Pregnancy following chemical activation of oocytes in a couple with repeated failure of fertilization using ICSI: Case report

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We report our attempts to achieve a successful pregnancy outcome with calcium ionophore A23187 and puromycin oocyte activation using sperm from a normozoospermic husband of a patient with previous repeated failed fertilization following ICSI. Oocytes from the female partner of a couple with a 4 year history of unexplained primary infertility with repeated failed fertilization following ICSI were used. In the latest ICSI attempt, oocytes were activated by treatment with calcium ionophore (5 min) and puromycin (5 h), then cultured. In this cycle, assisted oocyte activation with calcium ionophore and puromycin after ICSI resulted in a satisfactory fertilization rate (8/12; 66.7%); in prior cycles only one of 71 oocytes (1.4%) was fertilized. The outcome was a Caesarean section delivery of a healthy male infant without congenital abnormalities at 41 weeks, 2 days of gestation. In conclusion, the use of calcium ionophore and puromycin for oocyte activation was found to be a useful method in a case of repeated failed fertilization after ICSI.

Key words: calcium ionophore/failed fertilization/ICSI/oocyte activation/puromycin

Introduction

ICSI has enabled fertilization of oocytes from patients whose partners have extremely low numbers of viable sperm, and a very low probability of achieving fertilization in vitro. However, we occasionally encounter the unusual case in which ICSI fails despite normal sperm concentration and motility. The reason for this phenomenon may be a partial or complete inability of the sperm to activate oocytes, or an inability of the oocytes to decondense the sperm (Sakkas et al., 1996; Schmiady et al., 1996). Oocytes that were not fertilized with ICSI have been subsequently activated using chemical substances or electroporation, and these oocytes have been able to form pronuclei (Tesarik and Sousa, 1995; Rybouchkin et al., 1997; Battaglia et al., 1997; Zhang et al., 1999; Yanagida et al., 1999; Kim et al., 2001; Nakagawa et al., 2001; Eldar-Geva et al., 2003). Nakagawa et al. (2001) reported an oocyte activation model using sequential A23187 and puromycin, which produced human and mouse haploid parthenogenomes, in previously unfertilized oocytes after ICSI. Fertilization rates are often unsatisfactory in oocytes that are artificially activated after previous fertilization failure. It is extremely important to develop a model whereby sperm fertilizability for oocyte activation prior to ICSI can be evaluated, in order to enhance ICSI fertilization rates and to maximize conception rates. One such method, which shows promise, is the insertion of human sperm into mouse oocytes (Araki et al., 2004).

Case report

A 26 year old woman and her 37 year old husband were referred to our clinic (Kanayama Women’s Clinic, Nagoya); they had been married for >4 years. Her physical and gynaecological examinations were within normal limits, including hysterosalpingography and routine blood tests. Her menses were regular. However, the GnRH/thyrotrophin-releasing hormone (TRH) test was: LH 7.8/104.0 mIU/l; FSH 3.8/16.8 mIU/l; prolactin (PRL) 8.8/29.4 ng/l (baseline/30 min after GnRH/TRH administration). The thyroid hormone (T3) was 23 ng/ml. We diagnosed polycystic ovary syndrome (PCOS) after the GnRH/TRH test and ultrasound imaging of ovarian morphology. The mean of sperm examination was: volume, 4.7 ml; count, 95.8×10⁶/ml (one specimen had relatively low numbers); motility, 39.0%; and abnormal morphology, 76.0%.
We advised them in regard to timing of intercourse, and administered 21 cycles of artificial insemination, which were unsuccessful. Subsequently, after informed consent, we offered IVF. From April 2001 to January 2002, the couple received five treatment cycles: one IVF; one IVF plus ICSI; and three ICSI.

**Microinjection and activation procedure**

Injection of human sperm into mouse oocytes was performed using a piezo-drive unit following a modified procedure of Kimura and Yanagimachi (1995). A sperm tail was cut at mid-length with the application of a few piezo pulses, and then drawn up into the injection pipette. An oocyte was placed on a holding pipette and its zona pellucida was drilled by applying a few piezo pulses. The injection pipette was advanced mechanically until its tip almost reached the opposite side of the oocyte's cortex. The oocyte membrane was broken by applying one or two faint piezo-pulses, and the entire sperm was injected into the ooplasm. It usually took 20–30 min to inject a group of ~15 oocytes. Oocytes injected with sperm were kept for 20–30 min at room temperature. After sperm injection, the oocytes in the activation treatment group were activated in Ca-free Toyoda, Yohojama and Hoshi (TYH) medium containing 10 mmol/l SrCl₂ for 60 min at 37°C under 5% CO₂ + 95% room air. These oocytes were individually transferred into a 30 μl drop of the human follicular fluid medium (HFF99; Fuso Pharmaceutical Industries, Japan) with 10% synthetic serum substance (SSS; Irvine Scientific, USA) under mineral oil in a plastic dish, and incubated at 37°C under 5% CO₂ and room air. Approximately 7–11 h after injection or activation treatment, the oocytes were observed with an inverted microscope by rotating oocytes to determine if a 2nd polar body (2PB) and pronuclei (PN) were present (Araki et al., 2004).

In January 2002, we utilized the sperm activation test using mouse oocytes; the injection of the husband sperm into mouse oocytes did not achieve activation. However, when these oocytes were activated with SrCl₂ immediately after ICSI, the activation rate was 100% (31/31) (Araki et al., 2004). In view of the foregoing, in October 2002, we administered a sixth assisted reproductive technology cycle using ICSI. Ovarian stimulation was achieved using a combination of GnRH agonist (Nasanyl®, Yamanouchi, Japan) and hMG (Pergogreen®, Serono, Switzerland). An injection of 5000 IU of hCG (Profasi®; Serono, Switzerland) was administered when the dominant follicle reached a mean diameter of 20 mm. Vaginal ultrasound-guided follicle puncture was done 35 h after hCG injection. The retrieved oocytes were cultured for several hours in HUECM AH25 (Nipro, Japan) at 37°C in an atmosphere of 5% CO₂ under a humidified condition. All oocyte handling procedures were conducted on warm stages using conventional methods.

We retrieved 18 oocytes, and 15 of 18 retrieved oocytes were mature (metaphase II, MII), two were immature, and one was damaged. We attempted ICSI for all 15 MII oocytes; 12 were viable after 30 min, and were activated by calcium ionophore A23187 (5 μmol/l) for 5 min. Subsequently, the oocytes were activated with puromycin (10 μg/ml) for 5 h, rinsed several times in culture medium, and incubated for 48 h at 37°C in an atmosphere of 5% CO₂ under a humidified condition. Eight of the 12 oocytes developed to the 3–4-cell cleavage stage; the other four were unfertilized. Three days following oocyte retrieval, the serum estradiol (E₂) was 7456 ng/ml; therefore, we cancelled the embryo transfer for this cycle to avoid the ovarian hyperstimulation syndrome. All eight cleaved embryos were cryopreserved by the slow freezing procedure.

In November, 2002, we thawed three frozen embryos and transferred them to the patient; however, a pregnancy did not ensue. Subsequently, on January, 2003, we thawed two embryos (4-cell and 3-cell cleavage stage), and transferred them.

For luteal support during the frozen embryo transfer cycle, 1 day after cessation of menses, the patient applied one Estrana® patch (E₂ 0.72 mg/patch); this regimen continued until ovulation. Nine days after the cessation of menses, one additional Estrana® patch was applied. At 11 days, two patches were applied, and at 13 days, one more patch was applied (three patches in all). After ovulation, two Estrana® patches were applied every other day. In addition, the patient inserted a 300 mg progesterone suppository twice a day for the 3 weeks following ovulation.

Two weeks after embryo transfer, the urine hCG level was >25 mIU/ml by qualitative analysis. At 6 weeks of gestation, a singleton pregnancy and a fetal heartbeat was confirmed by transvaginal ultrasound. In October 2003, the patient delivered by Caesarean section due to cephalopelvic disproportion at 41 weeks, 2 days of gestation. The infant was a healthy male without congenital abnormalities (3256 g; 46,XY).

**Discussion**

IVF has proven beneficial for several types of infertile patients, and ICSI has been particularly helpful in cases of severe male infertility; however, a 100% success rate is unattainable because a small percentage of oocytes remain unfertilized despite the insertion of high quality sperm. It has been reported that most fertilization failures following ICSI manifest as non-activated oocytes (Sousa and Tesarik, 1994; Tesarik and Sousa, 1995a). However, it is unknown whether the oocyte or the sperm is defective. Conversely, several investigators have reported successes by artificially activating oocytes after prior fertilization attempts had failed (Tesarik and Sousa, 1995b; Battaglia et al., 1997; Rybouchkin et al., 1997; Zhang et al., 1999; Yanagida et al., 1999; Kim et al., 2001; Nakagawa et al., 2001; Elder-Geva et al., 2003; Tesarik et al., 2002).

The sperm of our infertile male patient possessed a minimal ability to form pronuclei even though these sperm were normal in number, however abnormal in both motility and morphology. This low rate of pronuclear formation...
might be attributable to a total or partial deficiency in the sperm’s capacity to activate oocytes, or to the inability of the oocytes to decondense the sperm. We evaluated the ability of the patient’s sperm to activate mouse oocytes, and almost all oocytes failed to form pronuclei; however, they formed 2PN after activation by SrCl₂. The results of the present study suggest that these sperm have a low potential to spontaneously activate oocytes and to form pronuclei; thus, artificial activation after ICSI may rescue oocytes fertilized with infertile human sperm that do not produce 2PN.

We did not know if SrCl₂ could be as useful an activator substance as calcium ionophore for human oocytes. It has been reported that 70–80% of unfertilized oocytes after ICSI displayed two pronuclei following electrical activation (Zhang et al., 1999). Eldar-Geva et al., 2003 reported that calcium ionophore oocyte activation appeared to be a useful method in cases of repeated failed fertilization after ICSI. Calcium ionophore, which induces a calcium transient in ooplasm, bypasses sperm factor function. Eldar-Geva et al., 2003 speculate that their male patient had a deficiency or defective sperm factor or some other sperm-associated oocyte-activating factor, despite normal acrosome morphology. Oocyte activation results in extrusion of the second polar body, decondensation of a haploid set of chromosomes, and the formation of a nuclear membrane around these chromosomes as the female pronucleus.

Nakagawa et al., 2000 reported an oocyte activation method, which effectively produces human and mouse haploid parthenogenones by the use of a combination of calcium ionophore and puromycin. The sequential treatment of calcium ionophore A23187 and puromycin activates unfertilized oocytes after ICSI; the resultant 2PN–2PB oocytes can cleave. Kimura et al., 1998 theorized that a sperm’s oocyte activation factor surrounds the sperm’s perinuclear materials; thus, if a sperm’s cell membrane is damaged, the oocyte activation factor may not be affected. The acrosomal abnormalities observed in their study suggest that the patient’s sperm lacked the oocyte activation factor present in normal sperm.

In our case, although the sperm count was high, electron microscopy revealed the sperm’s defective acrosomal membrane; this finding suggests that the sperm lack an oocyte activation substance despite their appearance (Araki et al., 2004).

Recently, Tesarik et al., 2002 reported that the oocyte activation defect was overcome by a simple modification of the micromanipulation technique (ICSI) with no need to use chemical agents, such as calcium ionophore. The possibility of using a simple modification of the standard ICSI micromanipulation technique instead of calcium ionophores alleviates concerns about the possible harmful effects on human embryos of these insufficiently tested drugs.

Chian et al., 1999 reported that 6-dimethylaminoprine (6DMAP), an analogue of puromycin, suppressed formation of the bovine male pronucleus, but not the female pronucleus. However, Nakagawa et al., 2001 reported the efficacy of calcium ionophore and puromycin for human parthenogenones with one haploid pronucleus. Further study of puromycin oocyte activation is indicated; therefore, we must inform patients of the risks and benefits of undergoing fertility treatment including this agent in regard to possible adverse effects on their future child. This couple gave their informed consent, and agreed to oocyte activation after ICSI. The incidence of chromosomal abnormalities increases with the degree of normal sperm morphology and the presence of these abnormalities impairs oocytogenesis (Yoshida et al., 1996). We recommend chromosome analysis for infants delivered after IVF with severe infertility; fortunately, in this case, the infant was found to have a normal karyotype and exhibited no congenital abnormalities. Calcium ionophore and puromycin oocyte activation seems to be a useful method in cases of repeated failed fertilization after ICSI. Appropriate counselling in cases such as this is as important as providing efficacious treatment. Obviously, we must inform these patients of the risks and benefits of undergoing fertility treatment and the possible implications for the future health of their child.

References


References
Successful pregnancy outcome using artificially activated oocytes


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