Artificial oocyte activation in severe teratozoospermia undergoing intracytoplasmic sperm injection

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Objective: To evaluate efficiency of Ionomycin on fertilization and cleavage rates, embryo development, and pregnancy rate after intracytoplasmic sperm injection (ICSI) in teratozoospermic patients.
Design: Laboratory study.
Setting: Isfahan fertility and infertility Center, and Royan institute, Tehran, Iran.
Patient(s): Eighty-seven couples with male factor etiology (severe teratozoospermia) undergoing ICSI.
Intervention(s): Ionomycin for artificial oocyte activation.
Main Outcome Measure(s): After oocyte collection, the oocytes were randomly divided into two groups: control and artificial oocyte activation (AOA). The injected oocytes in the control group were cultured in G1. The remaining oocytes were chemically activated by exposure to 10 μM Ionomycin for 10 minutes. Around 16 to 18 hours after ICSI, fertilization was assessed by the presence of pronuclei. The percentage of cleavage and high-quality embryos were calculated 48 and 72 hours after ICSI. Clinical pregnancy also was determined based on ultrasound observation of a fetal heart beat.
Result(S): There are significant differences in the mean of fertilization and cleavage rates 72 hours after ICSI between AOA and control groups. In patients who had no fertilization in the control group and all the embryos for transfer derived from the AOA group, two pregnancies were recorded. In the patients who had poor fertilization rate (1%–33%) in the control group (14.30%), there was a significant increase in mean fertilization rate (58.31%) because of AOA. The results of the present study also reveal that the samples had motile sperm, whereas in the chemically activated group this difference was not significant.
Conclusion (s): It can be concluded that in cases with severe teratozoospermia AOA may improve fertilization and cleavage rates, which in turn, affect the implantation and pregnancy rate. (Fertil Steril® 2008;90:2231–7. ©2008 by American Society for Reproductive Medicine.)

Key Words: Ionomycin, teratozoospermia, failed fertilization, oocyte activation, ICSI

Intracytoplasmic sperm injection (ICSI) in human reproduction provides possibilities for achieving fertilization even in cases of oligoasthenoteratozoospermia. Even though several critical steps in normal fertilization are bypassed in ICSI, fertilization rate remains within 60% to 70% (1, 2). Although, >80% of these oocytes contain spermatozoon (1), failed fertilization in these oocytes has been attributed to the inability of the sperm to activate the oocyte, or an inability of the oocyte to decondense the sperm (3–5). Many studies have suggested that failure of oocyte activation after aneuploidy is the principal cause of failed fertilization after ICSI (5–8).

During failed fertilization, meiosis-to-mitosis transition is impaired, and thus the fertilization failure is defined as failure of the M–G1 transition in metaphase II (MII) oocytes. The process of M–G1 transition in MII oocytes is called "oocyte activation."

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Activation of the oocyte results in a cascade of events including extrusion of the second polar body, decondensation of a haploid set of chromosomes, formation of a nuclear membrane around the chromosomes, and initiation of embryonic development (9, 10). Oocyte activation is also characterized by two main molecular events including an increase in intracellular Ca^{2+} concentrations followed by meiotic promoting factor inactivation for M–G1 transition (11).

Both sperm and oocyte factors are believed to be involved in failed oocyte activation after ICSI (12). Therefore, in failed fertilized oocytes sperm may have a different status including intact, condensed head, partially to completely decondensed, and premature chromosomal condensation to metaphase arrest (5, 13, 14). Sperm nuclear chromatin decondensation failure has been related to sperm quality, a high rate of DNA fragmentation, and perhaps because of inadequate calcium activation (15, 16). Lopes et al. (2) observed a correlation between the rate of DNA fragmentation and loss of oscilin activity. In addition, failed fertilization has also been attributed to acrosomal defects (17–19). Indeed, round-head spermatozoa (globozoospermia) lacking the acrosomal membrane and acrosin contents (20) not only fails to penetrate oocytes but also if injected into an oocyte fails

to initiate oocyte activation because of deficiency of sperm-associated oocyte-activating factor related with acrosome and perinuclear theca (21–23).

To overcome failed oocyte activation after ICSI or to induce parthenogenetic activation, different measures have been taken including electrical activation (24), chemical activation (25, 26), or modified ICSI (12, 27). Among these procedures, chemical activation is the most commonly used. Literature studies reveal that these activators are used mainly in case reports or used to activate failed fertilized oocytes around 24 hours after ICSI (26, 28, 29). Thus, the aim of this study was to evaluate the efficiency of Ionomycin on fertilization and cleavage rates, embryo development, and pregnancy rate after ICSI in teratozoospermic patients.

MATERIALS AND METHODS

Sperm Preparation and Patient Selection

Eighty-seven couples with male factor etiology (severe teratozoospermia) were initially consulted and selected based on sperm morphology of their previous semen analysis among patients referring to the Isfahan Fertility and Infertilirty Center. This study was initially approved in the ethical and scientific committee of the Isfahan Fertility and Infertility Center and Royan Institute, and there is no conflict of interest in this study.

The semen samples were collected by masturbation after 3–4 days of abstinence on the day of oocyte recovery. In some cases sperm were obtained from testicular sperm extraction (TESE) or by fine needle aspiration (FNA). In semen samples, sperm count was assessed by a Makler chamber, sperm motility was estimated by light microscopy according to the World Health Organization criteria (30), and sperm morphology was assessed by wet preparation during routine semen assessment. During the ICSI procedure, in some cases, semen samples were prepared for routine ICSI using discontinuous PureSperm gradients (80:40) (Nidacon, Gothenburg, Sweden), and in others, because of low sperm motility and density, semen samples were washed three times with G-rinse (Vitolife, Gothenburg, Sweden). In FNA, TESE, and cases with very low sperm count, we were not able to carry out morphologic assessment using Diff Quick staining. Therefore, the result of morphologic assessment using Diff Quick staining was not available for all the cases, so only the results of sperm morphology during the ICSI procedure at 400× magnification were presented, and patient selection was based on morphologic assessment at 400× under an inverted microscope during the ICSI. Cases were included for AOA that had over 98% abnormal sperm and their partners had at least four mature oocytes.

Sperm Morphology Assessment

Sperm were assessed according to Kruger's strict criteria, within limits of 400× magnification of the inverted microscope (31). Sperm were considered normal when the head was normal in size, shape, and acrosome, and lacking midpiece or tail defects.

Intracytoplasmic Sperm Injection

All of the media were purchased from Vitrolife, Gothenburg, Sweden, G3 series plus, unless otherwise stated. After oocyte collection, the oocytes were treated in hyaluronidase (Hyase) in G-MOPS medium. Oocytes were then washed in fresh G-MOPS and transferred to G-oocyte under oil in a Falcon 1006 dish prepared for ICSI. When possible, a PureSperm processed semen sample was introduced into ICSI-100 (a viscous sperm handling solution) in the same dish, or otherwise washed semen samples or washed FNA or TESE samples were introduced into G-oocyte. In each case, sperm were selected according to the best sperm present based on morphology and motility. Eppendorf micromanipulator mounted on a Nikon inverted microscope was used to perform ICSI. The injected oocytes were then washed in G-MOPS and were randomly divided to control and AOA groups. The injected oocytes in the control group were cultured in G1. The remaining oocytes were artificially activated by exposure to 10 μM Ionomycin for 10 minutes (5). Following oocyte activation, the oocytes were thoroughly washed in G1 and cultured in the same medium. Around 16 to 18 hours after ICSI fertilization was assessed by the presence of pronuclei. Oocytes with single, three, or higher pronuclei were very few and excluded from the data. Percentage of fertilization was calculated by multiplying the ratio of fertilized oocytes to the total number of survived injected metaphase II (MII) oocytes multiplied by 100 in two groups. To reduce the oocyte factor, any patients with fewer than four matured MII oocytes that had survived the ICSI procedure were excluded from the present study. Furthermore, immature, deformed, and post-mature oocytes, or any oocyte with certain types of abnormality, were also excluded from this study.

Numeric numbers were used to label dishes in each group. Assessment of fertilization and embryo scoring were performed by experience embryologists who were unaware of the labeling system.

Cleavage Rate and Embryo Quality

Forty-eight and 72 hours after ICSI, embryos were assessed for their cleavage and quality. Embryos with even-sized blastomeres, and $<$10% fragmentations were given a score of A. Embryos with even-sized blastomeres and between 10% and 50% fragmentations were given a score of B. Embryos with uneven-sized blastomeres and /or with $>$50% fragmentations were given a score of C (5). Grade A and B embryos were considered high-quality embryos, and their percentage was calculated. The percentage of cleavage for 48 hours post-ICSI was calculated as follows: sum of cleaved embryos/total number of zygotes × 100. The percentage of cleavage for 72 hours after ICSI was calculated as the number of embryos with greater than six cells over the total number of embryo present 48 hours after ICSI.

TABLE 1

Descriptive information on semen parameters and oocyte distribution in the control and AOA groups.

Mean ± SD	Maximum	Minimum	Parameters
15.09 ± 18.37	80.00	0.1	Concentration ($\times 10^6$/mL)
56.43 ± 87.53	400.00	0.1	Total density
19.56 ± 16.70	80.00	0.00	% Sperm motility
96.02 ± 3.28	100.00	90.00	% Abnormal sperm morphology[a]
30.04 ± 4.39	45.00	19.00	Male age
26.10 ± 4.27	40.00	18.00	Female age
15.94 ± 7.01	35.00	4.00	Total oocyte MII No.
7.61 ± 2.77	17.00	2.00	Oocyte in control group No.
7.73 ± 3.74	18.00	2.00	Oocyte in AOA group No.
1.22 ± 2.36	14.00	0.00	GV oocyte No.
0.75 ± 1.37	7.00	0.00	No. degenerate oocyte
0.24 ± 1.00	6.00	0.00	No. degenerate oocyte 18 hours after ICSI in control group
0.34 ± 0.64	3.00	0.00	No. degenerate oocyte 18 hours after ICSI in AOA group

Note: ICSI = intracytoplasmic sperm injection; AOA = artificial oocyte activation.
[a] Assessed during semen preparation.

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Embryo Transfer, Pregnancy Rate, and Patient Follow-up

During the present study a maximum of four embryos were transferred to each patient on day 3. The number of embryos transferred to each patient was according to age, number of previous failed cycles, and quality of embryos present at the time of embryo transfer. Embryos selection for transfer was based on embryo quality, irrespective of whether embryo(s) were from the control or AOA group. Following positive β-hCG, clinical pregnancy was determined based on ultrasound and observation of a fetal heart beat. Children's health was followed up by questionnaire through calling the patients.

Statistical Analysis

A Student's *t* test was performed to compare mean difference for each parameter between AOA and control groups, using the Statistical Package for the Social Studies (SPSS 11.5; SPS Inc., Chicago, IL) software.

RESULTS

Table 1 provides descriptive information regarding semen parameters and oocyte distribution in the control and oocyte activated groups. Table 2 shows the mean number of oocytes, fertilization rate, percentage of cleavage, and high-quality embryos 48 and 72 hours after ICSI. The statistical analysis of the data reveals that there is no significant difference in mean number of oocytes in the control and AOA groups, while fertilization rate and cleavage rate at 72 hours post-ICSI were significantly different between the two groups. Furthermore, there was no significant difference, in cleavage rate 48 hours after ICSI and the percentage of high-quality embryo 48 and 72 hours after ICSI between the two groups.

To evaluate the beneficial effect of AOA in different patients, patients were further divided according to fertilization rate in the control group. The results of Figure 1 show that in 10 patients no fertilization was achieved in the control group, whereas the AOA group resulted in a mean fertilization rate of 57.78%. In the patients who had poor fertilization rate (1%–33%) in the control group (14.30%), there was a significant increase in mean fertilization rate (58.31%) because of artificial activation, whereas in the patients who had a moderate fertilization rate (34%–65%) in the control group (47%), there was a significant increase in mean fertilization rate (63.4%) because of chemical activation. But unlike the latter groups, there was insignificant reduction in the mean fertilization rate between the patients with good fertilization rate (66%–100%) in the control group (85.83%) compared with the AOA group (77.9%).

Because of sociogeographic status of some couples, data on chemical and clinical pregnancy could only be obtained for 54 out of 87 patients. Twenty-five of 54 were pregnant in this study, resulting in overall pregnancy rate of 37.04%. In the patient who had no fertilization in the control group and all the embryos were transferred from the chemically activated group, two pregnancies were reported, whereas 4, 11, and 8 pregnancies were reported out of 9, 19, and 20 couples, resulting in 44%, 58%, and 40% pregnancy rate in poor, moderate, and good fertilization groups, respectively. Two multiple pregnancies were reported from the latter group. Regarding the health of children born through this procedure,

TABLE 2

Comparison of mean number of oocytes, fertilization rate, cleavage, and embryo quality score 24 and 48 hours after ICSI between AOA and control groups in 87 patients.

P-value	AOA group Mean ± SD	Control group Mean ± SD	Variables
.251	7.24 ± 3.04	7.73 ± 3.74	No. oocytes
.001	66.79 ± 23.57	52.73 ± 31.71	Fertilization rate
.15	95.73 ± 36.16	88.45 ± 30.87	Cleavage rate (48 hours after ICSI)
.001	74.77 ± 36.22	49.95 ± 38.58	Cleavage rate (72 hours after ICSI)
.309	75.12 ± 39.89	69.49 ± 39.94	% High-quality embryo (48 hours after ICSI)
.85	67.99 ± 36.24	67.16 ± 38.43	% High-quality embryo (72 hours after ICSI)

Note: ICSI = intracytoplasmic sperm injection; AOA = artificial oocyte activation.

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in the couples who could be contacted, no particular congenital anomalies were reported.

During this study patients were also divided according to motility status of their semen samples. Fertilization rate was compared between samples with immotile sperm and samples with motile sperm in the control and chemically activated groups. Figure 2 reveals that the sample with the immotile sperm have lower, but insignificant (P=.088), fertilization rate in the control group compared with the samples with motile sperm, whereas in the AOA group this difference was not significant.

DISCUSSION

Low fertilization rate in some of the ICSI cases has been attributed to total or partial deficiency in the sperm capacity to activate oocytes or to the inability of the oocytes to decondense the sperm (3, 4). Capacity of sperm to induce oocyte activation has been related to sperm-associated oocyte activating factor, which is present in the perinuclear theca, in close association with acrosome (7, 26, 32, 33). Sperm with abnormal acrosome or morphology has been shown to have lower fertilization potential in comparison to sperm with normal morphology (17, 34, 35). Previous studies suggest that sperm with abnormal morphology or small acrosome have lower capacity to induce oocyte activation (32, 33, 36).

To overcome failed oocyte activation different protocols have been used for artificial activation or for production of parthenogenic embryos. These protocols include electrical and chemical artificial oocyte activation (8, 25, 26, 37, 38). Clinical applications of these methods to human oocytes are very limited. They are mainly presented as case report studies regarding human oocyte activation in an assisted reproductive technique setting (26, 28, 34). Therefore, during this study the effect of Ionomycin on human oocyte activation was evaluated in ICSI cases with severe teratozoospermia.

FIGURE 1

Shows percentage of fertilization in control group, subdivided according to fertilization rate and their corresponding AOA group and their related pregnancy rate.

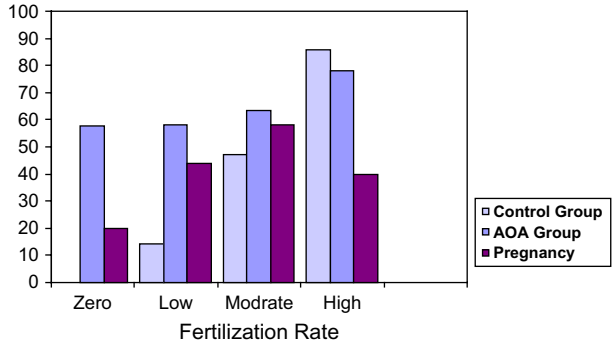

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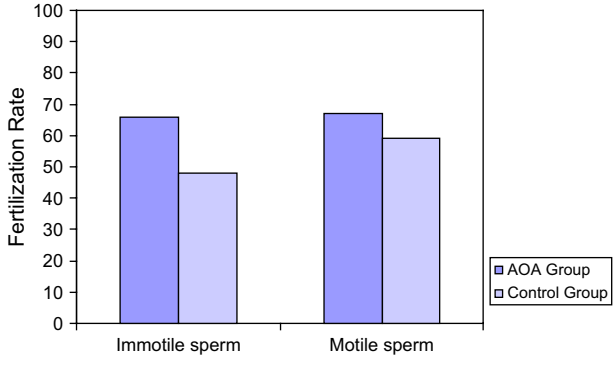
FIGURE 2

Comparison of fertilization rate in the control and chemically activated groups containing immotile and motile sperm.



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The results of this study reveal that fertilization rate has significantly increased in the AOA group compared with the control group. Even though the fertilization rate has significantly increased from 52.73 ± 31.71 to 66.79 ± 23.57, this may reveal that this difference may not be valuable at the clinical level. However, when patients were grouped according to the fertilization rate in the control group to zero, low, moderate, and high fertilization rates, the results show that in 10 patients in whom there was no fertilization, AOA resulted in 57.78% fertilization rate and two pregnancies. Furthermore, this treatment prevented cancellation of the ICSI cycle in these 10 patients. Although the overall pregnancy rate in this study was 37.04%, to evaluate the effect of AOA on ICSI pregnancy outcome further study with a higher number of patients is required. In addition, artificial oocyte activation increases the number of embryos or embryos with a higher cell number available for transfer (Table 2). The fertilization rate obtained with AOA was higher than the activation rate reported in other studies using calcium ionophore A23187 (41%–42%) on fresh oocytes (39, 40). Tesarik and Sousa (23) also showed that 88% of the oocytes that did not fertilize spontaneously 24 hours after ICSI developed 2PN after treatment with calcium ionophore, and 82% of treated oocytes reached the two-cell stage. The result of our previous study on the failed fertilized oocytes using Ionomycin is consistent with the aforementioned report (5).

Furthermore, with AOA, fertilization rate substantially and moderately, but significantly, increased in the low and moderate fertilization groups, respectively (Fig. 1). However, the fertilization rate in the high fertilization group was not significantly different compared with the AOA group. Even though all the patients in this study were selected according to severity of abnormal morphology, the results of Figure 1 reveals that not all the patients may benefit from this artificial activation protocol. Therefore, diagnostic tests, like the mouse oocyte activation test, may be useful to evaluate activation potentials of a semen sample before assisted artificial oocyte activation in clinical settings (41, 42). However, usage of the mouse oocyte activation test may be inconvenient in clinical settings, so other tests such as acrosome-related tests, like the gelationlysis test, may become useful or informative to assess integrity of acrosome, which may be related to sperm oocyte activation capacity (43, 44).

The results of Table 2 reveal that not only the fertilization rate has been improved by artificial oocyte activation, but in addition, cleavage rate 72 hours post-ICSI. Therefore, it can be concluded that in cases with severe teratozoospermia artificial oocyte activation may improve fertilization and cleavage rate 72 hours after ICSI. These results are consistent with the results obtained using different artificial activation protocol for bovine oocytes, which suggests that different activation protocols affect the developmental competence or blastocyst rate (45). Furthermore, addition of protein kinase inhibitor, like 6-DAMP, to activation protocol in bovine oocytes, significantly improves the blastocyst rate (45, 46). Thus, proper activation of oocytes may help the developmental competence of the oocytes and explain the increased cleavage and embryo quality rate observed in the AOA group.

The results of this study also reveal that in the control group, as expected, the fertilization rate is slightly, but insignificantly (P=.088) higher in the semen sample when motile sperm are available for ICSI, compared with cases where motile sperm are not available for ICSI. However, in the artificially activated oocyte subgroup with immotile sperm the fertilization rate improves and is not significantly different from the artificial oocyte activated subgroup with motile sperm, thus concluding that artificial activation has a greater effect on fertilization rate in the semen samples with immotile sperm compared with those with the motile sperm. Nakagawa et al. (25) found that 84.9% of failed fertilized oocytes after ICSI-exposed calcium ionophore A23187 and puromycin were activated, and about 64% were cleaved. They further reported that oocyte activation improves fertilization rate of semen samples with the immotile sperm, which is inconsistent with our results.

In the present study, sperm abnormality was not categorized; however, a recent study by Moaz et al. (47) subgrouped the sperm abnormality to the amorphous head, tapered head, and bent neck, and used a similar chemical activation protocol to this study; their results suggest that fertilization rate postchemical activation increased in the sperm with amorphous and tapered head and not in the subgroup with the bent neck.

Previous studies suggest that sperm morphology and normalcy of nucleus may effect fertilization and it subsequent development (35, 48). Failed fertilization in these cases may be related to failed oocyte activation. In addition, failed fertilization has also been reported in normospermic cases (29). The results of the present study and other studies suggest that failed fertilization can be overcome by artificial oocyte activation, even in failed fertilized oocytes after ICSI, termed "rescued ICSI." In rescued ICSI, the time of oocyte activation may be considered as an important factor on fertilization rate and subsequent development (38).

The results of the present study and other studies also suggest that artificial oocyte activation results in a high cleavage rate, and does not imposed inhibitory effect on subsequent embryo development (26, 28, 37, 38). In addition, this is the first study using Ionomycin on a large number of patients for AOA. The only concern regarding the AOA is the possible adverse affect on the health of babies born through this procedure. The results of the present study and other studies so far reveal that oocyte activation does not adversely affect the health of babies born through this procedure (28, 37, 38, 49). In addition, chromosomal analysis using FISH have shown that embryos derived through artificial oocyte activation have a normal chromosomal number (38).

However, we propose that further studies to evaluate the teratogenic and mutagenic effects should be studied in the animal model. Further, more group studies evaluating the health

of these babies might provide additional information to the future of artificial oocyte activation technology.

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