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Effect of Electrical Stimulation on Oocyte Activation after Intracytoplasmic Sperm Injection

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Abstract: *More than half of unfertilized oocytes after ICSI failed to activate in human subjects. We investigated whether the fertilization rate after ICSI can be increased by electrical stimulation. At first, the effects of electrical stimulation on the parthenogenesis of hamster oocytes were investigated. Delivering a single 375 V/cm DC pulse for 100 μsec was effective in inducing parthenogenesis. The pattern of Ca^{2+} transient increase in hamster oocytes induced by electrical stimulation was recorded with Indo-1/AM. It seemed that a single sharp increase in Ca^{2+} initiated parthenogenetic activation. Secondly, a 375 V/cm pulse was applied to human oocytes after ICSI. In the case of immotile sperm, the fertilization rate and cleavage rate were increased significantly by electrical stimulation. On the other hand, in the case of motile sperm, no increase in the fertilization rate was observed. This study demonstrated that electrical stimulation combined with ICSI may be expected to contribute to increasing the fertilization rate in the case of immotile sperm.*

Key words: *Electrical stimulation, Intracytoplasmic sperm injection, Human, Oocyte activation, Intracellular Ca^{2+}.*

When a cell is put into the electrical field, there occurs a disturbance of the molecular configuration phospholipid that constitutes the cell membrane [1, 2]. This phenomenon is called electroporation [3]. When electroporation occurs in unfertilized oocytes, extracellular Ca^{2+} flows into the cell through micro pores formed in the oolemma. With this as a trigger, the oocyte is activated to cause parthenogenesis [4–8]. With the intracytoplasmic sperm injection (ICSI) in human subjects, the fertilization rate after ICSI was 30–70% [9–12], there were some cases in which oocytes were not activated when oocytes were exactly injected with spermatozoa [13]. Especially in cases in which spermatozoa showed a total absence of motility, the fertilization rate was low. These unfertilized oocytes were not activated by the ICSI procedure. If we can induce the activation of such oocytes just after ICSI, we may be able to get good results in fertilization. So the effects of electrical stimulation on the parthenogenesis of hamster oocytes were investigated to apply the condition of electrical stimulation to human oocytes and the activation of human oocytes after ICSI were studied.

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Materials and Methods

1. Induction of oocyte activation by electrical stimulation in hamster

Hamster unfertilized oocytes were put into a high electrical field for electroporation and it was observed how the oocytes are activated.

Media: The medium used for the culture of hamster oocytes after electroporation was a modified Krebs-Ringer medium developed by Whitten [14] and Hoppe [15]. This medium was called WH medium [16]. Its composition was 109.6 mM NaCl, 4.8 mM KCl, 1.7 mM calcium lactate · $4H_2O$, 1.2 mM $Mg_2SO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 22.6 mM $NaHCO_3$, 5.6 mM glucose, 0.22 mM sodium pyruvate, 70 μg/ml K-penicillin G, and 3 mg/ml bovine serum albumin. Its pH was 7.4 when equilibrated with 5% CO_2, 5% O_2 and 90% N_2. The medium used for hamster oocyte collection and ICSI was a modified WH medium (mWH) [16]. It contained 10 mM HEPES and 12.6 mM $NaHCO_3$ instead of 22.6 mM $NaHCO_3$. The concentrations of other components were the same as those in the WH medium. Its pH was 7.4 when equilibrated with air.

Preparation of oocytes: Eight to twelve week old

female golden hamsters (about 90 g) were induced to superovulate by consecutive intraperitoneal injections 48 h apart of 30 IU pregnant mare serum gonadotropin (PMS; Teikokuzoki Co., Tokyo, Japan) and 30 IU human chorionic gonadotropin (hCG; Mochida Pharmaceutical Co., Tokyo, Japan). Unfertilized oocytes were collected from the fallopian tube 16 h after hCG administration. Oocyte-cumulus complexes were treated with a mWH containing 0.1% hyaluronidase for 5 min to remove cumulus cells by dispersion. Cumulus cell-free unfertilized oocytes were kept in the WH at 37°C for 10 min before use.

Electrical stimulation: An electrical pulse (DC pulse) was administrated under various conditions to hamster unfertilized oocytes. For electrical stimulation, phosphate buffered saline (PBS; p0261, Sigma, USA) was used as a pulsing medium. Oocytes were placed between parallel electrodes 2 mm apart in an electric chamber (FTC-03, Shimazu, Tokyo, Japan). Oocytes were placed in PBS and subjected to a DC pulse from an electric cell fuser (SSH-2, Shimazu, Tokyo, Japan). The type of square pulse used for the experiment was a single pulse, the field strengths were 150, 250, 375, 500 and 750 V/cm and the duration was 100 μsec. Three hours after electrical stimulation, oocytes were observed under a dissecting microscope. When the female pronucleous was formed and the presence of a second polar body was confirmed, the oocytes were judged to be activated. And in some stimulated oocytes, changes in the intracellular Ca^{2+} concentration were measured.

Measurement of changes in cytosolic-free Ca^{2+} concentration in hamster oocytes after electrical stimulation: Intracellular Ca^{2+} changes in hamster oocytes after the administration of the pulse were monitored fluorometrically with an Indo-1/AM (Ca^{2+}-sensitive indicator; Wako-Junyaku Co., Tokyo, Japan). Hamster unfertilized oocytes were loaded with 10 μM Indo-1/AM (in PBS) for 60 min at 37°C. Fluorescence measurements were performed in an Olympus OSP10-CA spectrofluorometer (Olympus, Tokyo, Japan) at an excitation wavelength of 380 nm and emission wavelength of 405 nm/480 nm with a pinhole size of 75 μm. After rinsing three times with PBS, Indo-I/AM loaded oocytes were put into an electric chamber containing PBS. Monitoring of intracellular Ca^{2+} changes was started and a single squared pulse with an electrical field was delivered to the oocytes from the SSH-2.

2. Effect of electrical stimulation on oocyte activation after ICSI in human

One hundred and sixteen treatment cycles by ICSI were carried out between January, 1994 and December, 1995 at the hospital attached to Fukushima Medical College. ICSI was applied in a case in which fertilization was not recognized with conventional *in-vitro*-fertilization and a case in which semen parameters were extremely poor. Seventy-six out of 116 patients had motile spermatozoa injected into the oocyte. And 29 had immotile spermatozoa injected because motile spermatozoa could not be found in the semen samples. The protocols were approved by the ethical committee of this hospital.

Gamete preparation: The patient were given buserelin acetate (900 μg/day: Suprecur; Hoechst Japan Co., Tokyo, Japan) after the day 21 in the previous luteal phase. Then they were given daily injection of 300 IU FSH (Phertinom P; Serono Japan Co., Tokyo, Japan) on days 3 and 4 in the treated cycle and 150 IU FSH daily on days 5 and 6 and 150 IU HMG (Pergonal; Teikokuzoki Co., Tokyo, Japan) daily from day 7 until the maturation of the follicles. When the leading follicle reached 18 mm in diameter, 10,000 IU hCG (HCG; Mochida Pharmaceutical Co., Tokyo, Japan) was administered. Oocytes were picked up by transvaginal ultrasound-guided puncture 35 h after. Oocytes were collected from follicular aspirates and placed into Human Tubal Fluid (HTF; Irvine Scientific Co., Irvine, CA). ICSI was carried out 3 to 11 h after oocyte retrieval. The cumulus cells were removed from oocytes just before ICSI by brief treatment with 0.025% hyaluronidase (Sigma type VIII) in hepes-buffered HTF (mHTF; Irvine Scientific Co., Irvine, CA) combined with pipetting. Only metaphase II oocytes were used for ICSI. Prophase I and Metaphase I oocytes were used for intracellular Ca^{2+} measurement at the time of electrical stimulation with permission of the patients. These immature oocytes were incubated for 24 to 48 h in HTF and only oocytes which developed Metaphase II were used for the experiment on Ca^{2+} measurement.

Semen samples were collected by masturbation and liquefied at room temperature. Spermatozoa was isolated either by rinsing once in mHTF or using the swim up method after isolating by centrifugation at 350 × g for 5 min.

Measurement of changes in cytosolic-free Ca^{2+} concentration in oocyte after electrical stimulation: Intracellular Ca^{2+} changes in human oocytes following the application of an DC pulse were monitored fluorometrically with Indo-I/AM. This method was the same as for hamster oocytes. The single DC pulse was at a field strength of 375 V/cm and duration of 100 μsec, because the number of oocytes examined was three. And the fluorescence images at the time of electrical stimulation were also observed in two oocytes.

They were loaded with the fluorescent Ca^{2+} indicator Fluo-3/AM (Wako-Junyaku Co., Tokyo, Japan) by incubating for 1 h at 37°C. The final concentration of Fluo-3/AM in HTF was 20 μM. After incubation, loaded oocytes were rinsed in HTF three times and introduced into an electric chamber containing PBS. Then the chamber was placed on the stage of a Nikon inverted microscope attached to a Bio-Rad MRC-600 confocal laser scanning microscope. The oocytes were scanned and then electrically stimulated by means of a 375 V/cm single square pulse for 100 μsec. The fluorescence images were observed.

Method of ICSI: Injection of spermatozoa was carried out according to the method of Perreault and Zirkin [17] and Yanagida *et al.* [16, 18]. The outer diameter of the injection needle was 5–6 μm and the inner diameter of the oocyte-holding pipette was 15 μm. A micromanipulator (MMO 22, Narishige, Tokyo, Japan) and a inverted microscope with Nomarsky modulation (Olympus IMT II, Tokyo, Japan) were used for ICSI. Briefly, spermatozoa were transferred into mHTF containing 10% polyvinylpyrrolidone (PVP-360, Sigma Co., Tokyo, Japan), and a single spermatozoon was then sucked into the injection pipette. An oocyte, held by a holding pipette in mHTF, was punctured with the injection pipette, a small amount of cytoplasm was sucked into the pipette, and then the spermatozoon was expelled into the oocyte. The pipette was withdrawn from the oocyte. When a motile spermatozoon was injected, it was immobilized just before injection. The method of immobilization for motile spermatozoa was achieved by drawing a spermatozoon in and out of the injection needle repeatedly [19].

Electrical stimulation of injected oocytes was carried out 30 min after ICSI. After electrical stimulation, oocytes were incubated in HTF for 42 h following by embryo transfer (ET).

Confirmation of fertilization: Eighteen hours after incubation, injected oocytes were observed under a dissecting microscope. When the female pronucleous was formed and the presence of second polar body was confirmed, the oocytes were judged to be activated. And when the female and male pronuclei with the second polar body were confirmed, the oocytes were judged to be fertilized. Forty-four hours after ICSI, a maximum of three cleaved embryos were transferred into the uterine cavity.

Statistical analysis: Statistical significance was assessed by an χ^2 test. At $P<0.05$, the difference was considered statistically significant.

Results

1. Induction of oocyte activation by electrical stimulation in hamster

The status of oocyte activation when single DC pulses at different field strengths were delivered for 100 μsec to hamster unfertilized oocytes is summarized in Table 1. When a single DC pulse with a field strength of 150–375 V/cm was delivered as the electrical stimulation, the survival rate (survived oocytes/stimulated oocytes) was more than 95%. At 500 and 750 V/cm, the survival rate was low and poor. The rate of activation (activated oocytes/stimulated oocytes) was higher in the 375 to 500 V/cm of field strength range.

Measurement of changes in cytosolic-free Ca^{2+} concentration in hamster oocytes after electrical stimulation: Changes in the intracellular Ca^{2+} concentration in hamster oocytes after stimulation with a single DC pulse are show in Fig. 1. There was an increase in the cytosolic-free Ca^{2+} concentration measured fluorometrically with Ca^{2+}-sensitive indicator Indo-I/AM in the oocytes at each

Table 1. The percentage of oocyte activation after electrical stimulation in hamster

Field strength (V/cm)	No. of stimulated oocytes (no. of exp.)	No. of survival oocytes (%)	No. of activated oocytes (%)
150	33 (4)	33 (100)[a]	3 (9)[c]
250	39 (6)	37 (95)[a]	25 (64)[d]
375	80 (8)	80 (100)[a]	66 (83)[e]
500	51 (6)	47 (92)[a]	41 (80)[d,e]
750	27 (4)	7 (26)[b]	6 (30)[c]

[a, b] Numbers within columns with different superscripts are different ($p<0.001$). [c, d, e] Numbers within columns with different superscripts are different [c-d] ($p<0.001$), [c-e] ($p<0.001$), [d-e] ($p<0.05$).

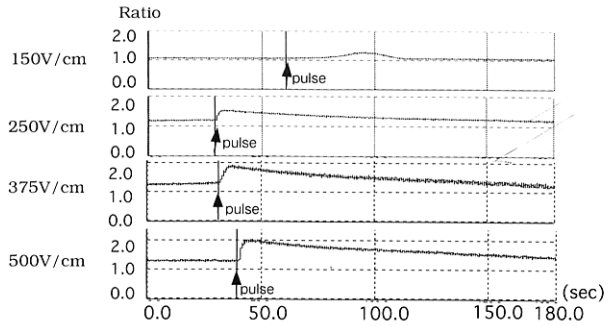

Fig. 1. Intracellular Ca^{2+} changes in hamster oocytes after administration of the DC pulse. Large arrows indicate times of stimulation. The intensity of fluoresence is shown as the ratio.

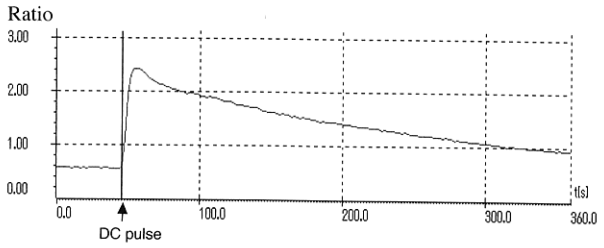

Fig. 2. Intracellular Ca^{2+} changes in human oocyte after administration of the DC pulse. Large arrow indicates time of stimulation. The intensity of fluoresence is shown as the ratio.

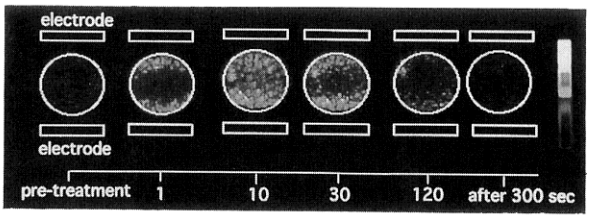

Fig. 3. Images of Ca^{2+} transient in a human oocyte following electrical stimulation. The stimulation was at a field strength of 375 V/cm and a duration of 100 μsec. The image in the second frame shows the light spots after 1 sec of electrical stimulation.

field strength. The fluorometrical intensity of intracellular Ca^{2+} is indicated by the ratio. As the field strength became strong, the peak ratio in each record increased. But the peak ratio that was measured at a field strength of 375 V/cm was the same as at a field strength of 500 V/cm. It took about 2–3 sec from the start of the rise to the peak. It took more than 3 min to come back to base line of the ratio curve after the peak.

2. Effect of electrical stimulation on oocyte activation after ICSI in human

From human subjects, we could not obtain sufficient oocytes. In only three oocytes the intracellular Ca^{2+} change was measured after the application of the DC pulse. The change in human oocytes was found to be the same as the change in hamster oocytes (Fig. 2). There was an immediate increase in intracellular Ca^{2+}, taking about 5 sec from the start of the rise to the peak. It took more than 6 min to come back to base line of the curve after the peak.

The fluorescence images of two oocytes were measured after electrical stimulation. The images are shown as pseudocolored Ca^{2+} images in Fig. 3. The accumulation of light spots during the stimulation was considered to be due to a change in the intracellular Ca^{2+} concentration. The first light spot was recognized near both electrodes, and the light spot spread throughout the whole oocyte within 10 sec after stimulation. Then the light spot faded by degrees, and had completely disappeared after 300 sec.

Results of ICSI: The results of ICSI in the case of immotile sperm are shown in Table 2. In group A only ICSI was performed and in group B ICSI was performed followed by electrical stimulation with a single DC pulse. The survival rates of both groups were same, but the fertilization rate in group B (60.0%) was significantly higher than that in group A (41.8%). The cleavage rates of group B were the same as the fertilization rate. But no case of pregnancy was obtained in either group.

Spermatozoon injected into cytoplasm was usually motile in ordinary ICSI. The results for the cases in which motile spermatozoa were injected are shown in Table 3. In group C only ICSI was performed, and in group D electrical stimulation was added. There were no significantly differences between these groups in survival, fertilization, cleavage and pregnancy rates.

Discussion

Microinsemination is an assisted reproductive technology that is applied in cases in which fertilization does not occur with conventional IVF or in which spermatological findings are extremely poor. Of the microinsemination techniques, ICSI is a method whereby fertilization can theoretically be achieved if one sperm is available. It is not inferior to conventional IVF in the fertilization rate.

In natural fertilization, an oocyte that is in the middle of the second meiosis and therefore biologically in a

Table 2. The results of ICSI in the case of immotile sperm

Group	Electro-poration	No. of oocytes (no. of case)	No. of survival oocytes (%)	No. of fertilized oocytes (%)[b]	No. of cleaved oocytes (%)[b]	No. of pregnancy
A	–	81 (14)	67 (82.7)	26 (38.8)[c]	20 (29.9)[c]	0
B	+[a]	46 (10)	40 (87.0)	24 (60.0)	20 (50.0)	0

[a]: Oocytes received a single DC pulse with a field strength of 375 V/cm and a duration of 100 μsec 30 min after ICSI. [b]: % is the % of survival oocytes. [c]: Values significantly different from those for group A ($p<0.05$).

Table 3. The results of ICSI in motile sperm case

Group	Electro-poration	No. of oocytes (no. of case)	No. of survival oocytes (%)	No. of fertilized oocytes (%)[b]	No. of cleaved oocytes (%)[b]	No. of pregnancy
C	–	467 (76)	396 (84.8)	276 (69.7)	247 (62.4)	10 (13.7)
D	+[a]	64 (11)	58 (90.6)	43 (74.1)[c]	37 (63.8)[c]	1 (9.1)

[a]: Oocytes received a single DC pulse with a field strength of 375 V/cm and a duration of 100 μsec 30 min after ICSI. [b]: % is the % of survival oocytes. [c]: Values not significantly different from those for group C.

static state is activated in the moment when the sperm gets fused with the cell membrane of the oocyte and starts cell division again. The intracellular Ca^{2+} concentration in the oocyte increases rapidly [20–23], second polar bodies are released and cortical granules disappear. The mechanism by which the oocyte is activated has yet to be elucidated [23–25]. It is not certain whether the sperm carries an oocyte-activating factor (sperm factor) into the oocyte or a factor present in the oocyte from the beginning brings its function into play. Because sperm oocyte fusion was bypassed in ICSI, it was thought that the sperm factor was important for oocyte activation. Recently Parrington *et al.* identified a soluble sperm protein (sperm factor) that exhibits Ca^{2+} oscillation-inducing activity in oocytes. And they reported that this protein exists as an oligomer with specific intracellular localization in the equatorial segment of the sperm head [26].

In the cases in which spermatozoa showed a total absence of motility, the fertilization rate was lower than the rate in cases in which motile spermatozoa were obtained. One of the reasons for poor fertilization was thought to be that dead spermatozoa had been picked up and injected into oocytes. The presence of dead spermatozoa means that the plasma membrane of the spermatozoa was broken a long previously. So dead spermatozoa may be lost sperm factor. Indeed, unfertilized oocytes after ICSI were observed to lack oocyte activation in spite of exact injection of immotile spermatozoa with acetolacmoid stain. If oocyte activation were induced artificially after ICSI in immotile spermatozoa cases, we thought that the fertilization rate might be increased. Two methods for activating oocytes are available: one is to physically stimulate the cytoplasm of oocytes and the other is to chemically treat the oocytes. As for physical stimulation, there is pricking an oocyte with a needle [27]. It has long been known that such a manipulation alone causes parthenogenesis. In the case of ICSI, insertion of a pipette itself for injection of the sperm serves as a stimulation, but presumably the stimulation is not brought into full play. Ca-ionophore [28] and ethanol [29] are known as the chemical substances which fascilitate activation of the oocyte. Ca-ionophore is a substance that acts on the Ca^{2+} channel of the cell membrane, thereby making extracellular Ca^{2+} flow into the cell. An increase in intracellular Ca^{2+} makes for activation of the oocyte [30]. Ethanol is said to act directly on the cell nucleus to release second polar bodies. Ca-ionophore and ethanol are able to induce oocyte activation, but there is concern about a possible chemical toxic effect on the oocyte. So we must wash the treated oocytes in culture medium several times. In electrical stimulation, basic culture medium can be used for treatment [31]. Basic medium is preferable, since electrolytes are suitable for fertilized oocytes. Then it is not necessary for the treated oocytes to be washed in the medium. So we adopted electrical stimulation to activate human oocytes and used PBS as a pulsing medium [31].

When a cell is put into an electric field of fixed intensity, the molecular configuration of the phospholipid double layer that constitutes the cell membrane is disturbed and there are formed very small pores with a diameter of 0.5 to 4 nm and numbering 1 to 1,000 per

membrane. This phenomenon is called electroporation and it is used in the study of cell fusion and parthenogenesis [32]. While the flowing electric current exerts no influence whatever on cells, the degree of disturbance of molecules in the cell membrane is dependent on the surrounding electric field strength, and when a certain level of voltage is exceeded (critical voltage), the cell membrane is irreversibly damaged [1, 2]. The voltage in the membrane is highest at the oocyte's poles (closest to the electrodes). The pores formed first at the poles. Figure 3 shows this characteristic of pore formation.

We applied electroporation to induce activation of human oocytes after ICSI and studied then desirable electrical stimulation conditions for human oocyte by using hamster oocytes. The conditions of stimulation were a field strength of 375–500 V/cm and a duration of 100 μsec, because the survival rate and the activation rate were significantly high. The results of measurements of changes in the Ca^{2+} concentration in oocytes treated by electrical stimulation showed that the ratio curve had immediate increase in intracellular Ca^{2+} just after electrical stimulation in hamster oocytes when the stimulation was effective for oocyte activation. We measured the changes in intracellular Ca^{2+} in hamster oocytes, and similar results have been reported for murine [33], rabbit [34] and porcine oocytes [35]. We predicted that stimulation with a field strength of 375 V/cm was effective for activation of human oocytes, because the change in human oocytes to which this DC pulse was applied was found to be the same as the change in hamster oocytes. And this condition proved effective for inducing the activation of human oocyte after ICSI in the case of immotile sperm. But there was no case of pregnancy in our study. Another report also showed that the pregnancy rate was very low [36]. The reason for this low pregnancy rate is unclear. Other possible causes are, for example, damage to the DNA.

When ICSI was conducted with motile spermatozoa after electrical stimulation, the fertilization rate and cleavage rate were not improved. It was thought that a spermatozoon was provided with oocyte activating factor and the oocyte was activated fully. So it was not necessary to add electrical stimulation. In the case of immotile sperm, it was thought that dead spermatozoa lost their sperm factor. When dead spermatozoa were picked up and injected into oocytes, oocyte activation did not occur and application of a DC pulse effectively assisted oocyte activation.

We concluded that electrical stimulation of injected oocytes was effective for fertilization in the case of immotile sperm.

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