Can Assessment of Total Acrosin Activity Help Predict Failed or Low Fertilization Rate ICSI for Implementation of Artificial Oocyte Activation?

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Abstract: Artificial oocyte activation (AOA), post intracytoplasmic sperm injection (ICSI) is considered as the only method to overcome failed fertilization post ICSI. However, there is still no prognostic test to foresee failed fertilization and to implement AOA. Therefore, the aim of this study was evaluation of total acrosin activity and its relation to failed fertilization and implementation of AOA post ICSI. 61 ICSI cycles were grouped to control ICSI (n=41), ICSI/ICSI-AOA (n=8) and ICSI-AOA (n=12) according to availability of sperm with normal morphology at time of ICSI. In the ICSI group all the oocytes were inseminated with sperm with normal morphology, while in the ICSI/ICSI-AOA at least 50% and the ICSI-AOA less than 25% were inseminated with sperm with normal morphology. The mean values of sperm parameters, percentage normal acrosome and acrosin activity were assessed for each group and these parameters were significantly higher in the ICSI group compared to the other groups. Half of the oocytes in the ICSI/ ICSI-AOA group underwent AOA (ICSI/ICSI-AOA+) and the other half were used as internal control (ICSI/ICSI-AOA-). Percentage fertilization rate were compared between the four groups and were significantly different between ICSI/ICSI-AOA+ with ICSI/ICSI-AOA- showing that AOA improves fertilization rate in the sibling oocytes. Unlike the treated oocytes (AOA+), a significant correlation was observed between density and percentage motility and acrosin activity with fertilization rate in the untreated oocytes (AOA-). Therefore, the results of this study indicate that evaluation of acrosin activity, along with morphology may help us to select semen samples with possible low oocyte activation potential for implementation AOA.

Keywords: ICSI, oocyte activation, acrosin activity, failed fertilization, sperm morphology.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is considered a powerful technique for treatment of various types of male factor infertility. Although this technique results in high fertilization rate in most ICSI cases, in globozoospermia, severe teratozoospermia, and in certain ICSI cases due to unknown reasons, percentage of fertilization rate remains low. Total and low fertilization rate results are not desirable and may lead to discontinuation of treatment [1-3].

Failed fertilization in an oocyte can have either a sperm or an oocyte etiology. Failure of the sperm to activate the oocyte or an inability of the oocyte to decondense the sperm head has been related to the sperm and to the oocyte, respectively [4]. Failure of sperm decondensation has also been related to sperm quality, high sperm DNA fragmentation and inadequate calcium activation [4-6]. It has been shown that factors involved in calcium activation is located in the perinuclear theca (PT) of sperm between the nuclear envelope (NE) and plasma membrane and it was proved that if the sperm's membrane is damaged, this can result in failed oocyte activation [5]. In addition it has been shown acrosome structure and post acrosome regions play significant roles in sperm nucleuse formation in spermatid stage and oocyte activation. Therefore abnormalities in two regions can result in failed fertilization in infertile men [7-9].

Further studies have revealed the presence of several molecules, localized in the perinuclear theca such as calicin, cylicin I, cylicin II, and acrosin, which have significant roles in the fertilization process [10-13] However, it is now believed that the main factor involved in oocyte activation is phospholipase C zeta (PLCζ). This factor induces interecelluar calcium oscillations and results in oocyte activation. Therefore abnormality in localization of PLCζ may lead to failed fertilization and absence of PLCζ in globozoospermia further accentuates this fact [14, 15].

Also acrosin is one of factors involves in sperm-oocyte penetration and is an acrosomal protease which is present in nonzymogen (active) and zymogen (inactive) forms. The inactive form is termed proacrosin and accounts for approximately 93% of the acrosin activity in human spermatozoa. During fertilization, proacrosin is converted into active acrosin [16]. Chaudhury *et al.* [16] reported that

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1876-827X/10

total acrosin activity may be considered a sensitive prognostic biochemical marker for evaluation of fertilization failure in the clinical evaluation of infertility. Therefore, it is common to observe absence of acrosin activity in globozoospermic individuals.

Considering the fact that failed fertilization post ICSI has been mainly attributed to failed oocyte activation therefore, artificial oocyte activation (AOA) has been proposed to overcome failed oocyte activation. AOA can be induced mechanically, chemically and electrically and it has been shown that these methods may not only lead to higher fertilization rates but also result in higher embryo quality, implantation and pregnancy rates [17-19].

Although AOA is gradually becoming used to overcome failed or low fertilization rate in a clinical setting, however, there are still no prognostic criteria for patient selection for AOA procedure prior to implementation of ICSI. Different authors have used different criteria such as acrosome size, shape of sperm head, status of sperm viability and motility for implementation of AOA [20, 21].

Considering the importance of AOA, the only test to evaluate the activation potential of human sperm is the mouse oocyte activation test (MOAT) [22]. This test has limitations, including requirement for an animal laboratory facility, expertise and its susceptibility to variations such as oocyte quality.

The best test for this purpose might be the assessment of the activity of PLC ζ [15], associated with the equatorial and post acrosome region [23]. Although recently, extensive research has been invested into the identification and evaluation of this protein, there is no such test so far available. Considering the associations between sperm acrosin activity and fertilization and positional association of acrosome and PT with acrosin and PLC ζ [23], therefore, the aim of this study was to evaluate the total acrosin activity test for activation potential of semen sample. In addition, we evaluated the correlation between acrosin activity, with semen parameters and fertilization rate.

MATERIALS AND METHODS

For acrosin activity all the chemicals were obtained from Sigma (Sigma, St. Louis, MO). For ICSI procedure all the media were purchased from Vitrolife G3 series plus. Chemical different from the aforementioned source are state in the text.

Experimental Design

This study received the agreement of the Institutional Review Board of Isfahan Fertility and Infertility Center and Royan Institute, Iran and was performed on 61 couples undergoing ICSI cycles between September 2008 and April 2009. Following the previously set criteria for artificial oocyte activation at the Isfahan Fertility and Infertility Center, the cycles were divided into three experimental groups according to morphologic assessment at 400 × magnifications under an inverted microscope at the time of ICSI according to Moaz *et al.* [24], 1) Control ICSI group; this included 41 couples whose sperm had normal morphology that were readily or effortlessly found during the ICSI procedure, therefore, sperm with normal morphology was used for insemination 2) ICSI/ICSI-AOA group; this included 8 couples with few sperm with normal morphology that could be found during the ICSI procedure to inseminate at least 50% of oocytes, but not all, of the oocytes, and 3) ICSI-AOA group; this included 12 couples with sperm with normal morphology that were hardly available or not found during the ICSI procedure, therefore, less than 25% of oocytes were inseminated by sperm with normal morphology. When sperm with normal morphology was not observed the sperm with closest morphology to normal morphology was chosen for insemination. In the latter group all the oocytes were artificially activated. In the ICSI/ICSI-AOA group, the inseminated oocytes were randomly divided into two groups, the oocyte from one group were left untreated (ICSI/ICSI-AOA-) while the oocytes in the other group were artificially activated (ICSI/ICSI-AOA+). It is important to note that bases of sperm selection for insemination are different from that reported for semen analysis in the Table **1**. In addition, the report in Table **1** is bases on semen analysis while analysis at time of selection is post density gradient centrifugation (DGC). Written informed consent was obtained from all the patients.

Semen Analysis

Semen samples were obtained from patients referred to Isfahan Fertility and Infertility Center. Semen samples were divided into three portions. The first portion was used for routine semen analysis by light microscopy according to World Health Organization (WHO) guidelines for sperm density and motility. Strict criteria were used for evaluation of sperm morphology [25, 26]. The second portion was used for the DGC procedure and the third portion was subjected for evaluation of total acrosin activity. Total acrosin activity was determined spectrophotometrically according to a modified method of Kennedy *et al.* [13].

Sperm Preparation by Density Gradient Centrifugation (DGC)

All procedures were conducted under sterile conditions. 1ml of a lower layer of 80% PureSperm (Pure Sperm, Nidacon, Sweden) gradient was transferred into a conical centrifuge tube, then 1 ml upper layer of 40% PureSperm gradient was gently dispensed onto the top of the lower layer. A liquefied semen sample was placed on top of the upper layer, and the tube was centrifuged for 20 min at 300g, then the pellet was carefully aspirated, washed and resuspended in Ham's F10+10% Human serum albumin.

Ovarian Stimulation and Preparation of Oocytes

A single-stimulation protocol was used for all patients. Briefly, ovarian stimulation was induced using burserelin (Aventis, Germany) from Day 21 of the previous cycle. Human menopausal gonadotrophin (Menogon, Ferring, Germany) in combination with recombinant FSH (Gonal-F, Serono, Switzerland) was administered daily from Day 2 of the cycle.

Ovulation was induced by 10 000 IU hCG (Organon, Holland). Oocytes were retrieved by transvaginal needle-guided ultrasound at 34–36 h post-hCG. After retrieval of oocytes, they were cultured in G-MOPS (VitroLife, Kungsbacka, Sweden) over layered with mineral oil

Table 1. Comparison of the Mean Value of Semen Parameters and Percentage Normal Acrosome in ICSI, ICSI-AOA and ICSI /ICSI-AOA Groups

Parameter	Mean ± SEM ICSI (41)	Mean ± SEM ICSI-AOA (12)	Mean ± SEM ICSI/ICSI-AOA (8)
Density (million/m)	81.91±9.02[ab]	20.53 ±7.81[a]	12.87±5.65[b]
% Sperm motility	50.75±1.67[ab]	20.86±3.53[a]	28.75±4.80[b]
% Abnormal sperm morphology (Strict criteria)	94.65±0.70[a]	98.66±0.68[a]	98.33±0.88
% Normal acrosome	62.59± 2.18[a]	42.11±8.32[a]	53.66±21.45

Common letters are significantly different in row ($P < 0.05$).

(Ovoil™, VitroLife) at 37°C and 6% CO_2. 80 IU/ml hyaluronidase (Irvine Scientific, Santa Ana, USA) was used to denude the oocytes. Oocytes were assessed for their nuclear status. Mature oocytes, with first polar body released, were used for ICSI.

For ICSI procedure, the best sperm based on normal morphology and motility were selected, and injected into the oocytes by Eppendorf micromanipulator mounted on a Nikon inverted microscope. Then oocytes were washed in GMOPS. The inseminated oocytes in the ICSI and ICSI/ICSI-AOA- groups were cultured in , while, the inseminated oocytes in the ICSI-AOA and ICSI/ICSI-AOA+ groups were artificially activated by exposure to 10 μM Ionomycin for 10 minute and then were washed in G1. About 16 to 18 hour after ICSI fertilization was assessed.

Female factors, such as oocyte qualities, were reduced by excluding patients with fewer than four matured MII oocytes that had survived the ICSI procedure. Furthermore, immature, deformed, and post-mature oocytes, or any oocyte with certain types of abnormality, were also excluded from this study.

Assessment of Fertilization, Embryo Quality and Embryo Transfer

The percentage of fertilization in each group were calculated by dividing the number of fertilized oocytes by the number of surviving metaphase II oocytes post ICSI, multiplied by 100. In this study only 5 one pronuclei (1PN) zygotes were observed at 16-18h post ICSI in the AOA groups; these zygotes were considered as unfertilized and were excluded from the results.

The ratio of cleaved embryos to the number of fertilized oocytes was considered as the cleavage rate. Embryo quality was evaluated at Days 2 and 3 post-oocyte retrieval, using a four-point score as described in the literature [27, 28]. Briefly, all cleaved embryos were assigned 1 point, and an additional point was added for each of the following features: absence of fragmentation (or fragmentation involving 25% of embryonic surface), absence of irregularities in blastomere size or shape, 4-cell stage on Day 2 and 8-cell stage on Day 3 . Maximum of 3 embryos were transferred based on embryo quality on the day 3. In ICSI/ICSI-AOA- and ICSI/ICSI-AOA+ group the bases of embryo transfer was also embryo quality irrespective of the AOA. Pregnancy rate was defined from ultrasonography findings showing at least one embryo with a fetal heart beat 5 weeks after transfer. Implantation rate was defined by the number of embryos with fetal heart beats per number of transferred embryos.

Total Acrosin Activity Assay

Two million spermatozoa were obtained from each semen sample, and washed thoroughly with PBS. Detergent buffer containing Triton X-100 (0.01%) and 23 mM N-aben-zoyl- dl-arginine p-nitroanilide hydrochloride (BAPNA), at pH 8.0 was used to allow activation of proacrosin into enzymatically active acrosin. Then the sperm pellet was suspended in 1 ml of detergent buffer for 3 hour at 37 °C. In this procedure, BAPNA is hydrolyzed by acrosin and con-verted to chromophoric product (4-nitroanilin) that was detected (405 nm) on a CE7250 spectrophotometer (CECIL, UK). Total acrosin activity (μIU/10^6 sperm) was calculated according to Kennedy *et al.* [13].

Assessment of Sperm Morphology

Washed semen samples were stained with a modified Papanicolaou technique and sperm morphology was evaluated according to strict criteria. In these criteria, sperm head size, shape, and covering acrosomal area was assessed. Sperm normal morphology characteristics included: length and diameter of sperm head of 4–5 and 2.5–3.5 μm respectively; a smooth oval configuration with a well defined acrosome involving about 40-70% of the sperm head; absence of cytoplasmic droplets of more than half the size of the sperm head; absence of neck, midpiece, or tail defects. Simultaneously, sperm acrosomal size was evaluated according to Menkveld *et al.* [29]. The normal acrosome has a smooth normal oval shape and covers 40-70% of the sperm head. An acrosome covering lower than 30% or higher than 70% of sperm head was considered to be an abnormal acrosome. In each sample, at least 200 cells were assessed [26,29].

Statistical Analysis

The percentage data were modeled by ArcSin transformation and the transformed data were analyzed by one-way ANOVA using SPSS software (SPSS Science, Chicago, IL, USA). Differences between all groups were compared by the Tukey multiple comparison post hoc test.

The correlations were computed by two tailed pearson correlation test of the same software. All data were presented as means ± SEM and differences were considered significant at $p<0.05$.

RESULTS

The mean ages of women included in the study were 31.09 ± 1.18, 30.46±1.08 and 28.42±2.33 in the ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups respectively. The mean ages of men were 35.71±1.18, 35.78±1.20 and 35.14±3.04 in the three groups respectively. The mean ages of men and women were not significantly different between the three groups.

Table **1** provides an analysis of semen parameters (sperm concentration, motility, abnormal morphology) in the three groups in neat semen. The mean of sperm density in the ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups was 81.91 ± 9.02, 20.53 ±7.81 and 12.87±5.65, respectively. Unlike the mean of sperm density between ICSI-AOA and ICSI/ ICSI-AOA groups ($p = 0.950$), this parameter was significantly higher in the ICSI group compared to the ICSI-AOA and ICSI/ ICSI-AOA groups ($P < 0.01$).

In addition, the mean percentage of motility in the ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups was 50.75±1.67, 20.86±3.53and 28.75±4.80 respectively. The percentage of motility was reduced significantly in the ICSI-AOA and ICSI/ ICSI-AOA groups compared to the ICSI group ($P < 0.01$).

The mean percentages of sperm abnormal morphology were 94.65±0.70, 98.66±0.68 and 98.33±0.88 in the ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups respectively. The percentage of abnormal morphology was significantly lower in the ICSI group compared to the ICSI-AOA group (P=0.005). However, significant differences was not observed between the ICSI/ ICSI-AOA group and the ICSI and ICSI-AOA groups ($P>0.05$). It is important to note that values of sperm morphology presented in the Table **1** is according to strict criteria based on pappinocola staining while the criteria for sperm selection was according to Moaz *et al.* [24].

In addition, in this study we compared the mean percentage of sperm having a normal acrosome between the three groups. The mean number of sperm with a normal acrosome was 62.59±2.18, 42.11±8.32 and 53.66±21.45 in the ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups respectively. The percentage of sperm with a normal acrosome was significantly higher in the ICSI group compared with the ICSI-AOA group ($P = 0.008$). However, no significant difference was observed between the ICSI/ ICSI-AOA group and the ICSI and ICSI-AOA groups ($P >0.05$).

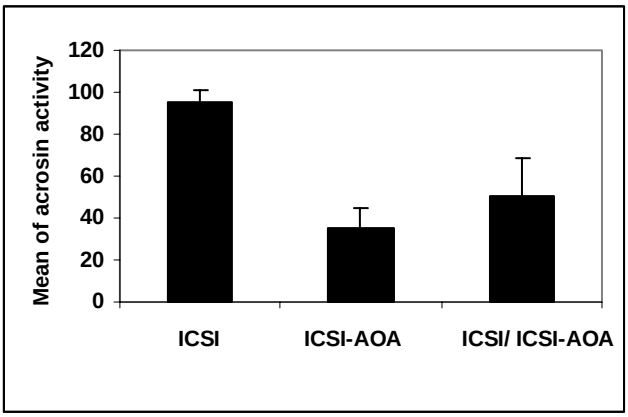


Fig (1). Comparison of the mean values of acrosin activity in ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups. The mean values for acrosin activity in ICSI-AOA and ICSI/ ICSI-AOA groups were significantly different with ICSI group $p<0.01$.

Fig. (**1**) shows comparison of the mean of acrosin activity in the ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups which were 89.13±6.17, 35.25±9.43 and 50.88±17.30 respectively. The mean acrosin activity in the ICSI group was significantly different compared to the ICSI-AOA ($P<0.01$) and ICSI/ ICSI-AOA ($P=0.034$) groups. However, no significant difference was observed between the ICSI-AOA and ICSI/ ICSI-AOA groups ($P=0.714$).

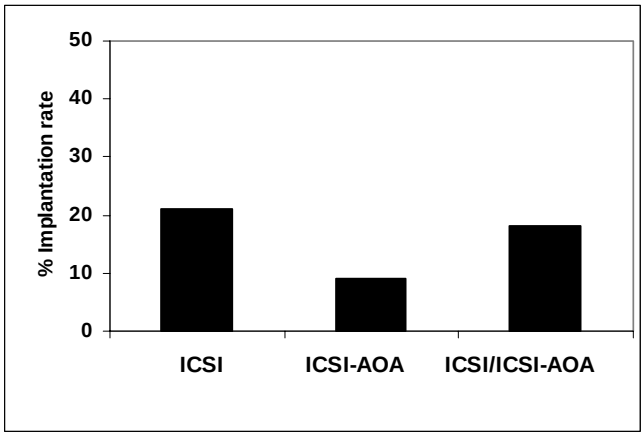
Table **2** show the fertilization rate in the ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups. Since the oocytes in the ICSI/ ICSI-AOA group were further subdivided into two groups, irrespective of type of sperm were used for insemination (normal *vs* abnormal), and one group underwent AOA (ICSI/ICSI-AOA+) while the other group remained untreated (ICSI/ICSI -AOA-); accordingly two fertilization rates are reported for this group. The mean percentage fertilization rate in the former group, that underwent oocyte activation, was 91.75±4.14 (ICSI/ICSI-AOA+), while in the latter group (ICSI/ICSI-AOA-), that were no activated, it was 64.00±9.8, the two values were significantly different from each other ($P=0.046$). The mean percentage fertilization rate between the ICSI/ICSI-AOA+ (91.75± 4.14) and the ICSI-AOA+ (70.14 ±5.6) groups and between the ICSI (76.16± 2.8) and ICSI/ICSI-AOA+ (91.75±4.14) groups were not significantly different at $P=0.099$ and $P=0.241$, respectively. The fertilization rate in the ICSI/ICSI-AOA- was in significantly lower than ICSI group, but it insignificantly improved compared to this group (ICSI) group upon oocyte activation (ICSI/ICSI-AOA+). Comparison of data between the three groups showed no significant difference for cleavage rate and embryo quality score.

Pregnancy was confirmed by ultrasound. In the ICSI group, out of 41 patients, 20 patients had successful

Table 2. Percentage Fertilization in ICSI, ICSI-AOA and ICSI/ ICSI-AOA Groups

ICSI/ ICSI-AOA		ICSI-AOA+	ICSI	Group
ICSI/ICSI-AOA+	ICSI/ICSI-AOA-			
91.75±4.14[a]	64.00±9.8[a]	70.14 ±5.6	76.16±2.8	**%Fertilization rate**

Common letters are significantly different ($P < 0.05$).



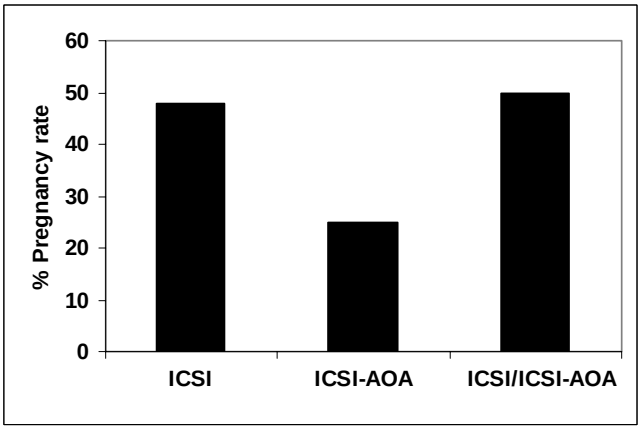


Fig. (2). Pregnancy and implantation rate were compared between ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups. Only, implantation rate was shown a significant difference between ICSI and ICSI-AOA group (p=0.042).

pregnancies (pregnancy rate=48%). Three sets of twins, 16 singletons and one set of triplets were reported. In the ICSI-AOA group, out of 12 patients, 3 patients had successful pregnancies with singletons (pregnancy rate=25%).The embryos derived from the ICSI/ICSI-AOA group, resulted in 4 pregnancies out of 8 patients (pregnancy rate=50%) (Fig. **2**). Comparison of pregnancies between groups showed no significant differences between the groups. Fig. (**2**) show the implantation rate in the ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups. The percentages of implantation were 21%, 9.1% and 18%, respectively. Only the implantation rate in the ICSI group showed a significant difference compared to the ICSI-AOA group (P=0.042).

Table **3** shows the correlations between semen parameters, percentage of normal acrosome and acrosin activity with fertilization rate in the oocytes that underwent AOA (AOA+) or those that did not undergo AOA (AOA-). The results of fertilization in the ICSI/ICSI-AOA+ and ICSI/ICSI-AOA- were added to the latter (AOA+) and former groups (AOA-) respectively. The results showed a significant correlation between density, percentage motility and acrosin activity with fertilization rate in the untreated oocytes or AOA- ($P<0.05$). Conversely, there was no significant correlation between these parameters with fertilization rate of the activated group (AOA+ , $P > 0.05$), except for motility which was close to significance (r=+0.40, P=0.06). In addition, in this study we observed a significant correlation between percentage of normal acrosome and fertilization rate in the oocytes that did not undergo oocyte activation (AOA-, P= 0.04). However, such significant correlations between this parameter with fertilization rate of the oocytes that underwent oocyte activation (AOA+) were not observed ($P > 0.05$).

In this study, a significant correlation were observed between percentage of small acrosome (r=-0.326, P=0.013), percentage of motility (r=0.528, P=0.000) and density (r=0.371, P=0.000) with acrosin activity and insignificant correlation was observed between percentage of abnormal morphology and acrosin activity (r=-0.200, P=0.065) . Not significant correlation was observed between percentage of small acrosome and fertilization rate of the oocytes that underwent oocyte activation (AOA+, r=+0.05, P=0.885). In contrary, in the oocytes that did not undergo oocyte activation (AOA-), a significant correlation between these two parameters were observed (r=-0.267, P=0.056).

DISCUSSION

In assisted reproduction technology (ART), intracytoplasmic sperm injection (ICSI) is the most efficient therapeutic procedure for treatment for male factor infertility. Even by passing barriers such as the acrosome reaction, sperm-zona binding and penetration, sperm-oolemma binding and fusion, a small percentage of oocytes remain unfertilized. Therefore, artificial oocyte activation has been recently introduced in ART to overcome defects in oocyte activation [30-33]. In this study, like our previous study, we showed that the fertilization rate in the ICSI/ICSI-AOA+ group whose half of the sibling oocytes were treated with AOA, was significantly greater than the ICSI/ICSI-AOA- group in which the sibling oocytes were not treated with AOA [21]. This once again suggests that failed oocyte activation may account for one of the underlying causes of failed fertilization. This conclusion is further confirmed by comparison of the fertilization rate in the ICSI-AOA group and ICSI control group. In the ICSI-AOA group, if AOA was not implemented, fertilization rate would have been significantly lower. However, the fertilization rate was not significant due to AOA. Although insignificant, the higher fertilization observed in the ICSI/ICSI-AOA+ compared just to ICSI-AOA is likely due to presence of higher sperm with normal morphology at time of insemination. This is in agreement with literature studies in this field which shows that sperm morphology has a dominant effect of fertilization rate. However, considering the fact that implementation of AOA for ICSI needs further evaluation in term of its safety, it is of paramount importance to develop a prognostic test whereby the sperm's ability to activate the oocyte can be evaluated prior to implementation of AOA in order to avoid possible unknown consequences of AOA.

In the previous study, we suggested that acrosomal anomalies, percentage of sperm showing a halo, or mean halo diameter using a gelatinolysis procedure for assessment acrosin activity, are related to failed fertilization after ICSI [12]. In addition, sperm acrosin activity has been shown to be a good prognostic test for predication of fertilization rate in the IVF procedure [13, 29, 34]. The results of this study also confirmed a significant correlation between sperm acrosin activity with fertilization rate in patients whose

Table 3. The Correlations Between Semen Parameters and Acrosin Activity with Fertilization Rate in the Untreated Oocytes (AOA-) or those that Underwent Artificial Oocyte Activation (AOA+)

Parameters	% Fertilization rate AOA-		%Fertilization rate AOA+	
	r	p-value	r	p-value
Density (million/ml)	0.365	0.004	-0.077	0.733
% Sperm motility	0.426	0.001	0.401	0.064
% Abnormal morphology	-0.203	0.148	0.494	0.123
Acrosin activity	0.334	0.012	0.256	0.305
% Normal acrosome	0.286	0.040	0.256	0.305

oocytes did not undergo AOA. In contrast, as expected, no significant correlation was observed between this parameter and fertilization rate in those oocytes that underwent AOA. This suggested a relationship between acrosin activity and oocyte activation potential.

Previous studies suggest that sperm morphology, especially the percentage of sperm with normal nuclear morphology, affects fertilization and subsequent development. It has been stated that failed fertilization in these cases was mainly related to failed oocyte activation [12, 21, 35, 36]. Therefore, in this study, we initially assessed sperm parameters such as density, motility and morphology in the ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups. Unlike with the percentage of abnormal morphology, sperm density and percentage of motility in the ICSI group were significantly higher than in the other two groups. In addition, the fertilization rate was higher in the ICSI group compared to when AOA was not implemented. This difference was not significant. As expected, according to the patient selection procedure based on sperm morphology assessed at the time of ICSI, the results of this study reveal that, to a certain extent, sperm morphology and percentage of sperm having a normal acrosome, on its own, might be sufficient to predict the necessity to implement AOA. These results are in agreement with previous reports which also suggest that semen samples containing sperm with abnormal acrosome or morphology have lower fertilization potential in comparison to sperm with normal morphology, thus suggesting that sperm with these criteria have a lower capacity to induce oocyte activation [5, 7, 37- 40] and these individuals may be good candidates for AOA. It is of interest to note that we did not observe a correlation between fertilization rate and abnormal sperm morphology assessed by strict criteria when using Papanicolaou staining. This difference may be due to the fact in during the ICSI procedure, one may not observe sperm abnormalities, such as symmetry and asymmetric of sperm, which may be recognizable after staining and such minor abnormalities may not have a profound effect on fertilization outcome. Considering the fact that assessment of sperm morphology is inevitably subject to inter and intra-assay variation, we aimed to assess acrosin activity to evaluate whether this parameter may predict fertilization post ICSI, in order to implement AOA when necessary.

Acrosin activity can be assessed by the gelationlysis test which evaluates non- zymogen form, but in order to assess total acrosin activity, which is more useful, we implemented the spectrophotometric test [13, 16, 41]. The results of this study show that acrosin activity is significantly higher in the ICSI or control groups, compared to the ICSI-AOA and ICSI/ ICSI-AOA groups. Fig. (**1**) shows that acrosin activity in the ICSI-AOA group was less than in the other two groups. Therefore, the results of this study revealed that both normal acrosome morphology and acrosin activity in patients undergoing AOA (ICSI-AOA), or whose half of their oocytes was treated for AOA (ICSI/ ICSI-AOA), were lower than in the control group. These results, along with correlation analysis, suggest that assessment of total acrosin activity, in addition to sperm morphology index, may have a prognostic value for prediction of fertilization rate prior to implementation of AOA. These results are also in agreement with other reports which show that the percentage of acrosome-reacted sperm and released acrosin activity of the teratozoospermic group was the lowest among oligozoospermic, asthenozoospermic and teratozoospermic infertile groups. In addition, previous studies reveal that individuals with teratozospermia show low capacity for zona pellucida induced acrosome reaction and are also defective in sperm-zona pellucida binding [42,43] which may equally account for failed fertilization post IVF in teratozooaspermia.

Acrosin activity and PLCζ are involved in sperm penetration and oocyte activation respectively. These two phenomena are independent and taking place in late stage of spermatogenesis [15, 43, 44]. On the basis of results of this study, there is a significant correlation between sperm acrosin activity and fertilization rate. Therefore semen samples with high acrosin activity have possibly higher chance of inducing oocyte activation and therefore resulting higher fertilization rate. This suggests that acrosin activity might be considered as independent factor for assessment of oocyte activation in human sperm. Since evaluation of PLCζ as a direct method for oocyte activation, is not available, therefore we suggest that evaluation of acrosin activity, as a factor for indirect assessment of oocyte activation, before AOA could be informative.

The mechanism of sperm head formation showed that formation of acrosome and perinuclear theca are meditated by the aid of microtubules, the manchette, which are believed to serve as conveyers of the perinuclear material [45], possibly PLCζ to the perinuclear region at the post acrosome region. Therefore, any defect in this system may lead to sperm abnormality and possibly abnormal

distribution of acrosome and perinuclear theca material, which inevitably lead to failed fertilization and oocyte activation. Indeed, recently Grasa *et al.* [23] showed a significant correlation between the acrosmoal subsidiary patterns of distribution of PLCζ with percentage intact acrosome, in addition, they showed variation in the localization of PLCζ between individuals and within an ejaculate.

Correlation between acrosin activity and semen parameters show a significant strong positive correlation exists between acrosin activity with motility and a weak correlation with sperm density and sperm morphology. Considering a significant correlation between sperm acrosin activity and no correlation between sperm morphology with fertilization, one may suggest that acrosin activity may have a higher value compared to sole sperm morphology for assessment of fertilization potential of a semen sample for ICSI. It is also important to note, despite insignificant relation between sperm morphology and fertilization rate, a significant relation was observed between percentage normal acrosome with fertilization rate, indicating that this parameter is important for sperm selection. But it is important to note the relation between acrosin activity with fertilization was higher that the relation between percentage normal acrosome and fertilization rate.

In addition to improved fertilization rate, it has been reported that proper oocyte activation effects embryo gene expression and post implantation embryo development. In this regard, Edson Borges *et al.* [46] showed that AOA increased the percentage of high-quality embryos and the implantation rate when it was used with ejaculated spermatozoa irrespective of sperm morphology. Therefore, in this study, we also compared cleavage rate, embryo quality score, pregnancy rate and implantation rate between ICSI, ICSI-AOA and ICSI/ ICSI-AOA groups. Pregnancy rate was insignificantly higher in ICSI and ICSI/ ICSI-AOA group compared to ICSI-AOA, however, a significant difference was obtained for implantation rates between the ICSI and ICSI-AOA groups ($p=0.042$). Since the fertilization rate is improved by AOA, the lower pregnancy in the ICSI-AOA group may suggest other factors such as sperm DNA fragmentation and sperm immaturity, which been shown to affect ICSI outcome, may account for this difference [47-51]. These results may confirm the previous report that suggests that AOA may improve the overall outcome of ICSI and may suggest more common use of AOA in the ICSI procedure is warranted; however this needs further scientific consideration [22].

We previously reported that AOA may be used to overcome failed or low fertilization rate. However, it was suggested that the safety of this procedure needs to be better assessed. Therefore, AOA should be used only when no or low fertilization rate is expected. Considering the absence of a pre-screen test for assessment of the activation potential of semen samples for the ICSI procedure the results of this study suggest that assessment of acrosin activity or related tests may help us to select semen samples with possible low oocyte activation potential. Therefore, further studies are recommended to assess the potential of this test as a screen tool.

ACKNOWLEDGEMENTS

The authors express their gratitude to the Royan Institute for its financial support, as well as the staff of Isfahan Fertility and Infertility Center for their kind collaboration.

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Received: March 13, 2010 Revised: April 15, 2010 Accepted: August 06, 2010