

Effect of oocyte activation with calcium ionophore on ICSI outcomes in teratospermia: A randomized clinical trial

Maryam Eftekhari¹ M.D., Sima Janati^{1,2} M.D., Mozhgan Rahsepar¹ M.D., Abbas Aflatoonian¹ M.D.

1. Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

2. Department of Obstetrics and Gynecology, Dezfoul University of Medical Sciences, Dezfoul, Iran.

Abstract

Background: Chemical activation is the most frequently used method for artificial oocyte activation (AOA), results in high fertilization rates.

Objective: This prospective, randomized, unblinded clinical study aimed to evaluate the efficiency of calcium ionophore on fertilization and pregnancy rate after intracytoplasmic sperm injection (ICSI) in infertile men suffer from teratospermia.

Materials and Methods: Thirty eight women with teratospermic partner underwent ICSI with GnRH-antagonist protocol. A total of 313 metaphase II (MII) oocytes were randomly divided after ICSI into two groups: In the oocytes of the control group (n=145), routine ICSI was applied. Oocytes in the AOA group (n=168) immediately after ICSI, were cultured in culture medium supplemented with 5 μ M calcium/100 μ M calcium ionophore (A23187) for 5 minutes and then washed at least three times with MOPS solution. In both groups, the fertilization was evaluated 16-18 hours after ICSI.

Results: The number of embryos obtained and fertilized oocytes were significantly different between two groups (p=0.04). There was no significant difference between the two studied groups regarding the fertilization and cleavage rate (95.33% vs. 89.56%; and 84.4% vs. 87.74%; respectively, p=0.06). Implantation rate was higher in AOA group than in control group, but the difference was not significant. (17.64% vs. 7.4%, p=0.14). No significant differences were observed in chemical and clinical pregnancy rate between groups (47.1% vs. 16.7%, p=0.07; and 41.2% vs. 16.7%; p=0.14, respectively).

Conclusion: Oocyte activation with calcium ionophore may improve ICSI outcomes in infertile men suffer from teratospermia. Although, we found no significant difference in the implantation and pregnancy rate between the two groups of patients. Further study with more cases can provide greater value.

Key words: Intracytoplasmic sperm injection, Calcium ionophore, Oocyte activation, Fertilization.

Registration ID in IRCT: IRCT2012112610328N1

This article extracted from fellowship course thesis. (Sima Jannati)

Corresponding Author:

Sima Janati, Research and Clinical Center for Infertility, Bouali Ave., Safaeyeh, Yazd, Iran.

Email: Sjanati@ymail.com

Tel: (+98) 9161407211

Received: 1 May 2013

Accepted: 29 June 2013

Introduction

Infertility influences one in seven couples generally and is a growing problem worldwide (1). Assisted reproductive techniques are currently accountable for up to 7% of childbirths in developed countries (2). Intracytoplasmic sperm injection (ICSI) has been developed as one of the best efficient therapeutic approach for male factor infertility (3). Whilst some crucial stages in normal fertilization are bypassed in ICSI, the average fertilization rate remains at 60-70% (4, 5).

Despite the continuous improvement of ICSI techniques, fertilization failure is a recurrent phenomenon in humans. Based on

initial observations that fertilization failure after ICSI is dependent on several variables including oocyte and sperm quality, as well as, ICSI technique, (4-6). When ICSI is used, total failure of fertilization after ICSI is a rare event, and more than 80% of oocytes contain a sperm (7). The cause of these failures is probably lack of activation of the oocyte, despite the injection of sperm (8-9).

Oocyte activation is one of the first events that occurs at fertilization and characterized by two major molecular events (5, 10): an elevation of intracellular calcium concentration, due to the initial release from the endoplasmic reticulum which occurs 1-3 minutes after binding of the sperm to the

oolemma, and a transient calcium elevation in oocyte as a main trigger of meiotic resumption during fertilization (6).

Many investigators have tried different protocols for artificial oocyte activation to overcome oocyte activation failure after ICSI, including chemical, electrical and mechanical methods (4, 6). Among them, chemical activation is the most commonly used method for artificial oocyte activation (4). Many chemical substances are known to induce an intracellular calcium surge and subsequently activate the oocytes, such as calcium ionophore (A23187), ethanol, ionomycin, puromycin, strontium chloride, probol ester and thimerosal (5-6). Several experiments have revealed that the artificial oocyte activation with calcium ionophore led to an increase in intracellular free calcium, mimicking physiologic mechanisms that cause oocyte activation (11-12). Therefore, this study was design to investigate the effect of artificial oocyte activation with calcium ionophore on fertilization, cleavage and pregnancy rate after ICSI in infertile men suffering from teratoospermia.

Materials and methods

This study was approved by Ethics Committee of Yazd Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Patient Selection

This prospective, randomized, unblinded, clinical trial was performed on 38 women with teratoospermic partner (normal morphology <14%) undergoing to ICSI cycles in Yazd Research and Clinical Center for Infertility, during the period of April to December, 2012. Teratoospermia in men confirmed based on their semen analysis. The patients were randomly allocated into two groups using a computer based randomization list. Written informed consent was obtained from all of the couples prior to the study. The women with aged more than 40, FSH \geq 12 IU/L, immature oocyte, oocyte deformity were eliminated from this study.

Ovarian stimulation and oocyte pickup

Controlled ovarian stimulation was performed by using the long GnRH-agonist down-regulation protocol. All patients received

low-dose oral contraceptive pills (Ocp LD) (30 μ g Ethinyl Estradiol and 0.3 mg Norgestrel, Aburaihan Pharmaceutical Co., Tehran, Iran) starting on 2nd day of the cycle until menstruation occurred. Once menses began, Gonal-F (150-300 IU/day, Serono, Italy) was given starting on 2nd day of the menstrual cycle.

When the down-regulation had been confirmed by serum E₂ levels of <50 pg/mL, Cetorelix (0.25 mg/day, SC, Merck-Serono, Germany) was started. The starting dose of hMG was 150-300 mIU/d, according to the patient's age and body weight. Monitoring was started on day 7 of hMG stimulation, with daily E₂ measurements and vaginal ultrasonography. When at least three follicles reached 14 mm in mean diameter, hCG (10,000 IU, IM, pregnyl, Daropakshsh, Iran) was given. Oocyte pickup was scheduled 36 hours after hCG injection.

Semen processing before ICSI

The semen samples were gathered by masturbation following 3-4 days of abstinence on the day of ovum pick up. Sperm concentration and total sperm count was assessed using a Makler chamber. Sperm motility and morphology analysis was performed under light microscopy according to the Kruger's strict criteria and World Health Organization criteria (13-14). After complete liquefaction, the semen was washed using density gradient sperm-separation techniques, and the final pellet was resuspended in 0.2 mL of medium.

ICSI and calcium ionophore treatment

After retrieval, oocytes were washed with G-MOPS medium (Vitrolife-Sweden) and incubated in culture medium (GIVF-plus; Vitrolife, Sweden) and covered with mineral oil (Ovoil; Vitrolife) for 2 hours at 37°C, 6% CO₂ and 5% O₂. Cumulus cells were removed mechanically by a 30-second exposure to Hyase medium containing 80 IU/mL of hyaluronidase (Vitrolife, Sweden), after which coronal cells were carefully removed. Denuded oocytes were then evaluated for nuclear status. Oocytes showing the release of the first polar body were considered mature and were used for ICSI. Oocytes with single, three, or more pronuclei, immature, malformed, and postmature were eliminated from this research. Eppendorf

micromanipulator mounted on a Nikon inverted microscope was used to perform ICSI. In the oocytes of the control group (n=145), routine ICSI was applied.

Oocytes of the artificial oocyte activation (AOA) group (n=168) were incubated in culture medium containing 5 μ M of calcium ionophore (A23187) (Sigma, St. Louis, MO, USA) for 5 minutes at 37°C and 6% CO₂, the oocytes were then washed with G-MOPS solution and placed in the same culture medium in the incubator under the same conditions. The oocytes were checked 16-18 hr after injection to determine the presence of pronuclei using a Nikon inverted microscope. Activated oocytes were defined with observation of at least one pronucleus or cleaved oocytes. The oocytes were cultured in vitro for 2-3 days to assess their development and cell division.

Evaluation of embryo quality and embryo transfer

Fertilization and embryo quality were assessed by a skilled embryologist. Cleavage stage embryos are graded according to the Hill's criteria(15): Grade A was considered the "optimal" embryo, with no or minimal fragments, equal-sized homogenous blastomeres (4-cell embryo on day 2 or 8- cell embryo on day 3) and light, homogeneous cytoplasm. Grade B: embryos with \leq 10% fragmentation, equal sized homogenous blastomeres. Grade C: embryos with $<$ 50% fragmentation, unequal sized blastomeres, and large granules. Grade D: embryos with $>$ 50% fragmentation, unequal sized blastomeres, and large black granules.

Embryo transfer was performed 48-72 hours after oocyte retrieval. For each couple, from one to three embryos were transferred, depending on the embryo quality and the age of the woman using a Labotect catheter (Labotect, Gotting, Germany). Remaining embryos were frozen in liquid nitrogen using verification method. All the patients received progesterone (100 mg/day, IM Aburaihan Pharmaceutical Co., Tehran, Iran) and estradiol valerate (6 mg/day, Aburaihan Pharmaceutical Co., Tehran, Iran) as luteal support, starting on the day of oocyte retrieval.

Outcome measures

The primary outcome, including fertilization rate, chemical and clinical pregnancy rate

were analyzed. The secondary outcomes, such as cleavage rate and percentage of high-quality embryos were also measured. The percentage of cleaved embryo was calculated as follow: total of cleaved embryos/number of zygotes X 100. Fertilization rate in both groups was calculated by multiplying the ratio of fertilized oocytes in the total number of survived injected metaphase II (MII) oocytes multiplied by 100.

Serum β hCG concentration was measured 2 weeks after embryo transfer. After pregnancy confirmation, progesterone and estradiol valerate were continued until the tenth week of pregnancy. Chemical pregnancy was defined as serum β hCG \geq 25 IU/L measured 2 weeks after embryo transfer. Clinical pregnancy was documented by the presence of a gestational sac with heart beat on vaginal or abdominal ultrasound at 4-5 weeks after embryo transfer. The implantation rate was characterized as the fraction of gestational sac(s) to the number of embryos transferred. Clinical abortion was defined as clinically recognized pregnancy losses before 20 weeks of gestation.

Statistical analysis

The SPSS 19 software package was used to perform all the statistical analyses. The normality of distribution of variables was tested by using the Kolmogorov-Smirnov test. Independent sample T-test was used for continuous variables which were normally distributed and Mann-Whitney U test for data not normally distributed. Chi-squared test or Fisher exact test were used for qualitative variables as appropriate. $p < 0.05$ was considered statistically significant.

Results

The results were reported in accordance with the Consort statement. Of 102 couples candidates for ICSI, 38 patients were enrolled in our study. There was no patient lost to follow-up. In two patients in the AOA group and one patient in control group the embryo transfer were cancelled because of ovarian hyperstimulation syndrome. Although, the women who had to cancel the embryo transfer, were participated in the final analysis. The Consort statement flow diagram is presented in Figure 1. Overall, 38 patients participated in this study.

The patients were divided randomly into two groups as study and control groups (n=19 each). A total of 202 MII oocyte were obtained from the AOA group. Of them, 168 oocytes were selected before ICSI. Moreover, 145 out of 171 MII oocytes were selected in the control group. Basic and demographic characteristics of patients are shown in table I. The demographic parameters were similar in both groups, including male and female age, basal FSH level, type and duration of infertility.

Table II shows descriptive information regarding semen parameters in control and AOA groups. There were no significant differences in sperm count, morphology, total and quick motility between groups. Table III presents a comparison of cycle characteristics between the two groups. As showed, there was no statistically significant difference in the number of oocytes retrieved and mature oocytes between two groups studied. Moreover, we found an insignificant difference in the endometrial thickness and the estradiol concentration in AOA group patients when compared to controls.

The data of embryo scoring in both groups are listed in Table IV. The number and grade of transferred embryos, as well as, the number of frozen embryo in AOA group showed no significant differences when compared to the control group. In the AOA group, 35.3% of embryos were grade C. In contrast, 5.6% of embryos in the Control

group were graded as C, although the difference was not significant (p=0.08). Interestingly, there was a significantly difference in the number of embryos obtained and fertilized oocytes between two groups (p=0.04 in two instances).

Table V shows pregnancy outcome in the two groups. The statistical analysis of the data revealed that there was no difference between the two groups in regard to cleavage rate. However, implantation and fertilization rates were insignificantly higher in AOA group when compared to control group. Whilst, there were no significant differences in chemical, clinical and ongoing pregnancy rates between two groups; there was a trend toward increased in these variables in AOA group. Multiple pregnancies and spontaneous abortion rate were similar in two groups. Patients in AOA group were also subdivided based on sperm morphology in semen samples. So, the clinical pregnancy rate was compared between the subgroups. Our results showed that the clinical pregnancy rate in patients with sperm morphology <4% was 25%; whereas patients with sperm morphology between 4% and 10%, and 11% and 13%, had the clinical pregnancy rate about 37.5% and 60%, respectively. Indeed, oocyte activation in patients with normal sperm morphology between 4 and 13% showed a beneficial effect on pregnancy outcome. It seems that the patients with sperm morphology <4% do not get benefit from oocyte activation.

Table I. Basic and demographic characteristics of patients

Characteristics	Study group (N= 19)	Control group (N= 19)	p-value
Male age (years) (Mean ± SD)	34.63 ± 3.94	35.1 ± 5.98	0.77
Female age (years) (Mean ± SD)	28.82 ± 3.35	29.94 ± 4.51	0.39
Basal FSH (IU/L) (Mean ± SD)	6.06 ± 1.9	6.89 ± 2.36	0.24
Duration of infertility (years) (Mean ± SD)	6.52 ± 3.9	6.39 ± 5	0.92
Infertility kind			0.66
Primary [n (%)]	15 (78.9%)	17 (89.5%)	
Secondary [n (%)]	4 (21.1%)	2 (10.5%)	

Study group: artificial oocyte activation. Student T-test, Mann-Whitney test, Fisher's exact test, and Chi-square test. p-value<0.05 was significant.

Table II. Semen parameters in two groups

Parameter	Study group (N= 19)	Control group (N= 19)	p-value
Sperm count/ml (Mean ± SD)	19.75 ± 15.38	21.73 ± 20.94	0.74
Total motility (Mean ± SD)	15.3 ± 13.89	19.52 ± 15.88	0.39
Quick motility (Mean ± SD)	2.05 ± 3.23	1.31 ± 2.66	0.38
Normal morphology			0.75
Normal morphology (<4%) [n (%)]	4 (21.1%)	5 (26.3%)	
Normal morphology (4-10%) [n (%)]	9 (47.4%)	10 (52.6%)	
Normal morphology (11-13%) [n (%)]	6 (31.6%)	4 (21.1%)	

Study group: artificial oocyte activation. Student t-test and Chi-square test. p-value<0.05 was significant.

Table III. Cycle characteristics

Characteristics	Study group N=19	Control group N=19	p-value
Follicles \geq 16 mm No.	11.57 \pm 2.96	10.73 \pm 3.98	0.46
Oocytes retrieved No.	10.63 \pm 5.26	9.1 \pm 4.12	0.32
Total mature oocyte No.	8.63 \pm 4.63	7.57 \pm 3.48	0.45
Injected oocyte MII No.	6.73 \pm 3.31	5.57 \pm 2.24	0.21
Endometrial thickness on day of hCG injection (mm)	8.42 \pm 0.99	8.86 \pm 1.41	0.27
Peak E ₂ on day of hCG injection (pg/ml)	1441.42 \pm 879.19	1605.94 \pm 688.19	0.52

Data are presented as Mean \pm SD. p-value<0.05 was significant.
Study group: artificial oocyte activation. Mann-Whitney test and student t-test.

Table IV. Embryo data of the study and control group

Characteristics	Study group (N= 19)	Control group (N= 19)	p-value
No. of embryos obtained (Mean \pm SD)	5.80 \pm 3.39	4 \pm 2.16 (*4.5)	0.04
No. of fertilized oocytes (Mean \pm SD)	6.36 \pm 3.02	4.57 \pm 2.14 (*5)	0.04
No. of embryos freeze (Mean \pm SD)	2.22 \pm 3.57	2.22 \pm 2.01	0.2
No. of embryos transferred (Mean \pm SD)	2.58 \pm 0.5	2.44 \pm 0.7	0.49
Grade of transferred embryos			0.08
A [n (%)]	6 (35.3%)	8 (44.4%)	
B [n (%)]	6 (29.4%)	9 (50%)	
C [n (%)]	6 (35.3%)	1 (5.6%)	
D [n (%)]	0 (0%)	0 (0%)	

Study group: artificial oocyte activation. Student t test and Chi-square test. *Median-p<0.05 was significant.

Table V. Pregnancy outcome

Characteristics	Study group (N= 19)	Control group (N= 19)	p-value
Fertilization rate	95.33%	84.4%	0.11
cleavage rate	89.56%	87.74%	0.76
Implantation rate	17.64%	7.4%	0.14
Chemical pregnancy rate/cycle [n (%)]	8 (47.1%)	3 (16.7%)	0.07
Clinical pregnancy rate/cycle [n (%)]	7 (41.2%)	3 (16.7%)	0.14
Ongoing pregnancy rate [n (%)]	6 (85.7%)	3 (100%)	1
Clinical abortion rate [n (%)]	2 (11.8%)	0 (0%)	0.22

Study group: artificial oocyte activation. Chi-square test. P-value<0.05 was significant.

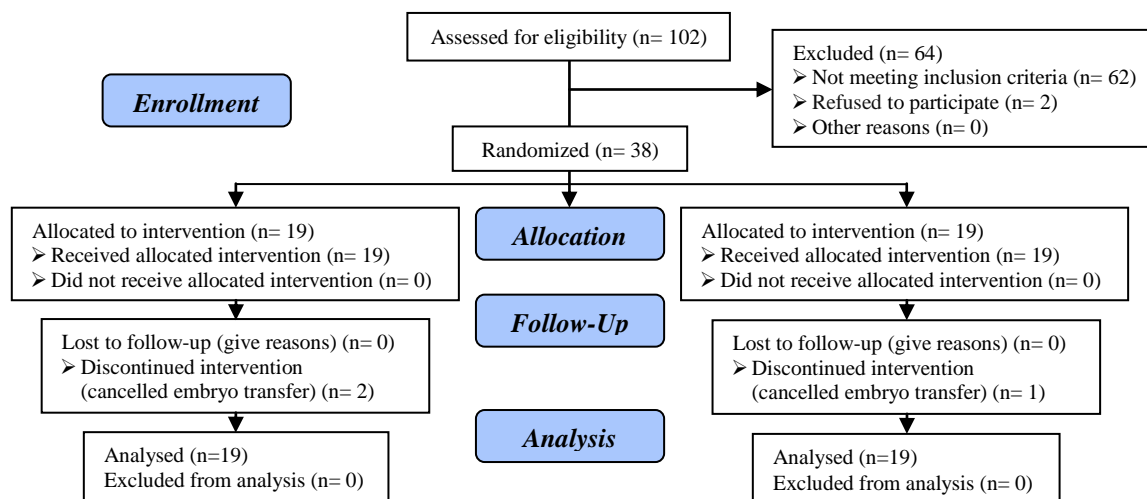


Figure 1. Recruitment follow-up and drop outs over the course of the study.

Discussion

Despite great advances in clinical and laboratory aspects of intra-cytoplasmic sperm injection (ICSI) procedure, fertilization failure still occurs in 2-3% of ICSI cycles (16, 5). The primary reason for failed fertilization after ICSI is lack of oocyte activation (4, 7, 16-18). In

these cases, various mechanical, electrical and chemical methods are known to use for artificial oocyte activation. Among these methods, chemical oocyte activation was currently used with substances (i.e. calcium ionophore). In ICSI cycles, oocyte activation with calcium ionophore has been effective at increasing the fertilization rate (11, 19).

In the present study, 168 selected oocytes were chemically activated with calcium ionophore (A23187) immediately after injection, to increase fertilization rate. Our results showed that there was no significant difference in fertilization and cleavage rate between two groups. Implantation, chemical and clinical pregnancy rate was higher in the AOA group compared with control group; although the difference was not significant. The results of this study are in contrast with the results of Borges and coworkers (11). They reported an improvement in ICSI outcomes following calcium ionophore oocyte activation in azoospermic patients. Although the researchers found that calcium ionophore treatment improved ICSI outcomes only when the epididymal, but not testicular spermatozoa, were injected.

In another study, Nasr-Esfahani *et al* evaluated efficiency of ionomycin on fertilization and cleavage rates, embryo development, and pregnancy rate after ICSI in teratozoospermic patients (8). They reported a significantly higher fertilization and cleavage rates, when oocytes were chemically activated by exposure to 10 μ M ionomycin about 16-18 hours after ICSI. Moreover, in their study, there was no significant difference in cleavage rate and percentage of high-quality embryo around 48 and 72 hours after ICSI between the two groups. The overall pregnancy rate was reported 37% in this study (4).

In a study by Eftekhar *et al*, the researchers analyzed the effect of artificial oocyte activation (AOA) on unfertilized oocytes in ICSI cycles using calcium ionophore (A23187) (5). In their study, fertilization and cleavage rate were 72.5% and 62.7%, respectively. However high quality embryos remained low. According to a study in 2006 by Lu *et al*, exposure of unfertilized oocytes to a combination of calcium ionophore and puromycin could effectively salvage them within 20 h after ICSI (20).

This study reported an improvement in rates of fertilization, fragmentation and of good quality embryo formation. They found that increase exposure time resulted in a decreased in fertilization and cell division rates, suggesting the effects of calcium ionophore exposure time on ICSI outcome. Our study results were in agreement with these studies by Eftekhar *et al* and Lu *et al* (5, 20). In addition, these studies reported a

similar fertilization rate after different exposure time with calcium ionophore demonstrating an optimal time of 24 h after ICSI to expose the oocyte with calcium ionophore.

The rate of cleavage in our research (89.56%) was comparable with the results reported by Nasr-Esfahani (74%); but was higher than the 62.7% reported by Eftekhar *et al* (4, 5). These discrepancies may be due to differences in time-course of oocyte activation. The results of our study indicated a no significant difference in the implantation, chemical and clinical pregnancy rates between two groups; there was a trend toward increasing these variables in AOA group. Although some inconsistency was observed, these findings add to previously reported evidence that artificial oocyte activation using calcium ionophore in ICSI cycles improved the rates of implantation, chemical and clinical pregnancy (5, 11).

The percentage of good quality embryos observed in our research were lower than that of reported by Nasr-Esfahani *et al* (35.3% vs. 70%) (6). It's known that some of the chemical agents can cause intracellular calcium elevation in the oocyte through single or multiple signaling pathways (21). So, there is no doubt that different chemical agent with different duration of exposure can result in various ICSI outcomes after oocyte activation. To stimulate oocyte activation in the present work, we applied calcium ionophore (A23187) for 5 minutes; nevertheless, in the study of Nasr-Esfahani *et al*, the investigators have used Ionomycin for 10 minutes (4). In addition, in Nasr-Esfahani *et al* study, Grade "A" and "B" embryos were considered high-quality embryos; but in our study, only Grade "A" embryos were defined as high-quality embryos (4). Our findings in this study showed a higher fertilization rate than those reported in Eftekhar *et al* study (95.33% vs. 72.5%), which is probably due to difference in the use of ICSI procedure or selected methods to activate oocytes.

Oocyte aging is the most common cause of poor results in IVF/ICSI procedures (22). Therefore, the time interval between oocyte retrieval and fertilization is critical to obtain good quality embryos. Long term culture of oocyte results in zona hardening, increase the rate of parthenogenesis, perturbed embryo development, and poor embryo quality (23). In our investigation, abortion rate in AOA group

was higher than control group; although, this rate was comparable with general population. This finding is in consistent with the prior researches that showed a normal chromosomal status of those embryos derived through artificial oocyte activation (20).

Conclusion

In this study, chemical oocyte activation with calcium ionophore resulted in an insignificant improvement in the implantation and pregnancy rate after ICSI, in men suffering from teratozoospermia. Although, activation of oocyte in this study could significantly increase the rates of fertilization and cleavage. Further study with more patients is therefore suggested to provide greater value.

Acknowledgments

We would like to express our gratitude to the Yazd Research and Clinical Center for Infertility for their great insights and invaluable assistance to realize the present research project. This study was supported by Shahid Sadoughi University of Medical Sciences.

Conflict of interest

The authors declare they have no conflict of interest.

References

1. Ramadan WM, Kashir J, Jones C, Coward K. Oocyte activation and phospholipase C zeta (PLC ζ): diagnostic and therapeutic implications for assisted reproductive technology. *Cell Commun Signal* 2012; 10: 12.
2. Skakkebaek NE, Jørgensen N, Main KM, Meyts ERD, Leffers H, Andersson AM, et al. Is human fecundity declining? *Int J Androl* 2006; 29: 2-11.
3. Devroey P, Van Steirteghem A. A review of ten years experience of ICSI. *Hum Reprod Update* 2004; 10: 19-28.
4. Nasr-Esfahani MH, Razavi S, Javdan Z, Tavalaei M. Artificial oocyte activation in severe teratozoospermia undergoing intracytoplasmic sperm injection. *Fertil Steril* 2008; 90: 2231-2237.
5. Eftekhari M, Mohammadian F, Yousefnejad F, Khani P, Aflatoonian A. Effect of calcium ionophore on unfertilized oocytes after ICSI cycles. *Iran J Reprod Med* 2012; 10: 83-86.
6. Nasr-Esfahani MH, Deemeh MR, Tavalaei M. Artificial oocyte activation and intracytoplasmic sperm injection. *Fertil Steril* 2010; 94: 520-526.
7. Flaherty SP, Payne D, Matthews CD. Fertilization failures and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod* 1998; 13 (Suppl.): 155-164.
8. Nasr-Esfahani MH, Razavi S, Mardani M, Shirazi R, Javanmardi S. Effects of failed oocyte activation and sperm protamine deficiency on fertilization post-ICSI. *Reprod Biomed Online* 2007; 14: 422-429.
9. Sakkas D, Uner F, Bianchi P, Bizzaro D, Wagner I, Jaquenoud N, et al. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. *Hum Reprod* 1996; 11: 837-843.
10. Tesarik J, Mendoza C, Greco E. The activity (calcium oscillator?) responsible for human oocyte activation after injection with round spermatids is associated with spermatid nuclei. *Fertil Steril* 2000; 74: 1245-1247.
11. Borges Jr E, de Almeida Ferreira Braga DP, de Sousa Bonetti TC, Iaconelli Jr A, Franco Jr JG. Artificial oocyte activation with calcium ionophore A23187 in intracytoplasmic sperm injection cycles using surgically retrieved spermatozoa. *Fertil Steril* 2009; 92: 131-136.
12. Wang E, Taylor RW, Pfeiffer DR. Mechanism and specificity of lanthanide series cation transport by ionophores A23187, 4-BrA23187, and ionomycin. *Biophys J* 1998; 75: 1244-1254.
13. Kruger TF, Menkveld R, Stander F, Lombard C, Van der Merwe J, Van Zyl J, et al. Sperm morphologic features as a prognostic factor in in vitro fertilization. *Fertil Steril* 1986; 46: 1118-1123.
14. World Health Organization. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 3rd Ed. Cambridge University Press, Cambridge; 1992.
15. Hill G, Freeman M, Bastias M, Rogers B, Herbert 3rd C, Osteen K, et al. The influence of oocyte maturity and embryo quality on pregnancy rate in a program for in vitro fertilization-embryo transfer. *Fertil Steril* 1989; 52: 801-806.
16. Kasai T, Ogawa K, Mizuno K, Nagai S, Uchida Y, Ohta S, et al. Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. *Asian J Androl* 2002; 4: 97-103.
17. Yamano S, Nakagawa K, Nakasaka H, Aono T. Fertilization failure and oocyte activation. *J Med Invest* 2000; 47: 1-8.
18. Battaglia DE, Koehler JK, Klein NA, Tucker MJ. Failure of oocyte activation after intracytoplasmic sperm injection using round-headed sperm. *Fertil Steril* 1997; 68: 118-122.
19. Mansour R, Fahmy I, Tawab NA, Kamal A, El-Demery Y, Aboulghar M, et al. Electrical activation of oocytes after intracytoplasmic sperm injection: a controlled randomized study. *Fertil Steril* 2009; 91: 133-139.
20. Lu Q, Zhao Y, Gao X, Li Y, Ma S, Mullen S, et al. Combination of calcium ionophore A23187 with puromycin salvages human unfertilized oocytes after ICSI. *Eur J Obstet Gynecol Reprod Biol* 2006; 126: 72-76.
21. Alberio R, Zakhartchenko V, Motlik J, Wolf E. Mammalian oocyte activation: lessons from the sperm and implications for nuclear transfer. *Int J Dev Biol* 2001; 45: 797-809.
22. Miao Y-L, Kikuchi K, Sun Q-Y, Schatten H. Oocyte aging: cellular and molecular changes,

developmental potential and reversal possibility.
Hum Reprod Update 2009; 15: 573-585.
23. Nagy ZP, Rienzi LF, Ubaldi FM, Greco E, Massey

JB, Kort HI. Effect of reduced oocyte aging on the
outcome of rescue intracytoplasmic sperm injection.
Fertil Steril 2006; 85: 901-906.