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Birth and Follow-up of Babies Born Following ICSI with Oocyte Activation Using Strontium Chloride or Calcium Ionophore A23187

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Abstract: We evaluated the efficacy and safety of two chemical oocyte activators, strontium chloride ($SrCl_2$) and calcium ionophore A23187 (A23187), after ICSI for patients with low fertilization (PLF) (less than 30%). Eighty-five PLF were randomly divided two groups: 35 patients in the $SrCl_2$ group (SG) and 50 patients in the A23187 group (AG). The control group (CG) was 530 patients who had undergone ICSI without artificial oocyte activation (AOA). The fertilization rate after AOA significantly increased from 24.7% to 54.5% in SG and from 20.9% to 62.4% in AG. Without AOA, there were no clinical pregnancies, but with AOA, 6 of 22 in SG and 9 of 37 in AG achieved pregnancies. Twenty-two babies (twenty singletons and one pair of twins) born after AOA had no abnormalities at birth [CG: 3.0% (16/530), SG: 0% (0/12), AG: 0% (0/10)]. Also, none of the infants' developmental characteristics showed any significant difference from the CG. Consequently, AOA using A23187 or $SrCl_2$ is beneficial for PLF following ICSI and does not adversely affect the growth or health of infants in their first 4 yr. Further studies of clinical tests for proper patient selection, efficacy and safety of AOA in larger samples are needed.

Key words: Artificial oocyte activation, Calcium ionophore A23187, Strontium chloride, Fertilization failure, Follow-up of babies

Introduction

Intracytoplasmic sperm injection (ICSI) has realized benefits for patients with severe male infertility. However, sometimes there are extremely low fertilization rates even after ICSI. The incidence of fertilization failure after

ICSI is 1–5% [1, 2]. In our unpublished data, the pregnancy rate is very low (2.1%; 4/190) in patients with low fertilization (PLF), less than 30%, following ICSI, and approximately 50% of fertilization failure cases are due to the sperm abnormalities. Oocytes unfertilised after ICSI have been activated using chemical [3–9], mechanical [10, 11], and electrical stimulation [12–15], and these oocytes have been able to form pronuclei. However, the optimal method and safety of artificial oocyte activation (AOA) have not been confirmed, despite the extreme importance of this technique for the rescue of unfertilized oocytes. The aim of this study was to evaluate the efficacy and safety of AOA with strontium chloride ($SrCl_2$) and calcium ionophore A23187 (A23187) for PLF or globozoospermia following ICSI. We also compared the clinical results of $SrCl_2$ and A23187 in the same patient and the same cycle. The safety of AOA was assessed by follow-up of the children up to 4 yr after birth.

Materials and Methods

Before treatment, we obtained approval from our Institutional Review Board and informed consent from eighty-five couples who received AOA following ICSI between 1 April 2004 and 31 October 2010 at Kyono ART Clinic. The treatment with $SrCl_2$ or A23187 was assigned to patients at random to assess the validity of oocyte activation (Study 1 and Study 2): 35 patients in the $SrCl_2$ group (SG), and 50 patients in the A23187 group (AG). We compared the clinical results of the patients without AOA (first treatment) and with AOA (second treatment). The control group (CG) was 530 patients for whom ICSI had been performed without AOA.

Ovarian Stimulation and Luteal Support

Ovarian stimulation was performed using a combina-

tion of gonadotrophin-releasing hormone (GnRH) agonist (Nasanyl; Yamanouchi, Japan), recombinant follicular stimulating hormone (rFSH) (Gonalef; Serono, Switzerland), and human menopausal gonadotrophin (hMG) (Pergogreen; Serono, Switzerland) or GnRH antagonist (Cetrorelix; Serono, Switzerland), rFSH, hMG, and clomiphene citrate (Clomid; Shionogi, Japan). An injection of 5000 IU of human chorionic gonadotrophin (hCG) (Profasi; Serono, Switzerland) was administered when the dominant follicle reached a mean diameter of 18 mm. Vaginal ultrasound-guided follicle puncture was performed 36 h after hCG injection. The retrieved oocytes were cultured for several hours in Queen's Advantage Cleavage medium (Sage, USA) at 37 °C in an atmosphere of 6% CO₂, 5% O₂, and 89% N₂ under humidified conditions. All oocyte handling procedures were conducted on warm stages using conventional methods. For luteal support during the fresh embryo transfer cycle, the patient was given 2 mg oral oestradiol (oestradiol tablets; Barr Laboratories Inc., USA) and 6 mg oral progesterone (Lutoral; Shionogi, Japan) per day until confirmation of pregnancy following embryo transfer. Embryo transfer was performed in most cases on day 3. Clinical pregnancy was determined transvaginal ultrasonography detecting by the presence of a gestational sac and fetal heartbeat.

Chemical oocyte activation using SrCl₂ and A23187

In Study 1, ICSI was performed on metaphase II (MII) oocytes. Thirty min after ICSI, these oocytes were activated in 10–20 mmol SrCl₂ · 6H₂O (Sigma-Aldrich, USA), 10% synthetic serum supplement (Irvine Scientific, USA), and Ca²⁺ free Dulbecco's Modified Eagle Medium (Gibco, USA) in 20 µl/drop at 37 °C, in 6% CO₂, 5% O₂, and 89% N₂ under humidified conditions for 60–120 min. Then the oocytes were rinsed and cultured in Universal IVF Medium or G-IVF Medium until the confirmation of fertilization [7].

In Study 2, 30 min after ICSI, the oocytes were activated in 10 µmol of A23187 (Sigma-Aldrich, USA) for 5–15 min. Subsequently, these oocytes were rinsed and cultured in Universal IVF Medium or G-IVF Medium for 16 to 18 h until confirmation of fertilization [9].

Some patients underwent AOA with both SrCl₂ and A23187 during the same cycle. In Study 3, we compared the two oocyte activators.

Child development assessment

Following AOA after ICSI, 17 patients delivered 22 babies (12 in SG; 10 in AG), and 13 patients (4 in SG; 9 in AG) have ongoing pregnancies at the time of writing. Prior to the child development assessment, we ob-

tained informed consent from all ICSI patients and sent written questionnaires to assess the children's physical and mental development according to the Maternal and Children's Health Handbook issued through local governments by the Ministry of Health, Labour and Welfare of Japan. In Study 4, we looked at the congenital abnormalities, heights, weights, and physical and mental development of children from birth to 4 yr of age, comparing cases in CG (n=530), SG (n=12), and AG (n=10) in the same period. In Study 5, we examined two children's congenital normality and development following a single vitrified-warmed blastocyst transfer after ICSI and AOA with A23187 using sperm from the same globozoospermic patient at different times.

Globozoospermia is a rare form of teratozoospermia, mainly characterized by round-headed spermatozoa that lack an acrosome. Semen analysis showed 100% round-headed sperm with abnormal morphology under light and electron microscopy.

Statistical analysis

Statistical analysis was performed using the chi-square test for independent and paired data, wherever appropriate. A *P* value of <0.01 was considered to be statistically significant.

Results

Study 1

Study 1 compared cycles of 35 patients without and with SrCl₂ oocyte activation after ICSI. There was a significant difference in fertilization rates between those without and with SrCl₂ oocyte activation, 24.7% (48/194) vs. 54.5% (97/178) (*P* < 0.01), respectively. There was no significant difference in clinical pregnancy rates per embryo transfer 0% (0/15) vs. 27.3% (6/22), respectively, or miscarriage rates, 0% (0/0) vs. 16.7% (1/6), respectively (Table 1).

Study 2

The cycles of 50 patients without and with A23187 oocyte activation after ICSI were compared. There was a significant difference in fertilization rates between those without and with A23187 activation, 20.9% (50/239) vs. 62.4% (131/210) (*P* < 0.01), respectively. There was no significant difference in clinical pregnancy rates, 0% (0/20) vs. 24.3% (9/37), respectively, or miscarriage rates, 0% (0/0) vs. 22.2% (2/9), respectively (Table 1).

Study 3

AOA using both SrCl₂ and A23187 was performed in the same cycle for 14 couples using their own oocytes

Table 1 Results of activation with and without SrCl₂ or A23187

n	SG			AG		
	Without Activation	With Activation	P value	Without Activation	With Activation	P value
	35	35		50	50	
Mean age of patients (mean±SD)	34.8 ± 4.8	35.4 ± 4.4		36.0 ± 4.4	36.1 ± 4.4	
Mean no. of previous cycles (mean±SD)	0.7 ± 1.3	2.2 ± 1.6		0.9 ± 1.4	2.2 ± 1.4	
Mean no. of oocytes collected (mean±SD)	8.9 ± 7.2	6.7 ± 6.6		7.2 ± 6.1	5.2 ± 4.3	
Mean no. of MII (mean±SD)	5.4 ± 4.7	4.3 ± 2.9		4.9 ± 3.9	4.3 ± 3.1	
Fertilization rate (%)	24.7 (48/194)	54.5 (97/178)	<0.01	20.9 (50/239)	62.4 (131/210)	<0.01
CPR* (%)	0 (0/15)	27.3 (6/22)	ns	0 (0/20)	24.3 (9/37)	ns
Implantation rate (%)	0 (0/21)	20.6 (7/34)	ns	0 (0/21)	20 (9/45)	ns
Miscarriage rate (%)	0 (0/0)	16.7 (1/6)	ns	0 (0/0)	22.2 (2/9)	ns

*CPR: Clinical Pregnancy Rate per Embryo Transfer.

Table 2 Comparison of results of SrCl₂ and A23187 activation

	SG	AG	P value
Fertilization rate (%)	50.0 (28/56)	59.3 (32/54)	ns
CPR* (%)	14.3 (2/14)	21.4 (3/14)	ns
Implantation rate (%)	14.3 (2/14)	21.4 (3/14)	ns
Miscarriage rate (%)	0 (0/2)	33.3 (1/3)	ns

*CPR: Clinical Pregnancy Rate per Embryo Transfer.

and sperm. There were no significant differences in the results: fertilization rate, 50.0% (28/56) vs. 59.3% (32/54) respectively; clinical pregnancy rate 14.3% (2/14) vs. 21.4% (3/14) respectively; and miscarriage rate 0% (0/2) vs. 33.3% (1/3), respectively (Table 2).

Study 4

Twelve babies (10 singletons and one pair of twins; 7 males, 5 females) were born to 9 patients after SrCl₂ oocyte activation following ICSI while 10 babies (10 singletons; 5 males, 5 females) were born to 9 patients after A23187 oocyte activation following ICSI. A comparison of the babies' data at birth was made among the 3 groups: CG, SG, and AG (Table 3). Babies in CG were singletons born to patients who received ICSI without AOA at our clinic. The babies born with AOA had no abnormalities at birth [CG: 3.0% (16/530), SG: 0% (0/12), AG: 0% (0/10)]. Also, none of the twenty singleton infants' developmental characteristics showed any significant difference from babies born without AOA (Fig. 1). The heights and weights of the twins were 47.0 cm and 2.3 kg, 43.0 cm and 2.0 kg at birth (cesarean section at 38 weeks 3 days gestation); 71.4 cm and 7.9 kg, 67.0 cm and 8.1 kg at 6 months; 81.8 cm and 9.9 kg, 77.3 cm and 9.3 kg at 18 months; and the two children were in the 50th percentile at 6 months after birth.

Study 5

In 2007, following GnRH agonist long protocol, twenty-one oocytes were retrieved from a patient whose husband was a globozoospermic patient, and eleven blastocysts were vitrified to prevent severe OHSS (Ovarian Hyperstimulation Syndrome). A single vitrified-warmed blastocyst (grade 4BB) [16] was transferred leading to the birth of a healthy boy, weighing 3.2 kg at 40 weeks 0 days gestation, on 12 April, 2008 [9]. The same mother gave birth to another healthy boy weighing 2.5 kg at 34 weeks 0 days gestation, on 28 July, 2010, following transfer of a single vitrified-warmed blastocyst (grade 4BA). The two boys (3 yr old and 8 months old) had no abnormalities at birth and normal physical and mental development up to the present.

Discussion

There have been several single case reports of electrical, mechanical, and chemical stimulation for PLF following conventional ICSI [3, 6–8, 17, 18] in case of oligoasthenoteratospermia [14, 18], nonobstructive azoospermia [14, 19], globozoospermia [4, 9, 15, 20, 21] and round spermatids [12, 22]. Recently, a few reviews [2, 23–25] related to AOA have been published. However, it is still unclear which methods are more effective, easier

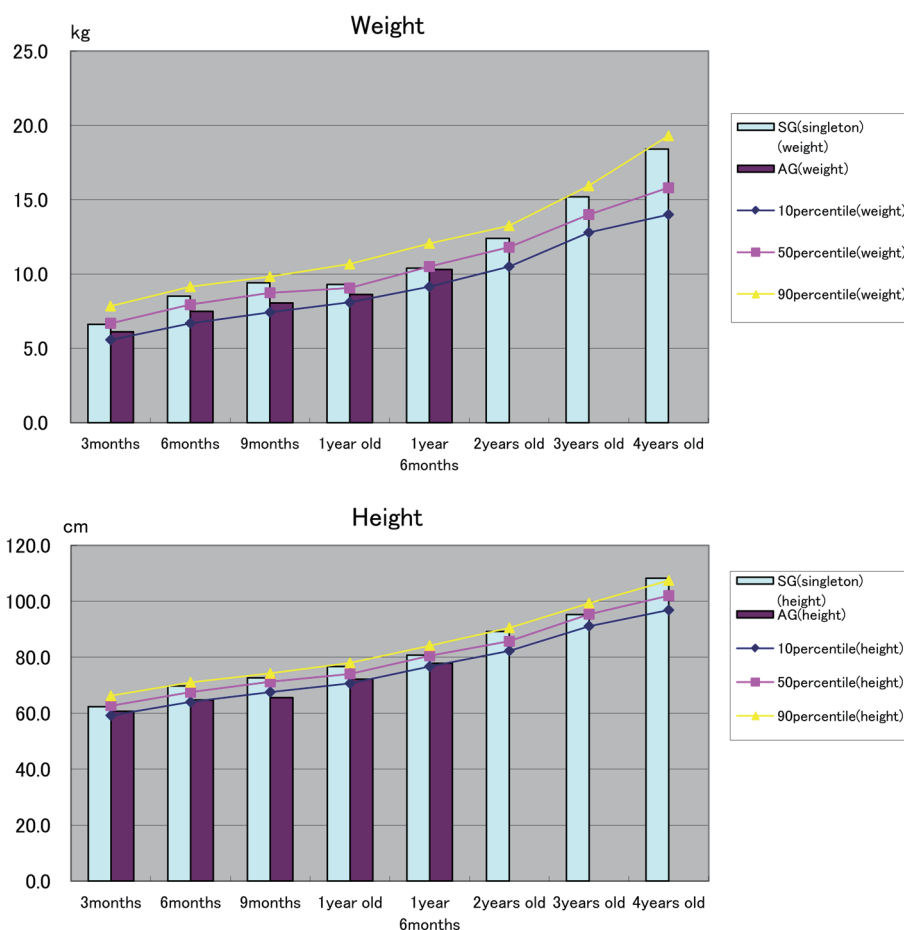


Fig. 1. Weight and Height: growth curve of children born after ICSI without AOA and bar graphs different developmental stages of children born after AOA with SrCl₂ or A23187.

Table 3 Comparison of children’s characteristics at birth (CG, SG, and AG)

outcome	CG*	SG		AG
	Singleton (n=530)	Singleton (n=10)	Twin (n=1)	Singleton (n=10)
obstetric outcome				
gestational age (weeks)	38.6 ± 5.2	39.0 ± 2.2	38.4	38.0 ± 2.5
neonatal outcome				
weight (kg) (mean±SD)	3.0 ± 0.5	2.9 ± 0.6	2.1 ± 0.2	2.8 ± 0.4
height (cm) (mean±SD)	48.3 ± 6.4	48.4 ± 2.7	45.0 ± 2.8	47.4 ± 2.2
head girth (cm) (mean±SD)	32.5 ± 5.9	33.3 ± 2.1	30.0 ± 2.8	33.3 ± 1.2
chest girth (cm) (mean±SD)	31.4 ± 13.7	30.9 ± 2.0	29.8 ± 3.9	30.6 ± 1.8
abnormality rate (%)	3.0 (16/530)	0 (0/10)	0 (0/2)	0 (0/10)

*Singletons born after ICSI without AOA from 1/4/2004 to 31/10/2010.

and safer for individual patients, according to their specific factors.

In this study, we compared fertilization and development of oocytes and pregnancy rates to examine the efficacy of AOA with SrCl₂ and A23187 in 85 PLF (<30%).

We also examined the 22 infants born after AOA following ICSI until 4 yr of age. Studies 1 and 2 indicate that SrCl₂ and A23187 activation are both beneficial for PLF following ICSI. In AG, clinical pregnancy rates and miscarriage rates (AOA vs. without AOA) were 24.3% (9/37)

vs. 0% (0/20) and 22.2% (2/9) vs. 0% (0/0), and in SG they were 27.3% (6/22) vs. 0% (0/15) and 16.7% (1/6) vs. 0% (0/0), respectively. In Study 3, SrCl₂ and A23187 gave similar positive results in the same PLF. Twenty-two children born after ICSI with AOA had no congenital abnormalities at birth, and showed no significant differences in physical development compared to 530 infants born without AOA after ICSI. The development of the six children whose data we received (turning over in bed, sitting up, extending hand to toy, vocal expression, and reacting to television at 6 months; walking with support, mimicking gestures, moving to music, understanding adults' words, and being pleased when meeting others by one year; running, using a spoon, scribbling with a crayon, and using more than one word at two years) were within normal ranges. The impact of SrCl₂ treatment on the chromosomes of gametes has been studied in mice and no side effects were reported [26].

Mansour *et al.* [14] reported 16 healthy babies were born to 12 patients following electrical activation of oocytes after ICSI. Clinical pregnancy rates and miscarriage rates (with AOA vs. without AOA) were 44.1% (15/34) vs. 47.8% (33/69) and 20.0% (3/15) vs. 9.1% (3/33), respectively. Heindryckx *et al.* (2008) reported 21 healthy babies were born to 17 patients following Ca ionophore²⁺ activation (Ionomycin) after ICSI. Clinical pregnancy rates and miscarriage rates (with AOA vs. without AOA) were 32.8% (20/61) vs. 0% (0/50) and 5.0% (1/20) vs. 0% (0/0), respectively. Ca ionophore treatment (A23187 or Ionomycin) might be the easiest and the fastest oocyte activation methods.

In terms of timing, AOA has mostly been performed after previous failure of ICSI treatments. However, AOA methods can also be used as a rescue procedure when fertilization failure is detected within 4 h of ICSI. Because the incidence of PCC (Premature Chromosome Condensation) is elevated at 4 h after ICSI when oocyte activation does not occur, it has been recommended that the fertilization status be evaluated and oocyte activation treatment be carried out on unfertilized oocytes within 4 h [27].

AOA after ICSI has been performed for PLF in case of globozoospermia, severe oligoasthenoteratospermia, nonobstructive azoospermia, and round spermatids; however, further studies are required to establish the clinical tests needed to evaluate the activation potential of semen samples for proper patient selection [24]. We tested two chemical oocyte activators with different activation mechanisms. Calcium ionophore A23187 increases Ca²⁺ permeability of the cell membrane, whereas strontium chloride releases endogenous Ca²⁺. PLC zeta,

the sperm-specific phospholipase C zeta, is now widely considered to be the physiological agent responsible for activating mammalian oocytes. It represents both a novel diagnostic biomarker of oocyte activation capability and a possible mode of treatment for oocyte activation failure patients [25]. However, the efficacy and safety of recombinant PLC zeta are not clear at present.

In conclusion, AOA using A23187 or SrCl₂ is beneficial for patients with low or no fertilization in ICSI. This study showed that AOA does not adversely affect the growth or health of babies. Further studies of clinical tests for proper patient selection, the efficacy and safety of AOA in larger samples of patients, and the ease, safety and efficacy of injection of recombinant PLC zeta protein as a more physiological oocyte activation agent are needed [28–30].

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