

Case report

Successful pregnancy after ICSI with strontium oocyte activation in low rates of fertilization



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Abstract

Fertilization failure (complete fertilization failure or low fertilization rates) after intracytoplasmic sperm injection (ICSI) can occur in rare cases. In the majority of these cases, the unfertilized oocytes are inactivated. Assisted oocyte activation was applied as a treatment option for a case of low fertilization rate as a clinical trial. A patient with a low fertilization rate (ranging from 0% to 33.3%; mean = 17.0%) after eight previous ICSI cycles at another hospital, was diagnosed with fertilization failure. The most likely cause of fertilization failure was failure of oocyte activation. Therefore, artificial oocyte activation by strontium treatment was combined with ICSI to achieve viable fertilized oocytes. Oocytes were stimulated with strontium (10 mM SrCl₂, 60 min) approximately 30 min after ICSI. Six injected oocytes were stimulated and all were then successfully fertilized. Two blastocysts were transferred into the uterus, resulting in a pregnancy and birth. A second pregnancy was achieved following implantation of two cryopreserved embryos (one blastocyst and one morula). In conclusion, strontium treatment was found to be an effective method for artificial oocyte activation in a case with a low fertilization rate after ICSI.

Keywords: fertilization failure, ICSI, pregnancy, oocyte activation, strontium

Introduction

Intracytoplasmic sperm injection (ICSI) is the most powerful tool in assisted reproduction technology. Even though the fertilization rate of ICSI is typically the highest of all assisted reproduction treatments, fertilization failure (complete fertilization failure or a low fertilization rate) after ICSI has been recognized in rare cases. The frequency of complete fertilization failure after ICSI has been reported at 1.29% (Esfandiari *et al.*, 2005) and 3% (Liu *et al.*, 1995). The definition of low fertilization rate is less clear. Low fertilization rates are more frequent than complete fertilization failure. Complete fertilization failure and low fertilization rates after ICSI can occur repeatedly. Repeated fertilization failure is not desirable, because both the stress and costs of oocyte retrieval are high. Investigation of oocytes remaining unfertilized after ICSI revealed that these unfertilized oocytes remain unactivated, despite proper

injection of spermatozoa (Wall *et al.*, 1996; Dubey *et al.*, 1997). As a potential solution, a number of clinical trials have attempted to determine the effectiveness of combining ICSI and assisted oocyte activation (AOA) (Vanderzwalmen *et al.*, 1997; Yanagida *et al.*, 1999; Eldar-Geva *et al.*, 2003).

In this report, strontium treatment was combined with ICSI for the treatment of a low fertilization rate case, achieving fertilized oocytes, pregnancy, and childbirth. This is the first report detailing the application of strontium for AOA in humans in a case with low fertilization rates after ICSI. In laboratory mice, the egg activation effect of strontium is superior to calcium ionophore, since strontium treatment induces calcium oscillations (Kline and Kline, 1992). Strontium is not used for conventional culture. Neither the toxicity nor the safety of strontium exposure for gametes and embryos are proven.

Materials and methods

The husband and wife enrolled in this study were 31 and 33 years old, respectively. As a couple, they had been infertile for 2 years after marriage before receiving a diagnosis of oligo-zoasthenozoospermia (sperm concentration: $9 \times 10^6/\text{ml}$; sperm motility = 10%). Several artificial inseminations using the husband's spermatozoa were attempted, but pregnancy could not be achieved. They were then treated at a university hospital for three years (1999–2002), during which time a total of eight ICSI cycles were performed using a long stimulation protocol. The results and semen analysis of each ICSI cycle are presented in Table 1. Fertilization rates for the eight ICSI cycles ranged from 0% to 33.3%, with a mean of 17.0%. Because of this, the couple were referred to the Fukushima Medical University hospital. A diagnosis of low fertilization rate in ICSI was made using the fertilization rates obtained in the eight previous ICSI attempts. As a result of low fertilization, good embryos could not be obtained. Therefore, it was thought that the number of oocytes fertilized should be increased and that an oocyte activation disorder was the cause underlying the low fertilization. The couple was informed that a larger number of fertilized oocytes might be obtained if AOA treatment was combined with ICSI.

The methods of calcium ionophore treatment, electrical stimulation and strontium treatment have been used by the authors previously for artificial oocyte activation. In 1994, calcium ionophore treatment with ICSI was applied in a clinical trial under the approval of an Ethical Review Committee. In 1999, electro-stimulation with ICSI was applied in the same way under the approval of the same committee. Strontium treatment as the method of choice was suggested by the authors due to its apparent safety and effectiveness. This study was performed with the approval of the Ethical Review Committee at Fukushima Medical University and with the informed consent of the patients. The Ethical Review Committee approved this

study entitled 'The clinical trial of the combination treatment of ICSI and artificial oocyte activation for fertilization failure cases after ICSI' as result of discussion, and calcium ionophore treatment, electrical stimulation and strontium treatment were included as methods of artificial oocyte activation.

Ovarian stimulation, preparation of gametes and ICSI

Gonadotrophin-releasing hormone (GnRH) agonist (buserelin acetate: Suprecur, Hoechst Japan Co., Tokyo, Japan) was administered at 600 μg per day beginning on day 21 of the previous treatment cycle. The patient was also given FSH (150 IU–300 IU, Fertinorm P, Serono Japan Co., Tokyo, Japan) and human menopausal gonadotrophin (HMG) (150 IU, HMG Teizo, Teikokuzoki Co., Tokyo, Japan) daily beginning on day 3 of the treatment cycle until follicular maturation. Human chorionic gonadotrophin (HCG) (10,000 IU, Mochida Pharmaceutical Co., Tokyo, Japan) was administered when the leading follicles reached a diameter of approximately 18 mm. Oocytes, retrieved transvaginally from the ovaries 35 h after HCG administration, were incubated for 2–5 h in P+ fertilization medium (SAGE In-Vitro Fertilization Inc., CA, USA). Immediately before ICSI, cumulus cells were removed by pipetting cumulus oocyte complexes in HEPES-buffered human tubal fluid (HTF) medium (sperm washing medium: SAGE In-Vitro Fertilization, Inc.) containing 0.25 mg/ml hyaluronidase (type VIII, H-3757; Sigma Chemical, St. Louis, MO, USA). Nine denuded oocytes were examined by light microscopy; only six oocytes were in metaphase II and therefore suitable for ICSI. None of these oocytes was evaluated as poor quality under microscopy.

Semen samples were allowed to liquefy for 30 min at room temperature before collection of spermatozoa by the swim-up method using P+ medium. Immediately before ICSI, motile spermatozoa were isolated individually using a micropipette. Piezo ICSI was performed as described by Yanagida *et al.*, (1998).

Table 1. The results of intracytoplasmic sperm injection (ICSI) and semen analysis of the couple's eight previous treatment cycles.

ICSI cycle	No. of oocytes retrieved	No. of oocytes injected	No. of oocytes fertilized (%)	No. of embryos transferred ^a	Sperm concentration ($\times 10^6/\text{ml}$)	Sperm motility (%)	Pregnancy
1	8	7	2 (28.6)	2 (1)	2.1	4.8	No
2	9	7	1 (14.3)	1 (1)	12.3	2.4	No
3	5	4	0 (0)	0 (0)	9.5	5	No
4	6	3	1 (33.3)	1 (0)	7.3	4	No
5	7	5	1 (20.0)	1 (0)	1.9	42	No
6	5	4	1 (25.0)	1 (0)	37.0	5	No
7	7	6	1 (16.7)	1 (1)	6.0	1	No
8	11	11	1 (9.1)	1 (0)	12.0	27	No
Total	58	47	8 (17.0)	8 (3)	-	-	None

^aAll embryo transfers were performed on day 3 after ICSI. Values in parenthesis indicate the number of good quality embryos available, defined as grades 1 and 2 using Veeck's classification (Veeck, 1991).

Strontium treatment of oocytes

Injected oocytes were activated by strontium treatment for approximately 30 min after ICSI. Strontium treatment was performed as described by Swann and Ozil (1994). Cultured oocytes were immediately transferred to Sr^{2+} -HTF (calcium-free human tubal fluid medium at pH 7.4 containing 10 mM SrCl_2 and 10% serum protein substitute [SPS; SAGE In-Vitro Fertilization, Inc.]). All inorganic and organic reagents in Sr^{2+} -HTF media were purchased from Sigma Chemical Co. unless otherwise specified. Oocytes were cultured for 60 min in Sr^{2+} -HTF under 5% CO_2 , 5% O_2 , and 90% N_2 . Oocytes were then transferred back to P+ medium.

Oocyte culture and embryo transfer

ICSI oocytes treated with strontium were cultured for 18 h in P+ medium containing 10% SPS. Oocytes with a second polar body and two pronuclei were considered to be normally fertilized. They were further cultured for 96 h to allow oocytes to develop into blastocysts, which were then transferred into the patient's uterus. The surplus embryos were cryopreserved with the vitrification method as described by Kuwayama *et al.*, (2005).

Results

Nine oocytes were retrieved by ICSI. Six of these were in metaphase II, allowing us to perform ICSI. All injected oocytes were stimulated using strontium treatment after ICSI. All treated oocytes possessed two pronuclei in the ooplasm with two clear polar bodies. All oocytes went on to be fertilized and were cultured for five days. Oocytes developed into three blastocysts, one morula, one two-cell stage embryo and one pronuclear stage oocyte. Two blastocysts were transferred into the uterus, resulting in the eventual birth of a healthy female (2903 g) at 40 weeks gestation. One blastocyst and one morula were cryopreserved by vitrification. The parents also wanted frozen embryo transfer 13 months later and two cryopreserved embryos were thawed and transferred into the uterus, resulting in a normal pregnancy (ongoing).

Discussion

Assisted oocyte activation (AOA) has been recommended as an efficient treatment option in cases of complete fertilization failure and low fertilization rates after ICSI (Heindryckx *et al.*, 2005). Several reports describe the efficacy of the combination of AOA and ICSI for the treatment of complete fertilization failure in previous treatment cycles of ICSI alone (Rybouchkin *et al.*, 1997; Yanagida *et al.*, 1999; Kim *et al.*, 2001; Eldar-Geva *et al.*, 2003; Murase *et al.*, 2004) and low fertilization rates (Chi *et al.*, 2004). AOA methodology applied to human oocytes includes calcium ionophores, electrical stimulation, and puromycin treatment (Lu *et al.*, 2006). Calcium ionophore treatment (Rybouchkin *et al.*, 1997; Kim *et al.*, 2001; Eldar-Geva *et al.*, 2003; Chi *et al.*, 2004; Murase *et al.*, 2004) and electrical stimulation (Yanagida *et al.*, 1999) in combination with ICSI have been reported to result in pregnancies and deliveries. Calcium ionophore treatment is the most commonly applied method of oocyte activation in clinical trials. Tesarik and

Testart (1994) reported the activation of human oocytes after ICSI by calcium ionophores. In 1995, Hoshi *et al.* reported the first pregnancy using ICSI with calcium ionophore treatment as AOA to increase the fertilization rate. Using calcium ionophore treatment, Rybouchkin reported the first pregnancy for a couple with complete fertilization failure due to a round-headed sperm defect associated with deficient oocyte activation capacity. Both calcium ionophore treatment and electrical stimulation resulted in a single transient increase of intracellular calcium (Ca^{2+}) in the oocyte; this transduction pathway induces oocyte activation through signal transduction mechanisms (Swann and Ozil, 1994). During physiological fertilization, a transient increase in intracellular Ca^{2+} occurs after spermatozoon-egg fusion; this is followed by calcium oscillations that continue for 3–4 h. One to several transient calcium increases are required for oocyte activation. Although the function of these calcium oscillations is not clear, they also affect the embryonic development (Cheung *et al.*, 2000; Alberio *et al.*, 2001). Bos-Mikich *et al.* (1995) demonstrated that calcium oscillations both during mitosis and exit from meiosis increased the number of inner cell mass cells of the blastocyst.

In mice, strontium treatment is used in place of calcium ionophore treatment and electrical stimulation as the method of oocyte activation. Calcium oscillations have been confirmed in the mouse model following strontium treatment (Kline and Kline, 1992). There are several reports concerned with the effects of strontium treatment on the chromosomes of fertilized oocytes (including parthenogenesis) and the achievement of offspring. The main reports are summarised in **Table 2**. O'Neill *et al.* (1991) undertook cytogenetic analysis of single-pronuclear haploid oocytes that were induced to parthenogenesis by strontium treatment in the mouse model and reported that strontium treatment did not induce chromosomal abnormality. Mouse sperm nuclei injected into mouse oocytes within 1 h after strontium exposure transformed normally into male pronuclei, and the number of chromosome aberrations did not significantly increase in the resultant zygotes (Tateno and Kamiguchi, 2005). In ICSI of mouse spermatozoa following strontium treatment, the incidence of diploid zygotes with chromosome aberrations was 12.3% (structural: 10.5%, aneuploidy: 1.8%) (Morozumi *et al.*, 2004).

There are few reports of animal experiments that have used ICSI and strontium oocyte activation together to obtain offspring. This is because animal experiment models in which an oocyte activation disorder is present are limited. Round spermatozoa and a sperm-linked oocyte activation factor disorder are included in an oocyte activation disorder model. In the mouse, injection of round spermatozoa in combination with strontium oocyte-activation led to live-birth results that were not significantly different to those outcomes obtained using mature spermatozoa (Loren and Lacham-Kaplan, 2006). Lacham-Kaplan *et al.* (2003) examined the capacity of embryos from the inbred C57BL/6J and 129Sv/ImJ mouse strains to term following ICSI with spermatozoa frozen without cryoprotectant. Activation of oocytes injected with frozen spermatozoa with strontium did result in the birth of pups in all strains. For the 129Sv/ImJ mouse, the number of pups born after activation of the oocytes injected with snap-frozen spermatozoa was the same as with fresh spermatozoa (control). For the C57BL/6J mouse, the number of pups from the snap-frozen spermatozoa with strontium activation was low, but the

Table 2. Reported studies concerning the chromosomal abnormality of oocytes/embryos and offspring after strontium treatment in mice.

Study	Strontium (Sr) treatment method ^a	Main result
O'Neill <i>et al.</i> (1991)	Sr (Parthenogenesis)	Chromosomes of 1PN oocytes: about 93.5–94.9% of evaluated oocytes had normal haploid set.
Morozumi <i>et al.</i> (2004)	ICSI + Sr ^b	Chromosomes of 1 cell zygotes: 87.7% had normal chromosomes.
Tateno and Kamiguchi (2005)	Sr + ICSI	Chromosomes of 1 cell zygotes: 94.8% ^c had normal chromosomes (control 91.7%, NS).
Lacham-Kaplan <i>et al.</i> (2003)	ICSI + Sr ^c	Offspring: 24% (control: 20%, NS).
Suganuma <i>et al.</i> (2005)	ICSI + Sr	Chromosomes of zygotes: 84% were of normal chromosome constitution (control = 91%). Rate of offspring = 49.2% (control = 69.6%, NS)
Loren and Lacham-Kaplan (2006)	ROSI + Sr ^d	Offspring: 19% (control 32%, NS).

Sr = strontium; ICSI – intracytoplasmic sperm injection; NS = not significant.

^aICSI + Sr = Sr treatment was performed after ICSI.

^bControl (ICSI without Sr) was not done. Mouse: 129Sv/Imj; spermatozoa = snap frozen spermatozoa. The value shows the offspring rate of transferred embryos.

^cICSI was performed within 0.5 h after Sr exposure.

^dROSI = round spermatid injection. The value shows the offspring rate of transferred 2-cell embryos.

number of pups from the snap-frozen spermatozoa without strontium was zero (Lacham-Kaplan *et al.*, 2003). Reversible infertility can be induced in male mice by oral administration of the alkylated imino sugars *N*-butyldeoxynojirimycin (NB-DNJ). Spermatozoa from cauda epididymis of NB-DNJ mouse have misshapen heads and low motility and a disorder of oocyte activating ability (Suganuma *et al.*, 2005). The oocytes injected with those spermatozoa were treated with strontium and resulted in normal fertilization (92.9%) and good offspring rate (49.2%) of transferred embryos (Suganuma *et al.*, 2005). This indicates that strontium treatment after ICSI did not affect the quality of embryos. As far as is known, there are no reports of strontium oocyte activation combined with ICSI in humans. The effect of oocyte activation by strontium was examined in 1-day-old unfertilized oocytes after IVF prior to the clinical trial. As a result, it was recognized that 10 mM strontium for 60 min was a necessary and sufficient treatment to result in 100% survival rate and oocyte activation rate.

The decision to use strontium after ICSI in this case of low fertilization was made carefully. There is evidence to suggest that there is an increased risk of abnormality in a child born following conception via IVF or ICSI (Hourvitz *et al.*, 2005; Ludwig, 2005). The in-vitro culture environment, which includes elements supplemented to the culture medium, may contribute to such abnormalities (Ludwig, 2005). Strontium added to culture medium is not used for conventional culture in animal experiments. There are no reports that strontium is safe for the gamete or embryo. However, neither are there reports about the toxicity of strontium to the gamete or embryo. The calcium ionophore has been used for oocyte activation in humans since the 1990s. In due course, it is hoped that the use of strontium for oocyte activation will be understood in the same way as calcium ionophores with regard to safety. It is not

possible to say that strontium is safe at present: however, there have not been any reports of problems in embryogenesis and offspring in the laboratory mouse to date.

The overall fertilization rate using ICSI ranges from 70–90% (van Steirteghem *et al.*, 1993; Ma and Ho Yuen, 2000; Ben-Yosef and Shalgi, 2001). Complete fertilization failure, however, was observed in 2–3% of ICSI treatments (Mahutte and Arici, 2003). Cases of low fertilization rates after ICSI have also been recognized. It is likely that complete fertilization failure and low fertilization efficacy after ICSI result from organic biological problems. As ICSI bypasses a number of the steps in the fertilization process, including capacitation, the acrosome reaction, hyperactivation, and spermatozoa–egg fusion, fertilization failure after ICSI likely is a result of disorders of the fertilization events that occur after spermatozoa–egg fusion.

Although sperm morphology was normal, the husband in this couple had been diagnosed with severe oligoasthenozoospermia by semen analysis. Three to 11 oocytes were injected with spermatozoon during each ICSI procedure. Fertilization rates commonly increase as the number of oocytes injected is decreased. In this case, an unspecified disorder of fertilization is likely to have existed, as fertilization rates were very low throughout multiple ICSI procedures.

Additional reports purport that oocyte activation does not occur in approximately 70% of unfertilized oocytes after ICSI (Yanagida 2004; Heindryckx *et al.*, 2005), despite accurate injection of the spermatozoon into the oocyte. In these cases, the causes of fertilization failure after ICSI are related to intrinsic spermatozoa or oocyte factors. Heterologous mouse ICSI (mouse oocyte activation test MOAT) is useful to evaluate the causes of ICSI fertilization failure. When MOAT demonstrates

sperm factor deficiencies, AOA is often an effective treatment option. MOAT could not be applied to the diagnosis in this case as the mechanism responsible for the low fertilization remained unclear. Volunteer spermatozoa that had previously been proven to be fertile were injected into 1-day-old unfertilized oocytes obtained from ordinary ICSI; fertilization occurred in 68.6% (24/35 oocytes) of oocytes (data not shown). It is often assumed that the majority of oocyte activation disorders are due to abnormalities in spermatozoa. It is important to remember, however, that disorders of meiotic maturation due to oocyte factors can also contribute to fertilization disorders (Ebner et al., 2006).

In this case, a strontium treatment was applied as AOA to improve the efficacy of ICSI. Chi et al. (2004) recently reported that oocyte activation by calcium ionophore treatment was useful to achieve fertilization in cases of low fertilization rates. In their study, the authors used split ICSI to demonstrate a significant difference in the rate of fertilization between treated and untreated oocytes. Although the split ICSI method was not used in this study, a fertilization rate of 75% using strontium treatment of spermatozoa was achieved, where as in the couple's eight previous (non-activated) ICSI cycles an average fertilization rate of only 17% was obtained. In this study, two pregnancies from four embryos were achieved, following two separate embryo transfers. Thus, strontium treatment may be as useful as calcium ionophore treatment or electrical stimulation for oocyte activation.

In conclusion, strontium treatment as AOA is reported to have been useful for a low fertilization case after ICSI, demonstrated by successful achievement of pregnancy in this case. As the mechanisms and genetic safety of oocyte activation induced by strontium treatment are not yet clear, further research is required before this technique can be clinically applied.

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