J Assist Reprod Genet
DOI 10.1007/s10815-013-0110-2

ASSISTED REPRODUCTION TECHNOLOGIES

Analysis of clinical outcomes with respect to spermatozoan origin after artificial oocyte activation with a calcium ionophore

Hye Jin Yoon · In Hee Bae · Hyoung Jun Kim · Jung Mi Jang · Yong Su Hur · Hae Kwon Kim · San Hyun Yoon · Won Don Lee · Jin Ho Lim

Received: 14 May 2013 / Accepted: 29 September 2013

Abstract
Purpose Fertilization failures have occurred repeatedly in reproductive centers after intracytoplasmic sperm injection (ICSI) and artificial oocyte activation (AOA) has been used to prevent it. This study was performed to investigate whether spermatozoan origin influences clinical outcomes of AOA with a calcium ionophore.
Methods A total of 185 ICSI cycles with a history of no or low fertilization was included in this retrospective study. The outcomes of AOA after ICSI were compared with ejaculated-normal, ejaculated-oligo-astheno-terato or extracted-testicular spermatozoa.
Results There were significant differences between the previous standard ICSI cycles and AOA cycles in the rate of fertilization and clinical outcomes among cases with different sperm origins. Thirty-eight healthy babies (20 singles and 18 twins, 29 cycles) were successfully delivered, and no congenital birth defects were observed.
Conclusions Most patients with a no or low fertilization history obtained an increased fertilization rate and a positive clinical outcome with AOA regardless of the origin of spermatozoa.

Keywords ICSI · Artificial oocyte activation · Fertilization failure · Origin of spermatozoa

Capsule Artificial oocyte activation with a calcium ionophore showed positive outcomes for patients with a history of no or low fertilization regardless of sperm origin.

H. J. Yoon (✉) · I. H. Bae · H. J. Kim · J. M. Jang · Y. S. Hur · W. D. Lee · J. H. Lim
Maria Fertility Hospital, 103-11, Sinseol-Dong, Dongdaemun-Gu Seoul 130-812, Republic of Korea
e-mail: dahamaria@naver.com

S. H. Yoon
Maria Research Center, 103-11, Sinseol-Dong, Dongdaemun-Gu Seoul 130-812, Republic of Korea

H. K. Kim
Department of Biotechnology, College of Natural Science, Seoul Women's University, 621, Hwarang-Lo, Nowon-Gu Seoul 139-794, Republic of Korea

Introduction

Since its introduction, intracytoplasmic sperm injection (ICSI) has been utilized as a means of rescuing various forms of male infertility, including oligo-astheno-teratozoospermia (OAT). The fertilization rate of ICSI is 70–80 %, but complete post-ICSI fertilization failure still occurs in 2–3 % of cases, which remains an unsolved problem in assisted reproductive technologies (ARTs) [1, 2].

Fertilization failure typically results from an insufficient number of mature, normal-morphology oocytes, irregular spermatozoan morphology, or poor motility characteristics. However, some patients with no such sperm defects and normal ovarian responses suffer from repeated fertilization failure [3].

The early steps of normal fertilization, up to sperm-oocyte fusion, can be by-passed through artificial techniques such as ICSI, but these ARTs cannot completely avert fertilization failure [4]. Oocyte activation is a crucial step in successful fertilization following sperm-oocyte fusion, and it induces calcium oscillations that raise the intracellular calcium levels in the oocyte [5]. The initial ooplasm calcium concentration increases within a few minutes of sperm-oocyte fusion, after which a sperm-derived factor (the oscillator) maintains this oocyte calcium level during normal fertilization [6]. Indeed, a lack of oocyte activation can lead to fertilization failure, as oocyte activation is triggered by the interaction between the oocyte and spermatozoa under normal conditions [7].

Published online: 10 October 2013

To rescue cases that encounter problems in oocyte activation immediately following sperm-oocyte fusion, certain artificial activation procedures have been recommended: mechanical activation by a modified ICSI technique [8, 9], electrical activation [10, 11], chemical compounds such as strontium chloride [12, 13], ionomycin [14], and calcium ionophores [15], and sequential treatment with calcium ionophore and puromycin [16].

Calcium ionophore is utilized to induce oocyte activation in many ARTs. Rybouchkin et al. (1997) reported a successful pregnancy and delivery using a combination of calcium chloride and ionophore injections following ICSI with globozoospermia [17]. Murase et al. demonstrated oocyte activation by injection of calcium ionophores and puromycin in a case of repeated post-ICSI fertilization failure [18]. Chi et al. verified that calcium ionophores can elicit effective oocyte activation in an ICSI cycle with a normal amount of sperm from a patient with a history of low fertilization [19]; Pinto and Check confirmed the same outcome for a patient with a history of OAT [20]. Finally, both Stecher et al. and Ahmady and Michael showed that calcium ionophores can be effective in cases of immotile frozen-thawed testicular sperm [21, 22].

Still, concerns have been raised about possible unfavorable effects of calcium ionophores injection and the positive correlation between calcium ionophore treatment and AOA with ICSI remains uncertain. Moreover, the question of whether sperm of different origins can affect outcomes remains controversial.

The aim of this retrospective study was to compare the effect of AOA following ICSI on fertilization rate and clinical outcomes according to the sperm origins for patients who had an experience of complete fertilization failure or low fertilization rate in previous standard ICSI cycles.

Materials and methods

Data was collected from patients who had complete fertilization failure or a low fertilization rate in previous standard ICSI cycles and subsequently had AOA cycles between 2007 and 2011 at Maria Fertility Hospital. A total of 185 ICSI cycles were reviewed, and the clinical outcomes after transcervical embryo transfer were compared depending on different sperm characteristics. We obtained Institutional Review Board approval (2013–003) for the administration of calcium ionophores to our patients.

The cycles were divided into two groups according to fertilization rate in the previous ICSI cycles; complete fertilization failure and low fertilization groups. Low fertilization was defined as less than 50 % fertilization rate after performing standard ICSI in previous cycle. Baseline investigations for the patients in this study noted a body mass index (BMI) of between 19 and 30, day 3 serum level of FSH < 10 IU/L and AMH > 1 ng/ml. Sperm were also classified according to the following origins and characteristics: ejaculated-normal, ejaculated-OAT, and extracted-testicular sperm.

The sperm samples were confirmed to be "normal" according to the following World Health Organization (WHO) criteria: total count ≥15 million, motility ≥50 %, and strict morphology ≥4 %, "OAT" was confirmed: total count <15 million, motility <50 %, and strict morphology ≤3 %. Testicular spermatozoa were retrieved by testicular sperm aspiration (TESA) using 21-gauge butterfly needle under local anesthesia from the male partner who had non-obstructive azoospermia.

All of the patients were submitted to a standard controlled ovarian stimulation (COS) by administration of gonadotropins using either long or short stimulation with agonist or antagonist cycles. Ovulation was triggered by the administration of 10,000 IU human chorionic gonadotropin (HCG) (IVF-C (LG Chemical, South Korea) or Ovidrel (Merckserono, Germany)). Oocyte retrieval was performed 36 h after HCG administration.

ICSI and embryos

Semen was prepared by centrifugation on a density gradient or by the swim-up method, depending on the number of sperm collected after liquefaction. Oocytes were injected by ICSI within 2 h of oocyte denudation under 200x or 400x magnification on a TE 2000 U microscope(Nikon, Japan) equipped with a warming plate (37 °C) and a manipulator (Narishige, Tokyo, Japan) [23]. Each oocyte was positioned with its polar body at the 12 or 6 o'clock position, and subsequently, an injecting pipette containing immobilized spermatozoa was introduced at the 3 o'clock position. The samples were examined 17–19 h after insemination for the presence and number of pronuclei (PN), which indicate fertilization. Over the five-year study period, three different culture media were used: YS [24], Cook media (Brisbane, Australia), and MRC [25]. Three days after oocyte retrieval and insemination, the embryos were transferred in the uterus of the female patient. The remaining embryos were cultured until they reached the blastocyst stage, at which time they were prepared for cryopreservation.

The serum HCG levels were measured 14 days after the oocytes were transplanted back into the uterus, and another 14 days later, clinical pregnancies were confirmed by vaginal ultrasound scans showing the presence of an intrauterine gestational sac. Implantation rates subsequently were determined by calculating the ratio of the number of fetal sacs to the number of embryos transferred.

Artificial oocyte activation

AOA was performed according to a methodology developed by Rybouchkin et al. [17] and Tesarik et al. [6]. Calcium

ionophore A23187 (Sigma Chemical, Sigma, USA) was used for post-ICSI AOA. The final solution, which was 10 μmol/L, was prepared just prior to ICSI. Thirty minutes after ICSI, the oocytes were exposed to the calcium ionophore for 30 min at 37 °C in 6 % CO_2 and 5 % O_2.

Statistics

The statistical analysis was carried out with SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). Quantitative data are presented as the mean ± standard deviation (SD) and properties are used for categorical variables. Mean values were compared by student t- test and proportions were compared by the chi-squared test or Fisher's extracted test. Values of $P<0.05$ were considered to be significantly difference.

Results

Data from a total of 185 cycles of post-ICSI AOA with a calcium ionophore over the course of 5 years were initially considered. For this retrospective study, the following 15 cases were excluded: 13 cases failed transfers (of these 8 cases failed fertilization and 5 cases were arrested embryo development) and 2 individuals who opted to cryopreserve all embryos. The eight fertilization failure cases in AOA cycles also had failed fertilization in the previous standard ICSI cycles. Two out of 5 arrested embryo development in AOA cycles had a complete fertilization and the other 3 AOA cycles also had arrested embryo development in the previous standard ICSI cycle.

A total of 98 patients, who presented with no fertilization in 98 standard ICSI cycles, returned for one more ICSI cycle in which AOA was performed (Table 1). There was a significant increase in the mean female age (36.3 years versus 37.9 years). The number of mature oocytes for ICSI was different between cycles before and after AOA (4.5 versus 8.4, $P<0.001$). However, following AOA, a fertilization rate of 75.7 % was achieved, which resulted in 100 % embryo transfers with a mean number of 3.2 embryos transferred. The implantation rate was 15.4 %, giving rise to a clinical pregnancy rate of 32.7 %.

A total of 72 patients presented with a low fertilization rate of <50 % in 72 standard ICSI cycles and returned for one more ICSI cycle, in which AOA was performed. When the characteristic features of the cycles without activation were compared with AOA cycles, there was a significant increase in the mean female age (34.2 years to 37.0 years, $P<0.01$; Table 1). The number of oocytes retrieved, as well as the number of mature oocytes for ICSI, was different (8.5 and 5.9 versus 13.5 and 9.4, $P<0.01$ and $P<0.001$, respectively). In the cycles with AOA, the fertilization rates were higher (68.2 % versus 34.8 %, $P<0.001$). An increase mean number of embryos were transferred after AOA (3.4 versus 2.3, $P<0.01$). The clinical pregnancy and implantation rates were significantly higher with AOA than without AOA (23.6 % and 5.6 % versus 9.4 % and 1.7 %, respectively, $P<0.05$).

Lost follow-ups occurred in situations where, over the course of the five-year study, patients were transferred to other clinics or hospitals for childbirth after an ultrasound-scan detection of a fetal heartbeat at 10 weeks in this center.

Table 1 Outcomes of artificial activation with calcium ionophore in patients with no- or low-fertilization history

Characteristic	No fertilization		P	Low fertilization		P
	Previous	AOA		Previous	AOA	
No. of cycles	98	98		72	72	
Female age	35.34±4.86	37.93±5.42	< 0.05	34.18±4.33	36.96±4.23	< 0.001
GnRH doses	2400.69±1591.35	2363.33±1277.56	0.871	2368.91±942.52	2575.00±1166.80	0.250
Retrieved oocytes	9.88±8.25	11.52±7.39	0.145	8.50±7.21	13.51±8.54	< 0.001
Mature oocytes per retrieved oocytes	4.48±.4.45	8.37±5.69	< 0.001	5.86±.4.65	9.43±6.87	< 0.001
Fertilized embryos	0	7.68±5.07 (75.7 %)	< 0.001	2.92±2.51 (34.8 %)	8.00±5.45 (68.2 %)	< 0.001
Cleavage embryos on day 2		6.44±4.36		2.58±2.29	6.65±4.48	< 0.001
High-quality embryos on day 3		2.39±2.70		0.24±0.49	2.25±3.78	< 0.001
Transferred embryos		3.18±1.23		2.31±1.79	3.40±1.10	< 0.001
Clinical pregnancy		32.7 % (32/98)		5.6 % (4/72)	23.6 % (17/72)	< 0.05
Implantation		15.4 % (48/312)		1.7 % (4/238)	9.4 % (23/245)	< 0.001
Abortions (n)		6			4	
Delivery cycles (n)		21			8	
Lost follow-up (n)		5			5	

Values are mean ± SD or rate; Clinical pregnancy: presence of G-sac/transferred cycle; Implantation: number of G-sacs/transferred embryos

To ascertain whether the spermatozoa characteristic affects clinical outcomes after ICSI with AOA, the outcomes with respect to the characteristics of sperm used for ICSI were compared (Table 2). These outcomes were evaluated for the following three sperm categories: ejaculated-normal (n=57), ejaculated-OAT (n=69), and extracted-testicular sperm (n=44). In the previous ICSI cycles, complete fertilization failure cycles and low fertilization cycles were 35 (61.4 %) and 22 cycles (38.6 %) of ejaculated-normal group and 31 (44.9 %), 38 cycles (55.1 %) of the ejaculated-OAT group, and 32 (72.7 %) and 12 cycles (27.3 %) of the extracted-testicular sperm group, respectively.

Although the age of patients increased significantly in the three groups, higher fertilization rates were obtained in the AOA groups than those of previous standard ICSI cycles in normal, OAT or Testicular-sperm groups. Additionally, more embryos were transferred in AOA cycles and resulted in higher clinical and implantation rates in AOA cycles compared to previous standard ICSI cycles (Table 2). In the previous ICSI cycles without AOA, the clinical pregnancy rates were 4.5 % (1/22) in the normal group, 7.9 % (3/38) in the OAT group, and 0 % (0/12) in the testicular group. All four clinical pregnancies resulted in abortion in the first trimester after ultrasound detection of an intrauterine gestational sac.

With the AOA treatment, 38 babies (20 singles and 18 twins, 29 cycles) were obtained from embryos produced with the post-ICSI AOA (Table 3). The average numbers of transferred embryos for single and twin births were 3.70±0.92 and 3.22±0.67, respectively. Maternal age (35.70±5.04 vs. 35.56 ±3.47) and gestational age at delivery (37.45±1.49 vs. 34.73 ±2.64) were comparable between single and twin births. The mean birth weights for single and twin babies were 3,210± 464 g and 2,389±437 g, respectively. No congenital birth defects were observed.

Discussion

Our results indicate that AOA with a calcium ionophore is efficient for patients who experienced total fertilization failure or low fertilization in their previous ICSI cycles.

Typically, a complete lack of fertilization occurs in approximately 1.5–10.5 % of IVF cycles [26, 27]. Specific sperm assays such as mouse oocyte activation tests [28] or hyaluronic acid binding assays [29] and sperm DNA fragmentation assays such as TUNEL assays [30] have been employed to overcome total fertilization failure; however, such failures still occur.

Chromatin staining has implicated oocyte activation failure in 70 % of cases of unfertilized oocytes, and half of post-ICSI fertilization failure cases have been attributed to low levels of sperm-derived oocyte activation factor [31].

Table 2 Comparison of clinical outcomes according to origin of spermatozoa

Values	Ejaculated normal		P	Ejaculated OAT		P	Testicular		P
	Previous	AOA		Previous	AOA		Previous	AOA	
No. of initiated cycles	57	57		69	69		44	44	
No. of transferred cycles	22 (38.6 %)	57 (100 %)		38 (55.1 %)	69 (100 %)		12 (27.3 %)	44 (100 %)	
Female age	33.72±4.51	37.39±5.51	< 0.05	34.40±4.02	37.74±4.75	< 0.001	34.11±4.76	37.34±4.63	< 0.05
GnRH doses	2027.58±854.08	2386.61±1071.89	0.048	2718.97±1908.66	2515.29±1171.04	0.454	2361.69±1144.26	2453.21±772.57	0.654
Retrieved oocytes	9.44±8.22	11.93±9.69	0.178	7.33±6.35	12.33±6.65	< 0.001	9.67±6.87	12.98±7.38	0.101
Mature oocytes per retrieved oocytes	5.51±4.46	7.53±7.34	0.098	6.53±4.47	9.09±5.35	< 0.05	3.53±4.41	10.07±5.73	< 0.001
Fertilized embryos	2.95±2.82 (29.9 %)	6.91±5.30 (71.5 %)	< 0.001	2.80±1.77 (38.2 %)	7.90±5.04 (74.6 %)	< 0.001	3.42±3.04 (37.4 %)	8.86±5.31 (76.3 %)	< 0.001
Cleavage embryos on day 2	2.56±2.89	5.75±4.63	< 0.001	2.71±1.77	6.48±3.98	< 0.001	2.37±1.95	7.61±4.59	< 0.001
High-quality embryos on day 3	0.13±0.34	1.88±3.75	< 0.05	0.24±0.48	2.43±2.9	< 0.001	0.32±0.58	2.75±2.82	< 0.001
Transferred embryos	1.83±1.85	3.16±1.33	< 0.001	2.71±1.63	3.33±1.04	< 0.05	2.32±1.92	3.34±1.18	< 0.05
Clinical pregnancy	4.5 % (1/22)	26.3 % (15/57)	< 0.05	7.9 % (3/38)	26.1 % (18/69)	< 0.05	0 %	36.4 % (16/44)	< 0.05
Implantation	1.4 % (1/72)	12.8 % (23/180)	< 0.05	2.5 % (3/122)	11.3 % (26/230)	< 0.05	0 %	15.0 % (22/147)	< 0.05

Values are mean ± SD or rate; Significant difference between groups, p<0.05, Clinical pregnancy: presence of G-sac/transferred cycle; Implantation: number of G-sacs/transferred embryos

Table 3 Comparison of characteristics between single- and twin-children births

Variable	Single	Twin
Delivery cycles (n)	20	9
Female age (years)	35.70±5.04	35.56±3.47
Gestational age (weeks)	37.45±1.49	34.73±2.64
Gender		
Male (n)	11	10
Female (n)	9	8
Birth weight (g)	3210±464	2389±437
> 2,500 (n)	20	10
1,500–2,500 (n)	0	8
Vaginal (n)	14	3
Caesarean (n)	6	6

Values are mean ± SD

Attachment of spermatozoa to the oocyte plasma membrane delivers the first activation signal, triggering a massive influx of Ca^{2+} into the oocyte. Intracellular Ca^{2+} oscillations are triggered by the sperm-specific phospholipase C and PLC zeta (located in the equatorial and post-acrosomal regions of the sperm head in humans), and these oscillations subsequently activate the oocyte [32]. Deficiencies in the ability of sperm to activate oocytes have been traced to an abnormal PLC zeta or to other mutations [33]. It is possible that the sperm selected for ICSI is deficient in some factor that is necessary for the fertilization procedure.

It is generally believed that an oocyte loses its fertilization capacity 24 h after retrieval; however, a recent report documented a live birth that resulted after calcium ionophore activation of one-day-old unfertilized oocytes with sperm from an OAT patient and repeated near-total post-ICSI fertilization failure [34]. In fact, artificial oocyte activation, which works by causing a Ca^{2+}influx into the oocyte regardless of whether a mechanical, electrical or chemical method is used, is thought to be a promising means of improving fertilization rates. Calcium ionophore, for example, is very commonly used for chemical activation of oocytes. Whereas the safety of artificial oocyte activation with calcium ionophores remains controversial, its application to a patient with globozoospermia resulted in a successful pregnancy [35]. Moreover, another case study on globozoospermia associated with a lack of PLC zeta reported that artificial oocyte activation with calcium ionophores successfully overcame repeated fertilization failures [36]. There have been two other favorable oocyte activation results by calcium ionophores: patients with oligozoospermia and OAT [37, 38] who had undergone repeated ICSI cycles, finally achieved fertilization and pregnancy when ICSI was coupled with calcium ionophore-mediated oocyte activation.

However, owing primarily to the relatively small number of cases worldwide, there is still no established gold standard for assisted oocyte activation with a calcium ionophore. Heindryckx [28] reported clinical outcomes for 17 patients with a history of fertilization failure, among whom there were four cases of total fertilization failure in the previous IVF cycle. To the best of our knowledge, the largest study including children born on AOA with calcium ionophores published to date, which involved 89 patients [39], concluded that AOA with calcium ionophores could be efficacious for patients with a fertilization rate that was below 30 % in their previous standard ICSI cycles. However, cases of testis-retrieved spermatozoa were excluded. The present study, by contrast, included patients for whom the spermatozoa was testicular (44 cases), and compared the clinical outcomes.

In the present study, a standard ICSI cycle with complete failed fertilization or less than 50 % fertilization in the previous cycle served as a control in the same patients to determine if AOA improves clinical outcomes. Our results demonstrated that AOA shows a benefit of achieving better clinical outcomes for patients who had low fertilization rate in previous ICSI cycles.

In order to know whether the spermatozoa characteristics affect clinical outcomes differently after AOA, ICSI outcomes with respect to different types of sperm were compared between ICSI cycles before and after AOA. In our study, AOA with a calcium ionophore after ICSI resulted in significant increases in fertilization rate and subsequent clinical outcomes in the three groups (ejaculated-normal; 26.3 %, ejaculated-OAT; 26.1 %, and extracted-testicular; 36.4 %, respectively). These results suggest that AOA after ICSI could help patients who had an experience of complete or low fertilization rate in previous ICSI cycles to increase clinical outcome without depending on sperm characteristics.

Borges [40] et al. (2009) reported that the usage of AOA may improve clinical outcomes in patients who were younger than 36 years old when they used ejaculate or epididymal spermatozoa. However, their study groups were patients undergoing ICSI cycles for the first time without any previous experience of low fertilization and control group was not the same patients. It seemed that, therefore, the effect of AOA after ICSI in the study was not significantly higher for all age groups.

This retrospective long-term study involved 185 ICSI cycles that had experienced complete failure or a low rate of fertilization in the previous standard ICSI cycle. The fact that there were 10 cases of lost follow-up somewhat compromises the overall results; nonetheless, we believe that if the scale of this study is taken into account, the data obtained can be considered valid and valuable. Possible damage resulting from prolonged exposure of the oocytes to calcium ionophore remains a concern, particularly considering that the longest exposure time reported thus far is 20 min [38]. However, it is well known that the calcium oscillations induced by sperm entry can last from 10 to 35 min. Accordingly, in the present study, oocytes were

exposed to a calcium solution for 30 min, which resulted in 38 healthy, defect-free babies. Based on this data, it is reasonable to conclude that the time of oocyte activation could be lengthened up to 30 min.

Although fertilization and clinical outcomes increased significantly after AOA regardless of sperm characteristics, 13 patients experienced fertilization failure even after AOA; in these cases, the average number of retrieved oocytes was six. For another nine patients, less than three oocytes were retrieved, and more than 4 oocytes were retrieved from four patients. The reported rate of post-ICSI fertilization failures, which generally occurs when only one or two oocytes are injected, is approximately 3 % [41]. To ascertain the cause of such fertilization failure, we examined the etiology of infertility. The total failure of fertilization in nine patients (who had less than three retrieved oocytes) represented female factor-mediated infertility and an additional suspected oocyte-related activation deficiency. However, the fertilization failure in four patients (who had more than four oocytes retrieved) is more difficult to explain because the etiology of infertility in all four cases was male factor-mediated infertility. Therefore, we postulated that artificial oocyte activation with calcium ionophores will not be effective for certain patients who have malefactor-mediated infertility, which supports an earlier report that post-ICSI artificial oocyte activation is not always beneficial for patients with a low fertilization rate [42]. Further studies are required to establish a method that can synergize with artificial oocyte activation with calcium ionophores.

In conclusion, artificial oocyte activation with calcium ionophores can have a positive effect on clinical outcomes for patients who experience no or low fertilization in their previous ICSI cycles. Furthermore, the sperm source will not affect the fertility potential or clinical outcomes after this treatment.

Conflict of interest None of the contributing authors (H.J.Y., I.H.B., H.J.K., J.M.J., Y.S.H., H.K.K., S.H.Y., W.D.L., or J.H.L.) has anything to disclose.

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