized oocytes that received AOA with A23187 on Day 1 after ICSI forming 2PN. Recently, we demonstrated that 88.9% of sperm heads show PCC in unfertilized oocytes at 24 h after ICSI, as determined by immunostaining for dimethylated histone H3K9 in the parental chromatin [4]. Furthermore, in the present study, chromatin staining of the unfertilized oocytes after Day 1-AOA showed a high frequency (92.3%) of sperm PCC formation (data not shown).

The present study demonstrated that AOA with A23187 on Day 1 by the conventional method of AOA immediately following ICSI (10 μM of A23187 for 5 min) induced 2PN formation in some unfertilized oocytes and development to the morula stage in a few embryos. However, it did not effectively activate unfertilized oocytes, as none of them developed to blastocysts. Therefore, it is suggested that for the rescue of one-day-old oocytes with fertilization failure after ICSI, other methods of AOA should be explored to determine the optimal support for embryo development.

Oocyte activation can be induced by either an increase in Ca^{2+} concentration in the oocytes or a subsequent decline in MPF [18]. The oocyte activation treatments used in clinical research include the Ca^{2+} ionophore A23187 [19], strontium treatment [20], and electrostimulation [12], all of which have successfully resulted in pregnancies and live births. A more effective decrease in MPF induced by a combination treatment of A23187 with the protein synthesis inhibitor puromycin, was reported to increase the rates of fertilization and pregnancy [11]. These results suggest that as an alternative strategy to the conventional activation method, usage of other substances should be examined for Day 1-AOA to support embryo development.

Oocytes arrested at the MII stage are fertilized soon after ovulation. If fertilization does not occur within the fertilization window, unfertilized oocytes in the oviduct (*in vivo*) or culture (*in vitro*) undergo a time-dependent deterioration in quality, a process called “oocyte aging”. “Aged oocytes” are those in which aging has already occurred. Functional changes associated with oocyte aging include decreased fertilization ability [21], chromosomal anomalies [22], and abnormal or retarded development of the embryo/fetus [23]. When performing Day 1-AOA, the oocytes are cultured for at least 18 h after ICSI, which suggests that the oocytes age *in vitro*, since they contain sperm in the ooplasm. A gradual decrease in MPF activity with aging was reported in *in vitro*-matured, aged COCs [24], and it was suggested that aged oocytes are more sensitive to active stimuli than newly matured oocytes [25]. In the present study, 2PN appeared at 8.2 ± 2.4 h after Day1-AOA, which is not so different to the time of 2PN appearance in normally fertilized oocytes after conventional ICSI (8.9 ± 2.2 h, preliminary analysis, data not shown), corresponding to No-AOA in this study. With regard to the PN appearance time, one-day-old fertilization-failed oocytes were not so sensitive to activation stimuli, and the conventional AOA method was seemingly effective, but not efficient, at inducing 2PN formation. However, zygotes of Day 1-AOA with 2PN formation regrettably did not develop to the blastocyst stage. Lu et al. reported a clinical pregnancy and a healthy baby after ICSI followed by one-day-old rescue AOA [14]. Importantly, their AOA method (5 μM of A23187 for 10 min) was different from ours (10 μM of A23187 for 5 min), suggesting that our conventional AOA method is not suitable for

one-day-old fertilization-failed oocytes, and that a different concentration of A23187 and processing time might determine the success of Day 1-AOA.

After Day 1-AOA, the rate of 2PN formation was significantly lower, whereas the rate of 1PN formation was significantly higher, than in the No-AOA group. This suggests that in oocytes with 1PN formation after Day 1-AOA, the 2PN might have already fused, and the 2PN oocytes look like 1PN oocytes at the second check for PN formation, since aged oocytes may form PN earlier than fresh oocytes. However, we dismissed this possibility by retrospectively tracking the PN formation over time with the EmbryoScope. Furthermore, the time of 1PN appearance in the Day 1-AOA group (7.0 ± 4.4 h) was not significantly different from that of 2PN (8.2 ± 2.4 h). In contrast, haploids of maternal origin significantly increased during the IVF of mouse oocytes that had aged after ovulation [26, 27], and human oocytes that had failed to fertilize after ICSI did not form the male PN, even after the oocytes had been activated [2]. Therefore, these results suggest that Day 1-AOA can activate the female PN but fail to induce the sperm nucleus to form the male PN, leading to the formation of 1PN. Conversely, the rate of ≥4PN formation in the Day 1-AOA group was significantly higher than in the No-AOA group. It has been established that chromosomes in human oocytes aged for two days are no longer aligned at the spindle equator, but are instead scattered within the degenerating spindle [28].

Typically, fertilization of one-day-old oocytes results in extremely low pregnancy rates. When rescue activation was carried out for such oocytes in 52 cases, the fertilization rate was 78% and two cases of pregnancy were obtained, but both resulted in miscarriage [29]. Therefore, the success of Lu et al. [14] might be a rare case. When oocyte activation does not occur following ICSI, the injected sperm-chromatin may undergo PCC over time. PCC is the abnormal condensation of chromatin and can induce damage in the chromosome or DNA. The incidence of PCC elevates at 4 h after ICSI, in which oocyte activation does not occur [15], and therefore oocyte activation methods can be used as a rescue procedure when fertilization failure is detected within 4 h of ICSI [30].

In conclusion, our results demonstrated that Day 1-AOA can induce activation in some oocytes with fertilization failure lacking PN at 18 h after ICSI, but the developmental competence of the 2PN zygotes to the blastocyst stage after AOA is low. Therefore, it is suggested that AOA with a lower concentration of A23187, with other substances, or at an earlier time may be better suited for the activation of aged oocytes and for embryo development, and that the optimal value of these factors should be explored for the rescue of oocytes with fertilization failure after ICSI.

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