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Electrical activation and in vitro development of human oocytes that fail to fertilize after intracytoplasmic sperm injection

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Objective: To determine whether electrically stimulated Ca^{2+} influx can "rescue" fertilization and early embryogenesis in human oocytes that fail to fertilize after intracytoplasmic sperm injection (ICSI).

Design: Prospective, randomized trial of a laboratory procedure.

Setting: A research laboratory at a university medical center.

Patient(s): Discarded oocytes from ICSI-IVF cycles.

Intervention(s): Oocytes (n = 104) that showed no evidence of fertilization 16–24 hours after ICSI were assigned to three treatment groups: group 1 (one direct current electrical pulse at 1.36–1.50 kV/cm for 40–60 μs), group 2 (three pulses every 15–20 minutes), or group 3 (treated the same as group 2 but with no electrical stimulation).

Main Outcome Measure(s): After stimulation, the oocytes were cultured in vitro for 3–5 days. Oocytes that displayed two pronuclei and a second polar body within 16 hours were considered to have fertilized normally. Fertilization and embryo cleavage rates were compared between groups.

Result(s): Fertilization occurred in 26 (70%) of 37 and 38 (78%) of 49 group 1 and 2 oocytes, respectively, but in only 5 (27%) of 18 group 3 oocytes. Within 3 days, group 2 embryos routinely developed beyond the two-cell to four-cell stage (61% versus 13% in group 1); 11% of these oocytes developed to the morula or early blastocyst stage. Sex chromosome analyses indicated 10 male and 8 female embryos.

Conclusion(s): Oocytes that fail to fertilize by 24 hours after ICSI can resume apparently normal fertilization and early embryonic development in response to electrical stimulation. Moreover, the degree of cytoplasmic activation as determined by the number of pulses applied affects fertilization efficiency and early embryonic development. (Fertil Steril® 1999;72:509–12. ©1999 by American Society for Reproductive Medicine.)

Key Words: ICSI, electrical activation, fertilization

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At the time of fertilization in mammals, the incoming sperm triggers oocyte activation by initiating a series of intracellular free Ca^{2+} transients (1). Two types of mechanisms, namely inositol 1,4,5-trisphosphate–induced calcium release and calcium-induced calcium release, have been suggested to be responsible for this release of intracellular Ca^{2+}. Recent studies indicate that soluble protein factor(s), such as oscillin, are released from the sperm head and induce inositol 1,4,5-trisphosphate–induced calcium release and calcium-induced calcium release to produce a transient increase in intracellular levels of free Ca^{2+} (2). This increase in free Ca^{2+} activates the oocyte by reducing metaphase-promoting factor and causes sperm swelling and male pronucleus formation.

Intracytoplasmic sperm injection (ICSI) has been used widely in the treatment of severe male factor infertility. However, many centers have reported fertilization rates of approximately 70%, even though most of the sperm injected are deposited into the cytoplasm. In these clinics, fertilization is defined by the appearance of two pronuclei within 24 hours of sperm injection. Further, when spermatids are

TABLE 1

Outcome of IVF for oocytes that failed to show evidence of fertilization within 24 hours of ICSI.

Outcome of ICSI	No. of oocytes (%)
Oocytes that were not fertilized by 24 hours	444
Oocytes that subsequently fertilized (two or three pronuclei)	41 (9)
Oocytes that subsequently cleaved in vitro	28 (6)

Note: ICSI = intracytoplasmic sperm injection.

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used, fertilization rates are even lower (3). Many investigators have postulated that the major reason for failed fertilization after ICSI may be suboptimal stimulation of inositol 1,4,5-trisphosphate–induced calcium release and/or calcium-induced calcium release as a result of insufficient release of the soluble proteins from the injected sperm heads (2).

Numerous studies in animals have shown that an electrical field can generate micropores in the cell membrane of gametes and somatic cells to induce sufficient Ca^{2+} influx through the pores to activate cytoplasm through Ca^{2+}-dependent mechanisms (4). Kimura and Yanagimachi (5) and Sasagawa and Yanagimachi (6) reported that mouse oocytes injected with secondary spermatocytes or spermatids are fertilized when stimulated by electroporation and develop into normal offspring when the resultant embryos are transferred to a recipient uterus. In this study, we examine whether human oocytes that show no evidence of fertilization after ICSI can be "rescued" to undergo normal fertilization by cell electroporation–generated Ca^{2+} signals.

MATERIALS AND METHODS

Source of Oocytes

Mature oocytes were subjected to ICSI; 16 hours later, they were evaluated for fertilization as defined by the presence of two pronuclei. Oocytes that showed no evidence of fertilization by 24 hours after ICSI were used with patient consent. The age of the patients ranged from 25–42 years and the primary diagnosis was male factor infertility. Intracytoplasmic sperm injection was performed with ejaculated, testicular or epididymal sperm in microdrops containing 6% Plasmanate (Bayer Corp., West Haven, CT) in HEPES-buffered human tubal fluid medium (Irvine Scientific, Santa Ana, CA).

In a preliminary study, we evaluated approximately 2,180 oocytes that were subjected to ICSI; 444 (20%) showed no evidence of fertilization within 24 hours after ICSI (Table 1). Fewer than 10% of these oocytes subsequently displayed pronuclei and even fewer showed evidence of further embryonic development. We were unable to document a single pregnancy from the transfer of an embryo that derived from such an oocyte. This population of oocytes therefore was selected for the present study, which was approved by the Institutional Board of Research Associates at the New York University Medical Center.

Experimental Design

Oocytes that failed to fertilize after ICSI were randomly assigned to one of three treatment groups irrespective of the indications for ICSI: group 1 was stimulated with one electrical pulse, group 2 was stimulated with three electrical pulses, and group 3 (controls) was treated the same as group 2 but with no electrical stimulation.

The oocytes were washed and electrical stimulation was performed in a buffer containing 0.3 M of mannitol, 0.1 mM of $CaCl_2$, 0.1 mM of $MgCl_2$, and 0.3 mg/mL of bovine serum albumin. One to five oocytes were loaded into a P-450 chamber (BTX Inc., San Diego, CA) consisting of two 100-mm-long platinum electrodes spaced 1 mm apart. Initially, nonfertilized oocytes were oriented in the chamber with an alternating current electrical pulse of 8 V for 6 seconds; this was followed by a direct current pulse (1.36–1.50 kV/cm for 40–60 μs) using the Electro Cell Manipulator 2001 (BTX Inc.). Fewer than 3% of these oocytes lysed; thus, these electrical parameters were used in the present study. Between and after each electrical pulse, the oocytes were washed four times with 6% (vol/vol) Plasmanate in human tubal fluid medium, which also was used for subsequent culture; they then were examined at 8, 12, and/or 16 hours. Oocytes that showed two pronuclei and a second polar body within 16 hours were considered to be fertilized normally. These oocytes then were cultured for 2–5 days to observe their potential for embryonic development.

Embryos that arrested at various stages of early embryonic development were fixed and 2–3 blastomeres were removed for sexing as assessed by fluorescent in situ hybridization (FISH) using α-satellite DNA probes for chromosomes X and Y (Vysis, Downers Grove, IL). The procedures for blastomere fixation, target DNA hybridization, and interpretation of signals have been described by Munné et al. (7) and by Dailey et al. (8). All signals were evaluated manually with an inverted microscope equipped with epifluorescence with multiple filters (Olympus, Melville, NY).

RESULTS

Seventy percent of the group 1 oocytes (one pulse) fertilized normally (Table 2); of these, 10 developed beyond the four-cell stage (Table 3). Seventy-eight percent of the group 2 oocytes (three pulses) fertilized normally (Table 2); 33 of these zygotes developed beyond the four-cell stage and 4 reached the blastocyst stage (Table 3). In all cases, the developing embryos had 2–4 cells at 24 hours and >4 cells at 48 hours after stimulation. The remaining oocytes in groups 1 and 2 either did not fertilize or had one or more than two pronuclei (Table 1). Finally, although 5 of 18 control

TABLE 2

Influence of electrical stimulation on fertilization outcome of oocytes that failed intracytoplasmic sperm injection.

Study group	No. (%) of oocytes with indicated fertilization outcome				
	None	2PN	1PN	3PN	4PN
Group 1 (n = 37)	5	26* (70)	2	2	2
Group 2 (n = 49)	4	38† (78)	2	4	1
Group 3 (n = 18)	13	5* (27)	0	0	0

Note: PN = pronuclei.
* P=.007 (group 1 versus group 3); determined by the χ^2 test.
† P=.005 (group 2 versus group 3); determined by the χ^2 test.

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oocytes in group 3 displayed two pronuclei after treatment, none of them cleaved after culture for 2 days.

Twenty-five multicellular embryos were processed for FISH analysis of sex chromosomes. Seven embryos displayed various sex chromosome anomalies; the remaining 18 were normal, with a sex ratio of 8 females to 10 males.

DISCUSSION

This study demonstrates that electrical stimulation can "rescue" oocytes that fail to fertilize after ICSI and stimulate them to complete the second meiotic division, form pronuclei, and undergo early embryonic development. These findings support the suggestion that the failure of fertilization after ICSI is due to suboptimal oocyte activation.

None of the oocytes used in this study showed evidence of any postfertilization event at 16–24 hours after ICSI. In a related study of 444 such oocytes, only 9% eventually displayed two pronuclei and only 6% cleaved. Moreover, 5 of 18 control oocytes fertilized after experimental treatment but not electroporation; although this "spontaneous" fertilization rate is higher than those reported in other studies, none of these zygotes cleaved. This poor outcome contrasts dramatically with that observed for the group 2 oocytes. When subjected to three pulses of electrical stimulation, 33 of these 49 oocytes progressed to at least the four-cell embryonic stage, and several continued to the blastocyst stage. Together, these findings suggest that the "rescue" of these oocytes was due to the electrical pulse(s) rather than to in vitro aging and/or handling.

TABLE 3

In vitro development of normally fertilized oocytes (two pronuclei) after electrical stimulation.

Study group	No. (%) of zygotes in indicated final stage of embryonic development in vitro*			
	<4 cells	4–7 cells	8 cells	8 cells to blastocyst
Group 1 (n = 26)	16 (61)†	6 (23)	2 (8)	2 (8)
Group 2 (n = 38)	5 (13)†	19 (50)	10 (26)	4 (11)
Group 3 (n = 5)	0	0	0	0

* Many embryos divided after stimulation and then experienced arrest. The most advanced stage of embryonic development achieved after 4 days of electrical stimulation is indicated.
† P=.0002 (group 1 versus group 2); determined by the χ^2 test.

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Although the fertilization rate was similar regardless of the number of electrical pulses applied, subsequent embryo development was dramatically improved in those oocytes that received three electrical pulses (Tables 2 and 3). This observation agrees with findings that rabbit embryos show improved development and implantation on repetitive stimulation (4). Together, these observations suggest that the degree of cytoplasmic activation before and during fertilization not only determines pronuclei formation but also establishes the potential for early embryonic development. Thus, one might speculate that multiple bursts of electrical stimulation may improve both fertilization efficiency and embryo cleavage when applied to oocytes injected with sperm of suboptimal quality. Many group 2 embryos in the present study had ≥4 cells within 48 hours of stimulation. Thus, despite a 24-hour "hiatus" in postfertilization development immediately after ICSI, these embryos cleaved at an appropriate rate after pronuclei formation.

Parthenogenetic activation and cleavage to the four-cell to eight-cell stage has been reported after the exposure of metaphase II human oocytes to an ionophore that causes Ca^{2+} influx (9). However, it seems unlikely that the embryos observed in this study resulted from this phenomenon. Rather, they appear to have derived from normal fertilization with haploid male and female gametes. This conclusion is based primarily on the sex ratio of 10 males to 8 females observed in the cleaving embryos subjected to FISH analysis.

Like any other new assisted reproductive procedure, the impact of electrical activation on nuclear configuration must be evaluated before this procedure can be considered for clinical purposes. Ideally, karyotyping or FISH analysis should be conducted to assess the incidences of aneuploidy and mosaicism in the resultant embryos. In this study, we noted sex chromosome anomalies in 7 of 25 embryos; however, this sample size is too small to allow for a meaningful statistical analysis, and prestimulation analyses were not conducted. Clearly, more extensive quantitative analyses of multiple chromosomes before and after electrical stimulation is necessary to establish the clinical usefulness of this procedure. Such studies are under way.

In conclusion, this study demonstrates that electrical activation can be used to facilitate fertilization and early embryonic development after ICSI. Clinically, this procedure

may be used to ensure fertilization in patients undergoing ICSI whose sperm or spermatids are suspected of being less competent to activate oocytes. Perhaps this clinical benefit could be maximized by performing electrical stimulation immediately after ICSI rather than 24 hours later, as was done in this study with discarded oocytes. Finally, this procedure also has potential as a research tool for investigating the intracellular events that underlie fertilization and early embryonic development.

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