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Three-Day-Old Human Unfertilized Oocytes after *In Vitro* Fertilization/Intracytoplasmic Sperm Injection Can Be Activated by Calcium Ionophore A23187 or Strontium Chloride and Develop to Blastocysts

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Abstract

Our objective was to observe the effectiveness of the calcium ionophore A23187 or strontium chloride on the activation and subsequent embryonic development of 3-day-old human unfertilized oocytes after *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). A total of 279 3-day-old unfertilized oocytes after IVF or ICSI were randomized to be activated by the calcium ionophore A23187 ($n = 138$) or strontium chloride ($n = 141$). The activated oocytes were cultured *in vitro* for 3–5 days. Activation rate, pronucleus formation, cleavage rate, and developmental potential of parthenotes during culture were evaluated. A total of 170 unfertilized oocytes were activated; 65 developed to cleavage stage, 19 developed to greater than the eight-cell stage, and five blastocysts were obtained. The activation rate of the calcium ionophore A23187 group was higher than that of the strontium chloride group (75.4% and 46.8%, respectively; $p < 0.05$); there was significant difference between two groups ($p < 0.05$). Among the 44 cleaved oocytes in the calcium ionophore A23187 group, eight developed to the two- to four-cell stage, 17 developed to the five- to eight-cell stage, 15 developed to greater than the eight-cell stage, and four blastocysts were obtained. Among the 21 cleaved oocytes in the strontium chloride group, six developed to the two- to four- cell stage, 10 developed to the five- to eight-cell stage, four developed to greater than the eight-cell stage, and one blastocyst was obtained. Three-day-old unfertilized human oocytes after IVF or ICSI could be activated by the calcium ionophore A23187 or strontium chloride, and a small part of parthenogenetic embryos developed into blastocysts. The treatment with the calcium ionophore A23187 was better than that of strontium chloride in respect to the activation rate of 3-day-old unfertilized human oocytes after IVF or ICSI.

Introduction

Parthenogenesis would provide an ethical tool for the objective assessment of oocyte developmental competence during the preclinical screening of experimental procedures in such areas as oocyte cryopreservation or *in vitro* maturation (Paffoni et al., 2007). Furthermore, it may be possible to eliminate the requirement to disaggregate normal competent human embryos for deriving human embryonic stem cell (hESC) lines *in vitro* (Kiessling, 2005; Vrana et al., 2003). We have established hESC lines derived from poor-quality embryos discarded during *in vitro* fertilization (IVF) procedures (Liu et al., 2011). Previous studies have reported that fresh human oocytes can develop to the blastocyst stage by using artificial activators (Cibelli et al., 2001; Lin et al., 2003). During IVF procedures, unfertilized human oocytes are routinely discarded. However, these unfertilized oocytes may provide rich sources of oocytes for parthenogenesis as well as for embryonic stem cell research. Until now, there is no report that 3-day-old unfertilized human oocytes after IVF/ICSI can be activated. Moreover, an optimized activation protocol may enhance better development of the embryo, which might in turn increase the chance of success in blastocyst formation from 3-day-old unfertilized human oocytes.

During fertilization, the sperm activates the oocyte by inducing a prolonged series of oscillations in the cytosolic free Ca^{2+} concentration (Ducibella et al., 2002; Stricker, 1999). Artificial stimuli also can activate the oocyte by elevating the cytoplasmic levels of calcium ions, even without the intervention of the sperm into the oocyte (Wang et al.,

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2008). Parthenogenesis can be induced with various methods of mechanical, chemical, and electrical stimuli in several species of mice, rabbits, goats, cows, and primates (Rougier et al., 2001). Chemical stimuli, such as ethanol (Loi et al., 1998), ionophore (Loi et al., 1998), calcium ionophore A23187 (Liu et al., 2002), and strontium (Cuthbertson et al., 1981) have been used to activate mammalian oocytes. Although many reports on human oocyte activation have been described (Cibelli et al., 2001; Krivokharchenko et al., 2003; Lin et al., 2003), until now no information is available on directly comparing the activation efficiency of the calcium ionophore A23187 or strontium chloride with large sample sizes.

SrCl2 is a known activator of mouse eggs (Kishikawa et al., 1999; Otaegui et al., 1999), but little is known regarding its effectiveness in activation of human eggs. Recently, it has been reported that SrCl2 effectively activated human oocytes and triggered development to the blastocyst stage (Krivokharchenko et al., 2003), and even resulted in birth of a baby (Yanagida et al., 2006), but these were case reports. However, no information was presented regarding the efficiency of the calcium ionophore A23187 compared with strontium chloride in large sample sizes. Furthermore, previous results indicated that better development rates were obtained when exposure of human oocytes to a calcium ionophore was followed by a protein kinase inhibitor 6-dimethylaminopurine (6-DMAP), than exposure to a calcium ionophore alone (Cibelli et al., 2001; Lin et al., 2003). Therefore, the objective of this study was to test the effectiveness of the calcium ionophore A23187 and strontium chloride on the activation and subsequent embryonic development of 3-day-old human unfertilized oocytes after IVF or intracytoplasmic sperm injection (ICSI).

Materials and Methods

Obtaining human oocytes

This study was performed with the approval of the Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University. Written informed consent was obtained from all participating patients in this study. This study was conducted at the Department of Reproductive Medicine of our hospital.

Human oocytes were obtained from patients whose gametes were unfertilized following conventional IVF or ICSI. For treatment, the patients underwent pituitary downregulation and controlled ovarian hyperstimulation. A gonadotrophin-releasing hormone (GnRH) analog (triptoreline, Decapeptyl 0.1; Ipsen S.p.A., Milan, Italy) was administered on day 21 of the previous treatment cycle. Ovarian hyperstimulation was achieved using recombinant follicle-stimulating hormone (rFSH; 150–300 IU, Gonal-F; Serono) daily beginning on day 3 of the treatment cycle until follicular maturation. Follicular development was monitored via ovarian ultrasonography and serum estradiol-17β assay. When the leading follicles had reached a mean diameter of 18 mm or more, human chorionic gonadotrophin (hCG; 10,000 IU, Gonasi; AMSA, Rome, Italy) was administered. Transvaginal follicular aspiration was performed 36 h after hCG administration, and 3–6 h later, oocytes were prepared for IVF or ICSI dependent upon semen analysis.

Following IVF insemination or ICSI, oocytes were cultured overnight and checked for signs of fertilization 19–20 h later and collected for further research 74–78 h later. Only metaphase II (MII) oocytes that showed no signs of fertilization but showed a normal morphology with uniform texture and clear cytoplasm were included in this study.

Activation treatment of oocytes

Denuded oocytes were randomly divided into two treatment groups: Oocytes in the first group were allocated to be activated by ionophore treatment, and oocytes in the second group were allocated to be activated by strontium treatment. Calcium ionophore A23187 activation was performed as described before with some modification (Paffoni et al., 2007). Oocytes were sequentially exposed to 5 μM calcium ionophore A23187 (Sigma-Aldrich, St. Louis, MO, USA) in Quinn's Advantage Human Tubal Fluid (HTF) medium (Sage BioPharma, Pasadena, CA, USA) for 5 min in the dark at 37°C 6% CO_2, washed five times, and incubated in 2 mM 6-DMAP (Sigma-Aldrich, St. Louis, MO, USA) in Quinn's Advantage Fertilization Medium (Sage BioPharma, Pasadena, CA, USA) for 3 h at 37°C, 6% CO_2. Then oocytes were washed five times, cultured in 40-μL microdrops of Quinn's Advantage Fertilization Medium under mineral oil at 37°, 6% CO_2 conditions.

Strontium activation was performed as described before with some modification (Swann and Ozil, 1994). Oocytes were exposed to 10 mM strontium chloride (Sigma-Aldrich, St. Louis, MO, USA) in Quinn's Advantage Fertilization Medium supplied with 10% serum protein substitute (SPS; SAGE In-Vitro Fertilization, Inc.) at pH 7.4 for 20 min at 37°C, 6% CO_2, washed five times, and incubated in 2.5 mM 6-DMAP in Quinn's Advantage Fertilization Medium for 1.5 h at 37°C, 6% CO_2. Oocytes were then washed five times and cultured in 40-μL microdrops of Quinn's Advantage Fertilization Medium under mineral oil at 37°C, 6% CO_2 conditions.

Oocyte and embryo culture

At 18–20 h after the exposure to ionophore or strontium, oocytes were evaluated for signs of activation under an inverted microscope. Oocytes with one or more enlarged pronuclei (PN) and extrusion of the second polar body were considered to be activated (Loi et al., 1998; Mitalipov et al., 2001). Parthenotes were washed and transferred to fresh Quinn's Advantage Fertilization Medium for 3 days, followed by a further 2 days of culture in Quinn's Advantage Blastocyst Medium (Sage BioPharma, Pasadena, CA, USA), and blastocyst formation was evaluated.

Statistics

Statistical analysis of data was performed with the use of the Statistical Package for Social Sciences (SPSS 13.0, USA). Differences between the two groups were determined by using a chi-squared test. Statistical significance was set at $p < 0.05$.

Results

Overall, 279 MII oocytes of 102 women were collected in this study: 138 were allocated to ionophore activation,

Table 1. Comparison of the Clinical Characteristics of the Donors Between Two Groups

	A23187 + 6-DMAP (n = *138*)	*Strontium + 6-DMAP* (n = *141*)	p *value*
Age	32.4 ± 3.76	32.6 ± 3.86	0.72
Duration of infertility (years)	3.1 ± 2.07	2.8 ± 1.79	0.06
BMI (kg/m^2)	21.9 ± 2.97	22.4 ± 2.99	0.78
Basic serum FSH level (mIU/mL)	6.3 ± 1.85	6.4 ± 1.73	0.43
Basic serum LH level (mIU/mL)	4.0 ± 1.74	4.3 ± 1.84	0.44
Basic serum T level (ng/mL)	29.6 ± 18.1	31.7 ± 18.8	0.60
Pregnancy/cycle (%)	38.1%	35%	0.10

Values are mean ± standard deviation (SD).
6-DMAP, 6-dimethylaminopurine; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

whereas 141 were allocated to strontium activation. Patients were comparable between the two groups. No statistical difference could be detected for age, duration of infertility, body mass index (BMI), pregnancy rate, or lutenizing hormone (LH), FSH, and testosterone plasma levels between the two groups of patients (Table 1). Oocytes treated with two different protocols were observed to determine the pronuclear formation rate and cultured for 5 days to assess embryo development.

A total of 170 oocytes were activated, 65 developed to cleavage stage, 19 developed to greater than an eight-cell stage, and five blastocysts were obtained. There was a significant difference in the activation rate between the two groups ($p < 0.05$; Table 2). The activation rate was significantly higher in the group of calcium ionophore and 6-DMAP treatment compared to that in the group of strontium and 6-DMAP treatment.

In the group of ionophore and 6-DMAP treatment, the proportions of activated oocytes presenting 1 pronucleus (PN), 2 PN, and poly PN were 26.0%, 56.7%, and 17.3%, respectively. In the group of strontium and 6-DMAP treatment, the proportions of activated oocytes presenting 1 PN, 2 PN, and poly PN were 13.7%, 54.5%, and 31.8%, respectively. There was no significant difference in 1 PN and 2 PN formation between the two groups ($p > 0.05$; Table 2), but there was a significant difference in 3 PN formation between the two groups ($p < 0.05$; Table 2).

The activated oocytes were cultured continuously for 5 days. In the group of ionophore and 6-DMAP treatment, 44 oocytes developed to the cleavage stage, eight to the two- to four-cell stage, 17 to the five- to eight-cell stage, 15 to greater than the eight-cell stage, and four to blastocysts. In the group of strontium and 6-DMAP treatment, 21 oocytes developed to the cleavage stage, six to the two- to four-cell stage, 10 to the five-to eight-cell stage, four to greater than the eight-cell stage, and one to a blastocyst. The differences were not significant between the two activation protocols in either the cleavage or development of embryos ($p > 0.05$; Table 2).

Discussion

During the process of natural fertilization, the entry of sperm into oocytes promotes a series of intracellular calcium oscillations and leads to oocyte activation (Rogers et al., 2004). The calcium oscillations result in the inactivation of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK), which enable the resumption and completion of meiosis, DNA synthesis, and pronuclear formation (Mitalipov et al., 2001). About 30% human oocytes fail to be activated after IVF or ICSI (Rawe et al., 2000). Assisted oocyte activation (AOA) has been regarded as an efficient treatment option in cases of low fertilization rates and complete fertilization failure after ICSI (Heindryckx et al., 2005; Yanagida et al., 2006). In this study, we chose calcium ionophore and strontium chloride as oocyte activators. Calcium ionophore treatment results in a single transient increase of intracellular calcium (Ca^{2+}) in the oocyte that induces oocyte activation through signal transduction mechanisms (Swann and Ozil, 1994). Strontium treatment, which promotes multiple calcium (Ca^{2+}) increases in the oocyte of mice as well as bovine (Bos-Mikich et al., 1995; Kline and Kline, 1992), was used to replace calcium ionophore treatment and electrical stimulation as methods of oocyte activation in mice (Yanagida et al., 2006). Although there are several reports concerned with the

Table 2. Comparison of Activation Rate and Embryo Development Between Two Different Activation Protocols on Unfertilized Human Oocytes after IVF

Activation treatment	*A23187 + 6-DMAP*	*Strontium + 6-DMAP*	p *value*	*Total %*
Number of oocytes	138	141		279
Oocytes activated %	75.4 (104/138)	46.8 (66/141)	0.00	60.9 (170/279)
1 PN (%)	26.0 (27/104)	13.6 (9/66)	0.08	21.2 (36/170)
2 PN (%)	56.7 (59/104)	54.5 (36/66)	0.84	55.9 (95/170)
3 PN (%)	17.3 (18/104)	31.8 (21/66)	0.04	22.9 (39/170)
Number of cleavage %	42.3 (44/104))	31.8 (21/66)	0.20	38.2 (65/170)
2–4 cells (%)	18.2 (8/44)	28.6 (6/21)	0.35	8.2 (14/170)
5–8 cells (%)	38.6 (17/44)	47.6 (10/21)	0.59	15.9 (27/170)
>8 cells (%)	34.1 (15/44)	19.0 (4/21)	0.26	11.2 (19/170)
Blastocysts %	9.1 (4/44)	4.8 (1/21)	1.00	2.9 (5/170)

IVF, *in vitro* fertilization; 6-DMAP, 6-diaminopurine; PN, pronucleus.

effects of strontium treatment of fertilized oocytes and the achievement of offspring (Krivokharchenko et al., 2003; Yanagida et al., 2006), data are limited to case reports and do not include large sample size reports.

In the present study, the activation of oocytes with calcium ionophores or strontium was followed by inhibition of protein phosporylation with 6-DMAP. This resulted not only in efficient oocyte activation, but also in parthenote development to the eight-cell stage or the blastocyst stage and thus further supported previous observations of oocytes of different species (Cibelli et al., 2001; Lin et al., 2003) and human oocytes (Paffoni et al., 2007). The reason why this combination is effective is that 6-DMAP caused the inhibition of the MPF reaction, following a kinetic similar to that occurring after fertilization (Paffoni et al., 2007), as observed in bovine (Liu and Yang, 1999) and pig (Grupen et al., 2002) oocytes.

As far as we know, this is the first study comparing the effects of these two protocols (strontium and 6-DMAP, ionophore and 6-DMAP) on activation of 3-day-old human unfertilized oocytes after IVF with a large sample size. The activation rate was significantly higher in the group of ionophore and 6-DMAP treatment compared to that in the group of strontium and 6-DMAP treatment. However, there was no significant difference in the pronuclear formation between the two groups, indicating that these two protocols were similar in inducing pronuclear formation. Compared with another report (Paffoni et al., 2007), the activation rate was higher and the cleavage rate of oocytes was lower in the group treated with ionophore and 6-DMAP. This was due possibly to the use of 3-day-old human unfertilized oocytes after IVF. With the extension of the eggs' age, ooctyes were easily activated due to the degradation of the material that maintained meiotic arrest, but the late developmental capacity decreased due to the degradation of the cytoplasmic ingredient, which maintained the further development of the oocyte (Web et al., 1986).

This study demonstrated that 3-day-old unfertilized oocytes after IVF/ICSI can be activated by protocols using the calcium ionophore A23187 or strontium chloride having the potential ability of formation of embryos and development of blastocysts *in vitro*. To our knowledge, these are the oldest oocytes successfully used in artificial activation. Previous studies have reported that fresh human oocytes can develop to the blastocyst stage by use of artificial activators (Cibelli et al., 2001; Lin et al., 2003). However, unfertilized oocytes are routinely discarded during IVF procedures. It is inconvenient to obtain 1- or 2-day-old unfertilized oocytes, but convenient to obtain 3-day-old unfertilized oocytes because of uterine embryo transfer or cryopreservation. Our study demonstrated that 3-day-old human oocytes can not only be activated by artificial activation methods, but also can develop to the blastocyst stage as reported using phospholipase activator on 1-day-old unfertilized oocytes (Rogers et al., 2004). The generation of such parthenogenetic blastocysts from oocytes, which can be more ethically acceptable than using embryos from fertilized zygotes, can provide a source of embryos for the creation of embryonic stem cells (Lin et al., 2003; Vrana et al., 2003). This can provide an experimental basis for the mechanism of human oocyte activation, solutions for the improvement of the fertilization rate of assisted reproductive technology, and an solution for limited embryo sources and ethical pressure problems for scientific experiments. However, further optimized conditions need to be found to generate viable parthenogenotes more efficiently. Moreover, it needs to be determined whether the parthenogenotes could be used to establish human embryonic stem cell lines.

In summary, 3-day-old unfertilized oocytes after IVF were artificially activated and a small part of parthenogenetic embryos developed into blastocysts. The treatment combination of the calcium ionophore A23187 with 6-DMAP was better than that of the combination of strontium chloride with 6-DMAP with respect to the activation rate of 3-day-old unfertilized oocytes after IVF or ICSI.

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Author Disclosure Statement

The authors declare that there are no conflicts of interest.

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