Can calcium ionophore "use" in patients with diminished ovarian reserve increase fertilization and pregnancy rates? A randomized, controlled study

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Objective: To determine whether calcium ionophore solution can improve the fertilization rate in patients with diminished ovarian reserve whose partners have normal sperm parameters.
Design: Between January 2014 and August 2014, patients with diminished ovarian reserve were randomized to make artificial oocyte activation with calcium ionophore solution.
Setting: University hospital.
Patient(s): A total of 296 patients who had diminished ovarian reserve and partners with normal sperm parameters were included in the study.
Intervention(s): Metaphase 2 oocytes were treated with calcium ionophore solution (GM508 Cult-Active) for 15 minutes just after intracytoplasmic sperm injection.
Main Outcome Measure(s): Fertilization rate, implantation rate, clinical pregnancy rate, ongoing pregnancy rate.
Result(s): Fertilization, implantation, pregnancy, and ongoing pregnancy rates for the calcium ionophore and control groups were 60.7% and 55.4%, 12.8% and 10.7%, 21% and 12.8%, and 10.9% and 6.1%, respectively.
Conclusion(s): This is the first prospective, randomized, controlled study to analyze the effect of calcium ionophore solution on fertilization rate in patients with diminished ovarian reserve. We did not observe any differences in fertilization, clinical pregnancy, or ongoing pregnancy rates between the groups. We propose that fertilization ratios could not be increased by artificial oocyte activation via application of calcium ionophore solution in patients with diminished ovarian reserve.
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Key Words: Calcium ionophore, fertilization failure, diminished ovarian reserve, ICSI

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Although success rates for IVF have increased to nearly 70% using the intracytoplasmic sperm injection (ICSI) method, persistent failures remain a problem for a large proportion of couples (1). During IVF treatment after ovarian hyperstimulation, the number of oocytes collected during oocyte pickup (OPU) may be diminished owing to empty follicles, early rupture of follicles, or inadequate maturation of the oocytes. This undesired drop in the number of oocytes after OPU becomes more worrisome in cases of low oocyte fertilization (LF) or total fertilization failure (TFF). Total fertilization failure is currently seen in approximately

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2%–3% of patients using the ICSI procedure (2), with our clinic reporting a failure rate of 4.3% (unpublished data). Another distressing issue in IVF is the patients with diminished ovarian reserve (DOR), whereby patients exhibit low oocytes numbers after ovarian hyperstimulation, even in cases in which higher gonadotropin doses were used. Patients with fewer oocytes per IVF/ICSI cycle exhibit lower fertilization rates (51%), along with higher rates of both fertilization failure (16%) and cycle cancellation (24.6%) (3, 4). Within our clinic, cycle cancellation was more frequently observed in patients with DOR, resulting in TFF rates of up to 32.5% (unpublished data). Although significant advances have been made in IVF therapy, many of the causes underlying fertilization failure remain poorly understood, thereby limiting the therapeutic options available for these individuals.

Calcium ionophore (CI) solution is used as an artificial oocyte activator in infertile couples affected by TFF or LF, as well as male-specific factors, including teratozoospermia, azospermia, cryptozoospermia, or globozoospermia (5–8). Here, we sought to investigate the effects of artificial oocyte activation (AOA) by CI after ICSI on the fertilization rate of patients with DOR. This study is the first to demonstrate the effect of CI on fertilization rates in patients with DOR.

MATERIALS AND METHODS

This prospective, randomized, controlled study was conducted at Baskent University, Adana, Turkey, in the IVF unit of the Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, between January and August 2014. All study-related protocols were approved by the institutional review board of our university (approval no. KA13/191) before initiation of the trial. The clinical trial registration number for this study is NCT02045914 (https://clinicaltrials.gov).

Study Design

Eligibility criteria for the study included DOR and partners with normal sperm parameters. Diminished ovarian reserve was defined according to the Bologna criteria for poor ovarian response (9). Participation was limited to patients who met two of the three Bologna criteria (i.e., abnormal ovarian reserve tests such as bilateral antral follicle count <5 or antimüllerian hormone (AMH) levels <0.5–1.1 ng/mL; poor ovarian response in the prior IVF cycle, defined as three or fewer retrieved follicles; and age ≥ 40 years). Sperm parameters were assessed as normal according to the World Health Organization criteria: sperm volume >1.5 mL, sperm pH >7.2, sperm count $\geq 15 \times 10^6$/mL, and progressive sperm motility $\geq 32\%$. Patients with globozoospermia or morphologically poor sperm were not included in the study. Sperm morphology was evaluated according to Kruger's criteria, with morphology $>4\%$ regarded as normal (10). Patients who had experienced a previous fertilization failure or who had a history of ovarian surgery, grade 3–4 endometriosis and endometrioma, adenomyomas, myomas, congenital uterine anomaly, and tubal pathology such as hydrosalphenx were excluded from the study.

Examination of 1,439 patients between January and August 2014 on the day of early follicular phase identified 408 patients with DOR (Fig. 1). Of these, 77 were excluded because of accompanying male infertility, with an additional 35 declining to participate in the study, resulting in a total of 296 patients. Informed consent was provided by all patients at the time of enrollment. Treatment protocols of ovarian hyperstimulation were then initiated, as described below. At the end of treatment, patient randomization was performed by a single researcher on the day of OPU, just before the process of the OPU with the support of a computer-generated program. Artificial oocyte activation by CI was applied to the oocytes of 148 randomly selected patients just after ICSI (CI group); the remaining randomly selected 148 patients did not receive AOA so were regarded as the control group.

Ovarian Stimulation, OPU

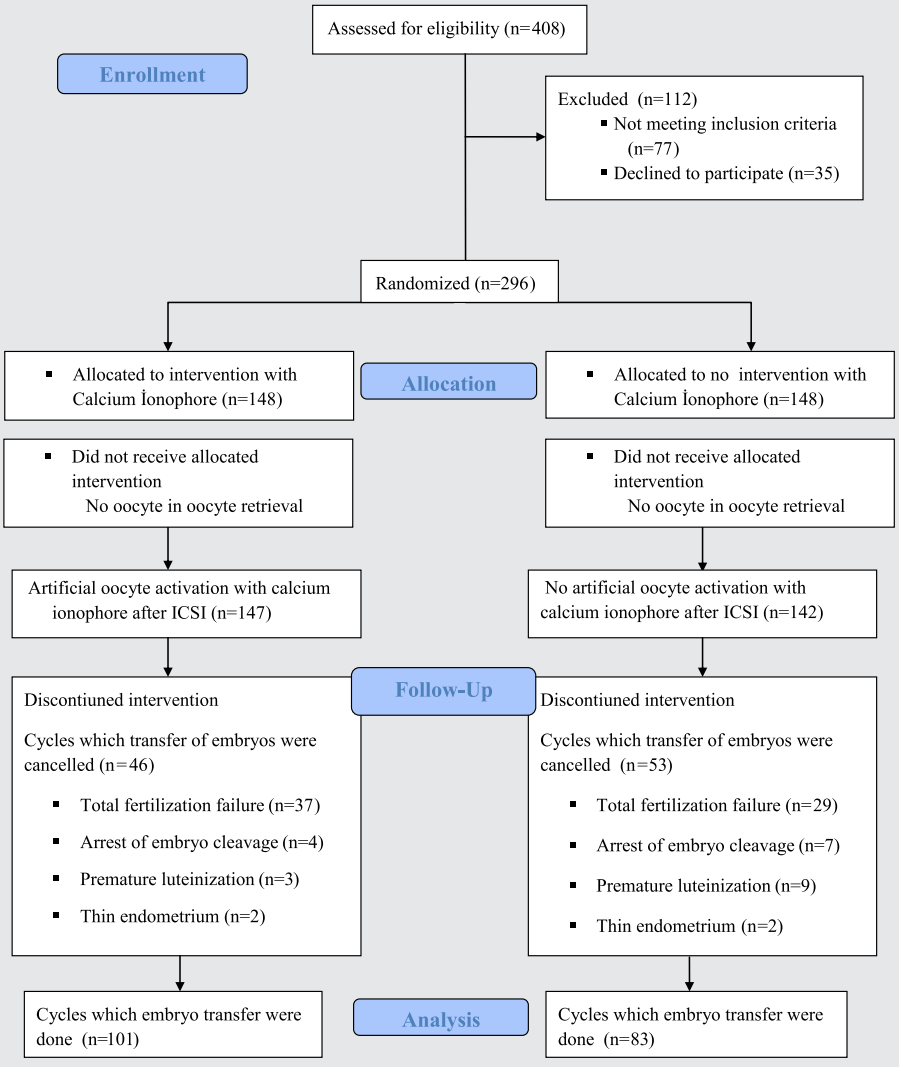
Ovarian hyperstimulation was carried out according to the long protocol, antagonist protocol, or clomiphene citrate–follicle stimulating hormone (CC-FSH) protocol. In the long protocol, down-regulation was achieved by GnRH analogues (Lucrin, Abbott; Gonapeptyl, Ferring), followed by hyperstimulation with recombinant FSH (Puregon, Merck Sharpe Dome; Gonal-f, MerckSerono). In the antagonist protocol, FSH analogues were started on the third day of the cycle, and GnRH antagonists (Orgalutran, Merck Sharpe Dome; Cetrotide, MerckSerono) were added when the leading follicles reached 12–13 mm in diameter or the E_2 level exceeded 300 pg/mL on the fifth, sixth, or seventh day of the cycle. In the CC-FSH protocol, clomiphene citrate (Klomen, Kocak) treatment at 50 mg twice daily was initiated on the third day of the cycle, and FSH in planned doses was added on the third day of CC treatment. In all protocols, ovulation was induced using 250 μg recombinant hCG (Ovitrelle, MerckSereno) administered after the leading follicle reached a minimum of 17 mm in diameter. Oocyte retrieval was performed 32–34 hours after hCG administration.

Oocytes were collected transvaginally under sedation anesthesia on the day of OPU. Metaphase I oocytes and germinal vesicles were eliminated, and only metaphase II (MII) oocytes were prepared for the ICSI procedure.

Artificial Oocyte Activation

Artificial oocyte activation was performed as described previously (7), unlikely with a lower concentration of CI solution. GM508 Cult-Active solution was incubated at 37°C in 5%–7% CO_2 for 4 hours before application. Following the ICSI procedure, all injected MII oocytes were incubated in 50 μL GM508 Cult-Active (Gynemed) CI solution for 15 minutes individually. Then, all oocytes were washed three times in 500 μL culture medium, and each injected oocyte was transferred into the culture medium and incubated for 16–20 hours until fertilization was achieved.

FIGURE 1



CONSORT statement flow chart of the whole study population.

Caglar Aytac. Calcium ionophore use in the patients with DOR. Fertil Steril 2015.

An oocyte was accepted as fertilized when two pronuclei and two polar bodies were detected. The fertilization rate was calculated as fertilized oocytes per number of MII oocytes injected.

Embryo Grading, Transfer, and Pregnancy

Embryos were graded according to the number and equivalence of cells and the percentage of vacuoles formed around them, the presence of multinucleation, and the status of the zona pellucida on the second and third days. Grades A, B, C, and D were assigned according to the Spanish Association of Reproductive Biology (La Asociación para el Estudio de la Biología de la Reproducción) consensus scoring of cleavage-stage embryos (11), and grade D embryos were excluded from further use. Turkish law allows patients younger than 35 years to receive only one embryo; two embryos can be transferred in patients aged 35 years and older, as well as in patients who have already undergone two unsuccessful ICSI cycles. Thus, one or two embryos were transferred on the third day after fertilization. All patients received luteal support with 90 mg/d P administered intravaginally (Crinone 8% gel, Serono) starting on the day of ET and continued for 12 days. Serum hCG levels were analyzed on day 12, with levels >10 mIU/mL suggestive of pregnancy. Patients exhibiting a ≥twofold increase in hCG levels over the next 2 days were then examined by transvaginal ultrasound to identify a gestational sac, a yolk sac, and the presence of a fetal heartbeat after 2 weeks. Clinical pregnancy was accepted as positive if the fetal gestational sac was apparent and fetal heartbeat was recorded regularly. Implantation rate was calculated as the number of gestational sacs seen on transvaginal ultrasound per number of transferred embryos. Clinically pregnant

patients were followed up in the perinatology unit until the end of the second trimester (20 weeks' gestation). An ongoing pregnancy rate was calculated for patients who progressed beyond the end of the second trimester.

Cycle cancellation was performed in patients exhibiting no oocytes after OPU (empty follicle), TFF, P levels >1.5 ng/mL, endometrial thickness <7 mm, or arrest of cleavage.

Patient characteristics, including ages of the patients and their partners, duration of infertility, causes of infertility, number of previous IVF cycles, bilateral ovarian antral follicle counts on the day of early follicular phase, AMH levels, smoking history, body mass index (BMI), E_2 and P levels and endometrial thickness on the day of hCG injection, duration of ovarian hyperstimulation, total dose and protocol of gonadotropin used for ovarian stimulation, number of cumulus–oocyte complex (COC), number of MII oocytes obtained on the OPU day, number of two-pronuclei zygotes obtained after fertilization, cleavage ratio, grade of the embryos, and number of the transferred embryos were recorded for each individual.

Statistical Analysis

The fertilization rate in normoresponders was calculated as 67% in our clinic (12). In the previous literature the fertilization rate was reported to be 51% in patients with fewer oocytes retrieved in IVF (3). In our study, we aimed to increase the fertilization rate from 51% to 67%–70% in patients with DOR. To this end, the estimated sample size calculated by the PS-Power sample-size calculation program to achieve 80% power ($1 - \beta$) and a type 1 error probability of 5% (α) was 296 patients, divided evenly between groups.

Demographic data for each group are presented as the mean ± SD, the median (range), or percentage values. A χ^2 test was used to compare categorical data, and the Mann-Whitney U test was used to compare continuous variables that were non-normally distributed. Student's t test was used to compare means of continuous data with normal distribution. P values of <.05 were considered statistically significant. Data were analyzed using SPSS version 18.0 for Windows.

RESULTS

The ages of patients and their partners, duration of infertility, BMI, smoking ratios, bilateral antral follicle counts, AMH levels, E_2 and P levels and endometrial thickness on the day of hCG injection, duration of hyperstimulation, total doses of gonadotropin used, numbers of COC and MII obtained on the day of OPU, and the number of previous IVF cycles were all similar between groups (Table 1). The majority of patients were experiencing their first IVF cycle (65.2%), whereas 20.9% had previously undergone one, and 13.9% had undergone two IVF cycles. Diagnosed causes of infertility before IVF included unexplained infertility (15%), tubal factor (6.3%), mild endometriosis (3.8%), and DOR (74.9%), which were statistically similar between groups (P=.29). The numbers of two-pronuclei zygotes, of oocytes that entered cleavage, of grades A, B, and C embryos, and of embryos transferred after fertilization were also similar between groups (Table 1).

The overall percentages of various ovarian stimulation protocols included 16% long protocol, 48.5% antagonist protocol, and 35.5% CC-FSH protocol. There were no significant differences in terms of the frequency of the antagonist or CC-FSH protocols between groups; however, the frequency of the long protocol was higher in the CI group (P=.004). Therefore, the total dose of gonadotropin used during ovarian stimulation in the CI group was slightly higher, though this difference was not statistically significant (P=.12).

The CI group exhibited a fertilization rate of 60.7% ± 4%, compared with 55.4% for the control group (P=.26). Cleavage rates were 93.8% for both the CI and control groups, and implantation rates were 12.8% and 10.7%, respectively (P=.61). The pregnancy rate per cycle was 21% in the CI group compared with 12.8% in the control group (P=.60), with per-transfer rates of 30.7% and 22.9%, respectively (P=.24). Clinical pregnancy rates were 13.6% per cycle and 19.8% per transfer in the CI group, compared with 8.1% and 10.9%, respectively, in the control group (P=.13). The ongoing pregnancy rate per cycle was 10.9% in the CI group and 6.1% in the control group (P=.14); the ongoing pregnancy rate per ET was 15.8% in the CI group and 10.8% in the control group (P=.38).

Reasons for cycle cancellation included higher P levels (≥1.5 ng/mL) on the day of hCG, empty follicle after OPU, thin endometrium on the day of ET, arrest of embryo cleavage, and TFF. The cancellation rate for ET was significantly lower in the CI group (31%) compared with the control group (43%; P=.05). This increased cancellation rate in the control group was found to be the result of higher empty follicle counts, higher levels of P on the day of hCG, and the tendency for more embryos to be frozen for other reasons. Therefore, the number of ETs in the control group was lower than that in the CI group. Next, patients who had cycles cancelled because of TFF were categorized into subgroups according to the method of AOA. The subgroups were similar in terms of age, duration of infertility, bilateral antral follicle counts, smoking rates, AMH levels, E_2 and P levels on hCG day, duration of hyperstimulation, and oocyte number obtained after OPU. The TFF rates were 25% in the CI group compared with 20.5% in the control group (P=.32; Table 2). Although the TFF rate was a somewhat higher in the CI group, this difference was not statistically significant.

DISCUSSION

We aimed to assess the effects of CI, a chemical oocyte activator, on fertilization rates in patients with DOR after implementation of the ICSI procedure. In this prospective, randomized, controlled study, fertilization and cleavage rates were similar in patients with DOR with or without AOA by CI after ICSI. Implantation, pregnancy, and ongoing pregnancy rates were also similar between groups.

The study by Krog et al. (4) demonstrated higher rates of TFF in IVF in patients with fewer oocytes (fewer than four), regardless of the woman's chronological age. Here, we observed TFF rates three times higher in patients with low oocyte numbers (fewer than four) relative to those with normal oocyte counts (41% vs. 12%). Although we

TABLE 1

Demographic characteristics of the two groups.

Characteristic	Calcium ionophore cycle	No calcium ionophore	*P* value
Patients			
No. of cycles	148	148	
Female age (y)	35 ± 4	36 ± 4	NS
Male age (y)	38 ± 6	38 ± 6	NS
Duration of infertility (y)	5.6 ± 4	5.2 ± 4	NS
Smoking (%)	14.9	22.5	NS
BMI (kg/m^2)	23.9 ± 5	24.8 ± 6	NS
AMH (ng/mL)	0.4 (0.01–1.19)	0.21 (0.01–1.10)	NS
Antral follicles (n)	2.4 ± 1.4	2.5 ± 1.7	NS
Previous IVF cycles of patients (n)	1.6 ± 1	1.7 ± 1	NS
Treatment			
E_2 level on hCG day (pg/mL)	529 ± 377	529 ± 394	NS
P level on hCG day (ng/mL)	0.3 (0.1–7.6)	0.3 (0.1–6.5)	NS
Endometrium thickness on hCG day (mm)	9 ± 2	9 ± 2	NS
COH duration (d)	8 ± 2	8 ± 2	NS
Total doses of gonadotropin (IU)	2,350 (300–3,525)	2,100 (300–4,125)	NS
COC (n)	2.4 ± 1	2.3 ± 1	NS
MII oocyte (n)	2 (1–4)	2 (0–4)	NS
Total oocytes treated with CI (n)	429	408	NS
After oocyte activation			
Two pronuclei (n)	1.5 ± 0.7	1.4 ± 0.7	NS
Cleavage (n)	1.6 ± 0.7	1.4 ± 0.6	NS
Grade A embryo (n)	0.4 ± 0.6	0.4 ± 0.5	NS
Grade B embryo (n)	0.8 ± 0.6	0.9 ± 0.6	NS
Grade C embryo (n)	0.009 ± 0.9	0.2 ± 0.1	NS
No. of transferred embryos	1.3 ± 0.4	1.3 ± 0.4	NS

Note: Values are presented as mean ± SD or median (range), unless otherwise noted. COH = controlled ovarian hyperstimulation; NS = not significant.

Caglar Aytac. Calcium ionophore use in the patients with DOR. Fertil Steril 2015.

consistently see fertilization rates of approximately 67% for normo-responders in our clinic (12), the fertilization rate of the patients with DOR described here was only 58%, with a significant decrease in overall fertility.

Despite the critical role of oocyte numbers in TFF, the most important reason for fertilization failure is a deficiency in oocyte activation (13). The fertilization process begins when a sperm fuses with an oocyte that has arrested in MII. Sperm-specific phospholipase C zeta (PCL zeta) is then secreted to induce calcium oscillations in the cytoplasm (14). Calcium is provided by the endoplasmic reticulum via inositol triphosphate (15); inhibition of this calcium has been shown to inhibit cleavage of the embryo in *Drosophila melanogaster* (16), indicating an essential role for calcium in oocyte activation. Calcium ionophore is a lipid-soluble molecule that permits calcium transport across the cell membrane, transiently increasing cytoplasmic calcium levels, thereby enabling oocyte activation and fertilization (17).

TABLE 2

IVF outcomes of the two groups.

Outcome	Calcium ionophore (+)	Calcium ionophore (−)	*P* value
Fertilization rate (%), mean ± SD (n)[a]	60.7 ± 4 (147)	55.4 ± 3.9 (142)	NS
Cleavage rate[a]	93.8 (106/113)	93.8 (106/113)	NS
Implantation rate/transfer[a]	12.8	10.7	NS
Pregnancy rate[b]	21 (31/148)	12.8 (19/148)	NS
Pregnancy rate/transfer[b]	30.7 (31/101)	22.9 (19/83)	NS
Clinical pregnancy rate/cycle[b]	13.6 (20/148)	8.1 (12/148)	NS
Clinical pregnancy rate/transfer[b]	19.8 (20/101)	14.5 (12/83)	NS
Ongoing pregnancy rate/cycle[b]	10.9 (16/125)	6.1 (9/143)	NS
Ongoing pregnancy rate/transfer[b]	15.8 (16/101)	10.8 (9/83)	NS
Total fertilization failure[b]	25 (37/147)	20.4 (29/142)	NS

Note: Values are presented as percentage (number), unless otherwise noted.
[a] Mann-Whitney *U* test for non-normally distributed quantitative data.
[b] *P* values according to the χ^2 test for categorical data.

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Previous studies of couples who had experienced fertilization failures in previous ICSI cycles revealed better fertilization rates using CI for oocyte activation in subsequent cycles, regardless of male factors (5, 18). Similar effects were also seen in patients exhibiting infertility due to male factors such as azospermia or cryptozoospermia, with the addition of CI resulting in better fertilization rates in subsequent ICSI cycles (7). In all of these studies, patients had experienced fertilization failure due to male and/or female factors in previous ICSI cycles. However, in one study, nearly a quarter of patients with TFF reported one or more previous pregnancies, suggesting that TFF is not indicative of definite infertility but may instead represent an episodic situation (4). Because neither TFF nor LF rates can be predicted using existing methodologies, we sought to lower the frequency of TFF and increase fertilization rates in patients with DOR in their specific cycle.

The treatment protocols for both groups were similar, with the exception of the long protocol, which was used more frequently in the CI group ($P=.004$). However, because the difference in treatment protocols in patients with DOR was shown to have no effect on live birth rates (19), we assumed that the difference in the use of the long protocol seen here would not affect ongoing pregnancy rates.

In the study by Ebner et al. (20), CI treatment during ICSI failed to improve fertilization rates of the embryos, but instead, amelioration was seen on the number of embryos going to the blastula formation in patients with previous embryo development problems. In our study, all embryos were transferred at cleavage stage, with no statistical differences observed in terms of the number of embryos of each grade. If we had followed the embryos to the point of blastula or morula formation, rather than the cleavage stage, we may have been able to better assess the effects of calcium on embryo quality.

The main concern in using CI as an oocyte activator in IVF cycles is the possibility of unknown effects on the embryos, with potential long-term consequences for the newborns themselves. Two recent studies revealed no chromosomal abnormalities in implanted embryos after IVF in which CI was used as an artificial oocyte activator, with no perinatal morbidities or mortalities evident in these patients (21, 22). Similar results have also been seen in children 3–10 years of age who were born after ICSI cycles with AOA via CI, with no differences seen in terms of neonatal, neurologic, or language development relative to their peers (23–25). Initial results from our study failed to identify any abnormality associated with this therapy, although ongoing monitoring continues. One report by Ebner et al. (26) described a case of anal atresia, a major malformation, in a baby conceived after an ICSI cycle with CI application; however, the gastrointestinal abnormality rates in babies born after CI application were found to be consistent with those in babies conceived using traditional ICSI.

As with all studies, this work is not without limitations. Ideally, it would have been better to split oocytes into two groups for use as CI and controls for each individual cycle; however, because of the low number of oocytes recovered in this cohort (four or fewer), this option was not feasible. Furthermore, although we selected patients exhibiting normal sperm parameters, the possibility of latent loss of sperm-activating capacity cannot be excluded. Such a possibility may have affected patient outcomes; however, this difference would have applied to both groups.

In conclusion, AOA by CI was not shown to affect fertilization rates in patients with DOR after ICSI. Similarly, AOA with CI did not decrease TFF rates in this population. Taken together, these results suggest that routine use of CI to increase fertilization, pregnancy, and ongoing pregnancy rates is not appropriate for patients with DOR.

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