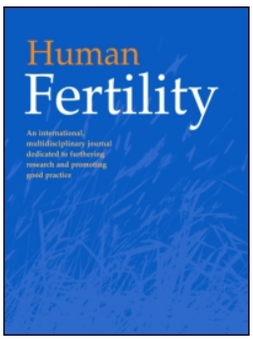

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ORIGINAL ARTICLE

Artificial oocyte activation after intracytoplasmic morphologically selected sperm injection: A prospective randomized sibling oocyte study

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ABSTRACT

This study aimed to evaluate the effect of artificial oocyte activation (AOA) by calcium ionophore after intracytoplasmic morphologically selected sperm injection (IMSI) on fertilization, cleavage rate and embryo quality. A total of 194 oocytes from 21 cycles from women with a history of low fertilization rate accompanying teratozoospermia were enrolled over a 3-month period. Mature oocytes from each patient were randomly allocated into two groups after IMSI. In the study group, half of the patients' oocytes ($n = 97$) were exposed to AOA, and in the control group ($n = 97$), AOA was not applied. The mean number of mature oocytes, fertilization and cleavage rates were similar between the study and control groups ($p > 0.05$ for each). However, fertilized oocytes of the AOA group were less likely to produce top quality embryos when calculated per fertilized oocyte (28/80; 35.0% versus 38/71; 53.5%, respectively; $p = 0.024$) and also per cycle (13/21; 61.9% versus 20/21; 95.24%, respectively; $p = 0.006$). Our study indicates that AOA may not improve fertilization rates after IMSI and may even reduce the ability of a successfully fertilized oocyte to develop into a top quality embryo. AOA should, therefore, be applied to cases with a defined oocyte activating deficiency.

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KEYWORDS
Oocyte; fertilization; intracytoplasmic sperm injection (ICSI)

Introduction

Since the introduction of intracytoplasmic sperm injection (ICSI) into assisted reproductive technologies (ARTs), there have been numerous studies on the effects of the male gamete regarding its morphology, quality and capacity to activate the oocyte for successful fertilization (Berkovitz et al., 2006; Moaz, Khattab, Foutouh, & Mohsen, 2006; Nasr-Esfahani, Razavi, Mardani, Shirazi, & Javanmardi, 2007). Since complete fertilization failures and low or moderate fertilization rates are still common in ART practice, sperm selection and oocyte activation are points of interest for improving clinical outcomes in male factor infertility (De Vos et al., 2003; Dozortsev & Nasr-Esfahani, 2012; Ebner et al., 2012).

Intracytoplasmic injection of spermatozoa selected according to fine morphology under high magnification (intracytoplasmic morphologically selected sperm injection, IMSI) is used to identify a morphologically normal spermatozoon by precise measurement of length and width of the sperm head and vacuoles. This technique has been used to improve assisted reproduction outcomes in cases of male factor infertility, in terms of improved fertilization rates and ART outcome (Antinori et al., 2008; Balaban et al., 2011; Berkovitz et al., 2006; De Vos et al., 2013).

The oocyte activating ability of the spermatozoon is a critical factor for fertilization. During natural fertilization, sperm-oocyte fusion is activated by introducing a de-membraned, "naked" sperm nucleus, which immediately becomes accessible to ooplasmic factors, but, following ICSI a whole sperm cell with an intact membrane is introduced into the ooplasm (Dozortsev & Nasr-Esfahani, 2012; Tesarik, Sousa, & Testart, 1994). Fertilization problems after ICSI are, therefore, related to the inability of a spermatozoon to release oocyte activation enzymes (Nasr-Esfahani et al., 2007). More than 80% of unfertilized oocytes are thought to contain an intact spermatozoon (Flaherty, Payne, & Matthews, 1998), and artificial oocyte activation (AOA) is considered useful in selected cases with deficiencies of sperm-associated oocyte activating factors related to the acrosome and perinuclear theca (Escalier, 1990).

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Currently, AOA by calcium ionophore (A23187) is the most commonly used technique to overcome failed oocyte activation after ICSI (Eldar-Geva et al., 2003). AOA is a carboxylic acid ionophore that transfers calcium and magnesium from an aqueous medium through the cell membrane into a cell (Reed & Lardy, 1972). Influx of calcium results in an increase in the concentration of calcium in the cytoplasm which is considered to act as a mitogenic signal to promote DNA synthesis and cell division (Luckasen, White, & Kersey, 1974). Many studies have demonstrated that AOA by calcium ionophore may increase free intracellular calcium and trigger oocyte activation (Heindryckx, Van der Elst, De Sutter, & Dhont, 2005) and it is considered a safe application (Vanden Meerschaut et al., 2014).

IMSI is an emerging assisted fertilization procedure which has become a preferred choice for the treatment of male factor infertility worldwide. Current literature, however, lacks robust evidence on whether AOA is effective when IMSI is used for assisted fertilization. We have, therefore, aimed to investigate the effects of AOA on embryological parameters, namely fertilization, cleavage rates and embryo quality in IMSI cycles. This study was designed as a randomized sibling split study, where each patient served as her own control, in order to eliminate the possible randomization disadvantages of inter-patient variations.

Materials and methods

Patients

This prospective, randomized and single blind study was conducted on infertile couples undergoing treatment at the IVF Clinic, Memorial Hospital, Antalya, Turkey, for a 3-month period between December 2013 and February 2014. The inclusion criteria comprised a diagnosis of teratozoospermia (<4% according to WHO 2010 reference limits) and a low fertilization rate (<50%) in the previous cycle. Complete fertilization failure was not observed in the previous cycles. Couples with a chromosomal abnormality in either partner and women with a poor ovarian reserve according to Bologna Criteria, 2011, were excluded (Ferraretti et al., 2011). In addition, cycles with <2 mature oocytes were excluded because splitting was not possible. The selected patients were carefully informed of the objectives and procedures of the study and signed a consent form. Only mature metaphase II oocytes were injected by IMSI. After injection, the oocytes of each patient were randomized to either AOA by calcium ionophore or the control group. Randomization was computer-generated, and the allocation list stored in a locked room by third parties not otherwise involved in the trial. The clinical and laboratory staff were blind to the intervention throughout the early embryo developmental period. The research project was approved by the regional ethics and research committee, under approval number 2013007.

Ovarian stimulation and oocyte pick-up

A GnRH agonist or an antagonist treatment was chosen for controlled ovarian stimulation according to the patient characteristics. Recombinant FSH regimens (GONAL-f, Merck Serono, Geneva, Switzerland) were used in all cases. hCG (Ovitrelle, Merck Serono, Geneva, Switzerland) was administered subcutaneously when the dominant follicle had reached a mean diameter of ≥18 mm.

Oocyte pick-up was performed 36 h after hCG administration, under general anaesthesia, with a 17 G dual lumen needle (Wallace, Smiths Medical, Sydney, New South Wales, Australia) through transvaginal ultrasound guidance.

Retrieved oocytes were initially placed in a MOPS-buffered medium (G-MOPS, Vitrolife, Gothenburg, Sweden) under oil (Ovoil, Vitrolife, Gothenburg, Sweden), supplemented with 5% (w/v) human serum albumin (HSA, Vitrolife, Gothenburg, Sweden) at 37 °C. After reduction of the excess cumulus oophorus with the help of two fine needles, oocytes were transferred to fertilization medium (G-IVF, Vitrolife, Gothenburg, Sweden) under oil (Ovoil, Vitrolife, Gothenburg, Sweden) supplemented with 10% (w/v) human serum albumin (HSA) and placed in a CO_2–O_2 incubator (HERAcell, Thermo Scientific, Waltham, MA) for 2 h until hyaluronidase treatment. All consequent incubation steps were handled in a CO_2–O_2 incubator under oil (Ovoil, Vitrolife, Gothenburg, Sweden).

Sperm evaluation and preparation

Semen samples were freshly collected on the day of oocyte retrieval. After allowing liquefaction at 37 °C, an initial evaluation was performed. Sperm concentration, motility, progression and morphology were evaluated according to the World Health Organization 2010 guidelines. Samples were prepared for IMSI by swim up from a washed pellet (Sperm Rinse, Vitrolife, Gothenburg, Sweden) technique.

A second evaluation after preparation was performed and recorded. Final sperm suspensions were diluted to 0.1×10^6 progressive motile spermatozoa per ml, and placed into micro droplets on Petri dishes

prepared for IMSI (WillCo-Dish, WillCo Wells, Amsterdam, Netherlands).

IMSI procedure

All laboratory procedures were carried out on Nunc IVF Product Line (Roskilde, Denmark) plastic-ware except IMSI (WillCo-Dish, Amsterdam, Netherlands). Oocyte denudation was performed in HEPES-buffered medium (G-Gamete, Vitrolife, Gothenburg, Sweden) 2 h after oocyte retrieval by using 40 IU enzymatic digestion by hyaluronic acid (HYASE, Vitrolife, Gothenburg, Sweden), followed by gentle pipetting.

IMSI was performed in HEPES-buffered medium (G-Gamete, Vitrolife, Gothenburg, Sweden). PVP was used for sperm selection and immobilization (ICSI, Vitrolife, Gothenburg, Sweden). For all oocytes, a single, motile spermatozoon exhibiting normal morphology according to the oval shape of the nucleus, length and width of the head and nuclear vacuoles was identified and selected in a glass bottom Petri dish (WillCo-Dish, Amsterdam, Netherlands), under an inverted microscope at 600 × original and 6000 × enhanced digital magnification (DMI3000 B, Leica, Wetzlar, Germany), equipped with an Integra TI micromanipulation set (Research Instruments, Cornwall, UK) and IMSI digital optics (Research Instruments, Cornwall, UK). Sperm injection was performed after aggressive sperm immobilization as previously described (Palermo et al., 1996).

Oocyte activation by calcium ionophore

Half of the injected oocytes from each patient were incubated for 15 min in the 30 µl of pre-equilibrated drops of medium containing Ca^{2+}-ionophore (GM508 Cult-Active, Gynemed, Lensahn, Germany), immediately after IMSI according to the instruction manual. After a series of rinsing steps, oocytes were transferred to the micro droplets of fresh fertilization medium (G-IVF), supplemented with 5% (w/v) human serum albumin (HSA). The remaining half of the oocytes of a given patient were directly transferred to fertilization medium (G-IVF), supplemented with 5% (w/v) human serum albumin (HSA) to serve as control.

Embryo culture and evaluation

All embryos were cultured to the cleavage stage (3 d) in a 37 °C incubator (HERAcell, Thermo Scientific, Waltham, MA) with 6% CO_2, 5% O_2, 89% N_2 and humidified atmosphere. Sibling oocytes of the same patient were cultured in the same compartment of the incubator.

Fertilization was evaluated blindly by two embryologists 16–18 h after IMSI. Two clearly visible pronuclei were considered evidence of normal fertilization. Fertilization rates were calculated as the number of normally fertilized oocytes/the number of metaphase II oocytes injected ×100. Fertilized oocytes were transferred to the pre-equilibrated micro droplets of fresh cleavage medium (G1) supplemented with 5% (w/v) human serum albumin (HSA) under oil and placed in a CO_2–O_2 incubator for embryo culture.

On day 3, a second evaluation was performed by the same embryologists to assess embryo quality. Cleavage rates were calculated as the number of cleaved embryos on day 3/the number of normally fertilized oocytes ×100. Embryo grading was classified according to the ASEBIR consensus scheme (ASEBIR, 2008). Embryos with less than 10% fragmentation and consisting of 7–8 even-sized blastomeres on day 3 without any vacuolization, granulation and multinucleation were considered grade A (top quality). The percentage of top quality embryos on day 3 was calculated as the number of top quality embryos on day 3/the number of normally fertilized oocytes ×100, and the number of cases with at least one top quality embryo was calculated as the percentage of cases holding at least one top quality embryo on day 3.

Statistical analysis

We calculated that an appropriate sample size for statistical analysis would be 42 patients, with 21 patients in each group, with the type I probability of error being fixed at 5% and the type II error 20%. This sample size was able to detect if AOA could change fertilization rates, assuming an expected 10% difference between the groups.

All data were extracted from the patients' electronic medical records. The statistical analysis was done with SPSS 22.0 software for windows (IBM Software, New York, NY). For independent samples, the Mann–Whitney *U* test was used for analyses involving continuous variables, including difference in fertilization rates, the number of Grade A (top quality) embryos on day 3. The χ^2 (or Fisher exact test where appropriate) was used for analyses involving categorical variables. A p value <0.05 was selected as the level of statistical significance.

Results

During the study period, 21 couples meeting the inclusion criteria underwent 21 IMSI cycles. The mean age at treatment was 30.2 years (range 23–35). All the

patients had a history of previously unsuccessful IVF-ET attempts (mean 1.47 ± 0.68). The numbers of oocytes retrieved and MII oocytes were 13.0 ± 6.4 and 9.2 ± 4.7, respectively.

Ninety-seven oocytes were injected in both groups and the mean number of AOA-treated and non-treated metaphase II oocytes was 4.6 ± 2.4 and 4.6 ± 2.3, respectively. The number of oocytes fertilized was comparable between the groups ($p > 0.05$). The total number of cleaved embryos on day 3 was similar between the AOA-treated and non-treated groups ($p > 0.05$) (Table 1).

The number of top quality embryos produced on day 3 was 28 (38.4%) in the AOA-treated group and 38 (58.5%) in the non-treated group. The difference was statistically significant ($p = 0.024$). Consequently, in the AOA-treated oocyte cohort, the number of cases with at least one top quality embryo was statistically significantly lower than that in the control group (13/21; 61.9% versus 20/21; 95.24%, respectively; $p = 0.006$) (Table 1).

There were no significant differences in pregnancy, ongoing pregnancy and implantation rates between study and control groups, when single-embryo transfer cycles were compared ($p > 0.05$ for all). The outcomes of double-embryo transfer (DET) cycles were not calculated because tracing the implanted embryo back to study or control groups was not possible.

Discussion

Numerous studies have been performed to evaluate the efficacy of AOA after standard ICSI (Borges, de Almeida Ferreira Braga, de Sousa Bonetti, Iaconelli, & Franco, 2009; De Vos et al., 2003; Hsu et al., 1999; Moaz et al., 2006; Nasr-Esfahani, Razavi, Javdan, & Tavalaee, 2008) but the effects of AOA on fertilization rates have not been studied following IMSI in a sibling oocyte design within the same cycle. The present findings do not reveal an improved effect of AOA on fertilization rates. Similarly, cleavage rates and number of cleaved embryos were found to be comparable between the groups. Our results are in contrast with a previous study demonstrating improved fertilization rates by AOA compared with IMSI alone (Yoon et al., 2014), but the former study compared two different cycles of the same couple. Our study was designed to include sibling oocyte by splitting into study and control groups, in order to eliminate the possible randomization problems of inter-cycle variations. Other studies have reported that the use of AOA with calcium ionophore in patients with a history of fertilization failure is a safe and reliable tool in cryptozoospermic cases (Ebner et al., 2012; Nasr-Esfahani, Deemeh, & Tavalaee, 2010). However, these studies also compared different cycles and thus the possible disadvantage of inter-cycle variations within the patient.

IMSI was introduced as a valuable tool to identify and select a spermatozoon with a high fertilizing ability (Yoon et al., 2014). AOA is considered to be useful in selected patients who have low fertilization potential (Sermondade et al., 2011; Taylor et al., 2010) and a beneficial effect of AOA was implied for patients with a compromised fertilization due to globozoospermia

Table 1. Comparison of IMSI treatment outcomes between AOA and control groups.

		AOA group	Control group	*p* Value
Total number of MII oocytes		97	97	
MII oocytes per patient		4.6 ± 2.4	4.6 ± 2.3	0.929
Total number of fertilized oocytes		80/97 (82.5)	71/97 (73.2)	0.125
Fertilized oocytes per patient		3.8 ± 2.5	3.4 ± 1.8	0.817
Total number of cleaved embryos		73/80 (91.3)	65/71 (91.5)	0.999
Embryos per patient		3.5 ± 2.4	3.1 ± 1.6	0.938
Total number of top quality embryos		28/80 (35.0)	38/71 (53.5)	**0.024**
Top quality embryos per patient		1.3 ± 1.5	1.8 ± 1.2	0.112
Number of cases with ≥1 top quality embryo		13/21 (61.9)	20/21 (95.2)	**0.006**
Number of transferred embryos	in DET	4	6	
	SET	6	10	0.170
Pregnancy outcome[a]				
Implantation		2 (33.3)	4 (40.0)	0.608
Biochemical pregnancy		0 (0.0)	0 (0.0)	–
Clinical pregnancy		2 (33.3)	4 (40.0)	0.608
Spontaneous abortion		0 (0.0)	1 (10.0)	0.625
Ongoing pregnancy[b]		2 (33.3)	3 (30.0)	0.654

IMSI: intracytoplasmic morphologically selected sperm injection; AOA: artificial oocyte activation; MII: metaphase 2; SET: single embryo transfer; DET: double embryo transfer. Mann–Whitney *U* test or Pearson Chi-squared test was used for the analysis. Data are mean ± SD or *n* (%) unless otherwise specified. Boldface data indicates statistical significance ($p < 0.05$).
[a]Data for pregnancy outcomes are expressed per embryo transfer and only for SET.
[b]Defined as progression beyond 12 weeks of gestation.

(Dirican, Isik, Vicdan, Sozen, & Suludere, 2008; Ebner et al., 2012; Montag, Köster, van der Ven, Bohlen, & van der Ven, 2012; Nasr-Esfahani et al., 2010). Consistently, high fertilization rates were obtained in our study with IMSI in both AOA-treated and non-treated oocytes, and AOA did not seem to confer any beneficial effect on improving fertilization rates.

No adverse effects of calcium ionophore on *in vitro* or *in vivo* embryo development have previously been noted (Ebner & Montag, 2016; Heytens et al., 2008; Kyono, Takisawa, Nakajo, Doshida, & Toya, 2012; Vanden Meerschaut et al., 2012). However, the safety and efficacy of AOA has recently been questioned. It is considered to affect cell homeostasis by mediating export of potassium through the plasma membrane which results in consecutive acidification of the cytosol (Managò et al., 2015) and may have long-term effects on gene expression and epigenetic modifications (Santella & Dale, 2015). In addition, the artificial calcium oscillations created by AOA are suspected of having undesirable downstream consequences, such as effects on mitochondrial metabolism or other developmentally critical pathways (van Blerkom, Cohen, & Johnson, 2015).

In our study, the total number of top quality embryos was significantly reduced in the AOA-treated group. This is clinically important because selection and transfer of top-quality embryos is an essential element of success in assisted reproduction. These findings might be related either to effects of calcium ionophore on oocyte homeostasis or mitochondrial metabolism (Santella & Dale, 2015; van Blerkom et al., 2015), or through disturbance in the embryo culture due to the addition of an extra oocyte culture and manipulation step.

As a ready-to-use calcium ionophore solution (CultActive) was used in our study, it was not possible to control for any solvent effect. In addition, the present study did not evaluate the effects of the calcium ionophore in a dose-dependent manner, where there might be non-specific effects due to additional rinsing and transferring steps. These are the main limitations of the study.

The primary outcomes were fertilization rates and embryo quality, but not pregnancy. Consequently, due to low sample size of our study, we were unable to compare the differences in implantation, clinical and ongoing pregnancy rates.

Our study reveals that AOA after IMSI does not improve the fertilization rates and may reduce the ability of successfully fertilized oocytes to develop into top-quality embryos. Further studies including ongoing pregnancy and livebirth outcomes with larger sample sizes are required.

Disclosure statement

All authors declare that they do not have any conflict of interest. All authors state that this study follows the principles of the Declaration of Helsinki.

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