RESEARCH PAPER

Calcium Ionophore–Assisted Oocyte Activation Is Associated with Higher Fertilization Outcomes in Women with Endometriosis Undergoing ICSI: A Prospective Observational Study

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Received: 09th June, 2026; Revised: 30th June, 2026; Accepted: 11th July, 2026; Available Online: 20th July, 2026

ABSTRACT

Background and Aim: Endometriosis may impair oocyte competence and calcium-mediated activation, limiting fertilization after ICSI. This study evaluated whether calcium ionophore-assisted oocyte activation (AOA) associated with improved fertilization and embryological outcomes in women with endometriosis undergoing fresh ICSI cycles. **Patients and Methods:** This prospective observational cohort included 108 women with endometriosis-associated infertility at Cairo University Hospital. Fifty-four underwent ICSI followed by calcium ionophore AOA using GM508 Cult-Active, and 54 underwent standard ICSI. The primary outcome was fertilization rate. Secondary outcomes included normal fertilization, cleavage, blastocyst formation, high-quality embryos, implantation, and clinical pregnancy. **Results:** AOA significantly increased normally fertilized oocytes (3.41 ± 0.92 vs 2.87 ± 0.80; p=0.003) and fertilization rate (75.99 ± 12.79% vs 59.10 ± 15.94%; p<0.001). It also improved cleaved embryos, blastocyst number, blastocyst formation rate, and high-quality embryos. Adjusted analysis confirmed higher normal fertilization (adjusted OR 2.34, 95% CI 1.80–3.05), blastocyst formation (adjusted OR 1.40, 95% CI 1.08–1.82), and high-quality embryo production (adjusted IRR 1.43, 95% CI 1.28–1.60). Clinical pregnancy was higher but not significant (33.3% vs 24.1%; p=0.288). **Conclusion:** Calcium ionophore AOA was associated with higher fertilization and embryo-development outcomes in selected endometriosis ICSI cycles but did not significantly improve implantation or clinical pregnancy. Because allocation was non-randomized, residual indication bias cannot be excluded. These findings apply mainly to young women with surgically confirmed endometriosis, acceptable ovarian reserve, normal semen parameters, and fresh ICSI cycles, and require confirmation in larger randomized trials assessing pregnancy and live birth.

Keywords: Endometriosis; ICSI; Calcium ionophore; Assisted oocyte activation; Fertilization; Embryo quality; Clinical pregnancy.

How to cite this article: Elmasri N.A, Gabr R.M, Kapil T.Y, Azmy O.M, El-Khayat W.M, Elbanna W.S, Shaker A.N, Calcium Ionophore–Assisted Oocyte Activation Is Associated with Higher Fertilization Outcomes in Women with Endometriosis Undergoing ICSI: A Prospective Observational Study. 2026;16(70s): 359-376. DOI: 10.25258/ijddt.16.70s.37

Source of support: None

Conflict of interest: None

INTRODUCTION

Endometriosis is a chronic estrogen-dependent inflammatory disorder defined by endometrial-like tissue outside the uterine cavity. It affects women during their reproductive years and is a frequent contributor to infertility. Its reproductive impact is multifactorial: advanced disease may distort pelvic anatomy and impair tubo-ovarian function, whereas even mild disease may reduce fertility through inflammation, oxidative stress, altered follicular fluid, impaired oocyte competence and disturbed endometrial receptivity (1,2).

ICSI is widely used for endometriosis-associated infertility because it bypasses several barriers to fertilization, including sperm-zona binding and sperm-oolemma fusion. However, ICSI cannot correct intrinsic defects within the oocyte. This may explain why outcomes in endometriosis remain heterogeneous: some studies report fewer retrieved and mature oocytes, reduced fertilization or poorer embryo development, while others show comparable pregnancy outcomes after controlling for age, ovarian reserve, previous surgery and disease phenotype (3-5). These conflicting findings suggest that endometriosis may not

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impair all cycles equally, but may identify a subgroup of patients with functionally compromised oocytes.

Normal fertilization after ICSI still requires oocyte activation. Physiologically, sperm-derived phospholipase C zeta induces repeated intracellular calcium oscillations, which trigger resumption of meiosis, second polar body extrusion, cortical granule exocytosis, pronuclear formation and early embryonic development.6 Failure of this calcium-dependent process is a recognized cause of low or total fertilization failure after ICSI. In endometriosis, activation competence may be vulnerable because inflammatory cytokines, reactive oxygen species and iron-mediated oxidative injury can affect mitochondrial function, cytoplasmic maturation, endoplasmic reticulum calcium stores and meiotic spindle organization (2,6).

Calcium ionophore–assisted oocyte activation (AOA) has been introduced to artificially increase intracellular calcium after ICSI and support oocyte activation. Previous studies and meta-analyses have shown improved fertilization and, in selected populations, improved reproductive outcomes, particularly among patients with previous low or total fertilization failure, globozoospermia, severe male factor infertility or suspected activation deficiency (7,8). Nevertheless, these studies mainly evaluated mixed infertility populations or classic fertilization-failure indications. Direct evidence in women with endometriosis remains limited.

This represents the main research gap addressed by the present study. Endometriosis is biologically associated with impaired oocyte competence, while calcium ionophore AOA is biologically intended to overcome activation failure; however, the clinical intersection between these two concepts has been insufficiently investigated. Therefore, this prospective observational study aimed to evaluate whether calcium ionophore–based AOA improves fertilization rates in women with endometriosis undergoing fresh ICSI cycles compared with conventional ICSI without AOA. Secondary objectives included assessment of normally fertilized oocytes, cleavage, blastocyst formation, high-quality embryo production, implantation and clinical pregnancy outcomes.

The present study differs from previous work by focusing specifically on endometriosis rather than mixed infertility or male-factor cohorts, using fertilization rate as the primary endpoint, and examining whether any fertilization benefit is accompanied by improved downstream embryological outcomes.

PATIENTS AND METHODS

Study Design and Setting

This prospective observational cohort study was carried out at the Assisted Reproductive Technology Unit, Department of Obstetrics and Gynecology, Cairo University Hospital. The study enrolled women with endometriosis-associated infertility who were scheduled to undergo fresh intracytoplasmic sperm injection (ICSI) cycles.

Participants were recruited after assessment of eligibility criteria. Official permission was obtained from obstetrics and gynecology department, faculty of medicine, Cairo University and the study was approved by the Local Ethics Committee of the Faculty of medicine, Cairo University under number (MS-573-2025) dated 25-11-2025. All participants provided written informed consent before enrollment.

The study was conducted with respect for patient autonomy, confidentiality, and voluntary participation. Data was coded and stored securely. Participants were informed that they could withdraw at any time without affecting their medical care. No additional harmful procedures were performed solely for research purposes, and calcium ionophore AOA was used only when clinically indicated or accepted by the patient after counseling.

Study Population and Group Allocation

A total of 108 women with endometriosis undergoing fresh ICSI cycles were included in the final analysis. Of these, 54 patients underwent ICSI followed by calcium ionophore-assisted oocyte activation, while 54 patients underwent standard ICSI without assisted oocyte activation. Eligible women were classified into two groups according to the clinical decision and laboratory protocol used during the ICSI cycle. The calcium ionophore group included women who underwent ICSI followed by assisted oocyte activation (AOA) using calcium ionophore solution, GM508 Cult-Active. The control group included women who underwent conventional ICSI without calcium ionophore exposure.

Because of the observational nature of the study, treatment allocation was not randomized. The decision to use AOA was based on predefined clinical or embryological indications, physician recommendation, previous cycle history, and patient preference after counseling. All clinical, stimulation, laboratory, embryological, and pregnancy-related data were collected prospectively.

Women were assigned to the AOA group if they fulfilled the eligibility criteria and had at least one of the following: previous low fertilization rate, defined as fertilization of less than 30% of injected metaphase II oocytes in a previous ICSI cycle; suspected risk of fertilization failure based on clinical or embryological assessment; abnormal oocyte appearance or previous impaired fertilization; or acceptance of AOA after counseling.

Women were assigned to the control group if they had no previous history of low fertilization, no clinical or embryological indication for AOA, or declined calcium ionophore activation after counseling and preferred to continue with standard ICSI.

The reason for allocation was documented for each participant, including previous fertilization impairment, physician recommendation, oocyte-related indication, or patient preference. These variables were considered during statistical analysis to reduce allocation-related bias.

Women were eligible for inclusion if they met all of the following criteria: confirmed diagnosis of endometriosis, previous history of endometriosis surgery, age between 25 and 35 years, planned fresh ICSI cycle, anti-Müllerian hormone level between 1.0 and 3.0 ng/mL, antral follicle count of at least seven follicles, availability of mature metaphase II oocytes after retrieval, and written informed consent.

Women were excluded if they had poor ovarian reserve, advanced maternal age, polycystic ovary syndrome

diagnosed according to Rotterdam criteria, abnormal male partner semen parameters according to WHO 2021 criteria, use of surgically retrieved sperm, donor oocytes or donor sperm, severe medical disease that could affect ovarian stimulation or pregnancy outcome, cycle cancellation before oocyte retrieval, absence of retrieved metaphase II oocytes, refusal to participate, or withdrawal of consent.

All participants underwent full pre-cycle assessment. A detailed history was obtained, including age, residence, occupation, education, smoking status, weight, height, and body mass index. Infertility history included type and duration of infertility, previous investigations, previous ovulation induction, previous IVF/ICSI trials, and prior fertilization outcomes.

Endometriosis-related history included symptoms, duration of disease, dysmenorrhea, chronic pelvic pain, dyspareunia, previous medical treatment, history of endometrioma, previous endometriosis surgery, type and date of surgery, and available operative findings. Menstrual, obstetric, medical, surgical, drug, and relevant family histories were also recorded.

General examination included assessment of general condition, body mass index, blood pressure, pulse, signs of endocrine disturbance, and signs of chronic medical disease. Gynecological examination was performed when clinically indicated to assess uterine size, adnexal masses, pelvic tenderness, and findings suggestive of endometriosis.

Pre-cycle investigations included serum anti-Müllerian hormone and, when indicated, basal follicle-stimulating hormone, luteinizing hormone, estradiol, thyroid-stimulating hormone, and prolactin. Routine preoperative investigations were performed according to the unit protocol.

Semen analysis was performed for all male partners according to WHO 2021 criteria. Semen volume, sperm concentration, total motility, progressive motility, morphology, and total motile sperm count, when available, were recorded. Only couples with normal semen parameters were included to minimize the confounding effect of male factor infertility on fertilization outcomes.

Baseline transvaginal ultrasound was performed, usually on day 2 or 3 of the menstrual cycle. Uterine morphology, ovarian morphology, antral follicle count, baseline ovarian cysts, and the presence, size, and laterality of endometrioma were documented.

Endometriosis stage was recorded according to the revised American Society for Reproductive Medicine classification whenever staging was available from previous operative reports. Patients were categorized as stage I, II, III, or IV based on documented surgical findings.

Study intervention

Controlled ovarian stimulation was performed according to the ART unit protocol. Either a gonadotropin-releasing hormone antagonist or agonist protocol was used, according to clinical judgment.

The starting gonadotropin dose was individualized based on age, ovarian reserve, body mass index, previous ovarian response, and physician decision. Follicular monitoring was performed by serial transvaginal ultrasound, with serum estradiol assessment when indicated.

The stimulation protocol, starting and total gonadotropin dose, duration of stimulation, follicle number and size on trigger day, trigger type, and serum estradiol level on trigger day, when available, were prospectively recorded. Final oocyte maturation was triggered when adequate follicular development was achieved. Oocyte retrieval was performed approximately 34–36 hours later by transvaginal ultrasound-guided follicular aspiration under anesthesia.

Retrieved cumulus–oocyte complexes were identified by the embryologist and cultured under standard laboratory conditions. Oocytes were denuded according to the ART unit protocol, and nuclear maturity was assessed after denudation.

Only metaphase II oocytes, identified by the presence of the first polar body, were injected. The total number of retrieved oocytes, number of metaphase II oocytes, number of immature oocytes, oocyte morphology when documented, and number of injected mature oocytes were recorded.

Semen samples were obtained by masturbation after an appropriate abstinence period according to laboratory policy. Sperm preparation was performed using the standard technique applied in the unit, such as density-gradient centrifugation or swim-up preparation. Prepared spermatozoa were used for ICSI after assessing motility and morphology.

ICSI was performed for all mature metaphase II oocytes using standard micromanipulation. A single morphologically suitable spermatozoon was immobilized and injected into each mature oocyte. Injected oocytes were then cultured under standard incubator conditions according to the laboratory protocol.

In the AOA group, injected metaphase II oocytes were exposed immediately after ICSI to calcium ionophore solution using GM508 Cult-Active, following the manufacturer's instructions and the ART laboratory protocol.

Oocytes were incubated in the calcium ionophore solution for 15 minutes after sperm injection. They were then washed thoroughly in culture medium to remove residual activation solution and transferred to fresh culture medium for incubation under standard conditions.

In the control group, injected oocytes were cultured after standard ICSI without exposure to calcium ionophore. Apart from calcium ionophore exposure, culture conditions and laboratory procedures were kept similar between groups. All embryology procedures were performed by trained embryologists in the same ART laboratory to minimize technical variability.

Fertilization was evaluated approximately 16–18 hours after ICSI. Normal fertilization was defined as the presence of two pronuclei and two polar bodies.

The number of normally fertilized oocytes, unfertilized oocytes, and abnormally fertilized oocytes was recorded. Fertilization rate was calculated per injected metaphase II oocyte. Abnormally fertilized oocytes were not used for embryo transfer.

Normally fertilized oocytes were cultured according to the ART unit protocol. Cleavage-stage assessment was performed on day 2 and/or day 3 after ICSI. When extended culture was performed, blastocyst development was assessed on day 5 or day 6.

Cleavage-stage embryos were evaluated according to blastomere number, symmetry, fragmentation, and multinucleation. Blastocysts were assessed according to expansion grade, inner cell mass quality, and trophectoderm quality. High-quality embryos were defined according to the unit grading criteria and included embryos suitable for transfer or cryopreservation.

Fresh embryo transfer was performed according to clinical and embryological decision-making. The day of transfer, number of embryos transferred, and embryo quality were recorded.

Luteal phase support was prescribed according to the ART unit protocol, usually starting after oocyte retrieval and continuing until pregnancy testing. If pregnancy occurred, luteal support was continued according to the treating physician's decision.

Serum β-hCG was measured approximately 14 days after embryo transfer. Clinical pregnancy was confirmed by transvaginal ultrasound showing an intrauterine gestational sac, with or without fetal cardiac activity depending on the timing of ultrasound assessment.

The following data were prospectively collected: maternal age, body mass index, type and duration of infertility, previous IVF/ICSI history, previous endometriosis surgery, endometriosis stage, anti-Müllerian hormone level, antral follicle count, stimulation protocol, gonadotropin dose, number of retrieved oocytes, number of metaphase II oocytes, semen parameters, number of injected oocytes, number of normally fertilized oocytes, cleavage rate, blastocyst formation rate, number and quality of embryos, number of embryos transferred, implantation outcome, and clinical pregnancy outcome.

Outcome Measures

Primary Outcome

The primary outcome was fertilization rate, calculated as:

Number of normally fertilized oocytes / Number of injected metaphase II oocytes × 100.

Normal fertilization was defined as the presence of two pronuclei and two polar bodies 16–18 hours after ICSI.

Secondary Outcomes

Secondary outcomes included the number of normally fertilized oocytes, cleavage rate, blastocyst formation rate, number of high-quality embryos, embryo transfer outcomes, implantation rate, and clinical pregnancy rate.

Cleavage rate was defined as the percentage of normally fertilized embryos that underwent cleavage. Blastocyst formation rate was defined as the percentage of normally fertilized embryos reaching the blastocyst stage. Implantation rate was calculated as the number of gestational sacs divided by the number of transferred embryos. Clinical pregnancy was defined as the presence of an intrauterine gestational sac on transvaginal ultrasound.

Sample Size Calculation

Sample size was calculated using Epi Info STATCALC based on data from Kaur et al. (9) The calculation assumed a two-sided 95% confidence level, 80% power, an expected fertilization rate of 50% in the standard ICSI group, and an expected fertilization rate of 70% in the calcium ionophore group.

The required sample size was 49 women per group. To compensate for possible dropout, cycle cancellation, or incomplete laboratory data, the sample was increased to 54 women in each group, giving a total sample size of 108 women. Although all eligible metaphase II oocytes were assessed, the patient was considered the primary unit of recruitment and group allocation.

Statistical Analysis

Data were coded, checked, and entered into a computerized database. Statistical analysis was performed using IBM SPSS software. Additional advanced analyses, including propensity score weighting, inverse probability of treatment weighting, overlap weighting, generalized estimating equations, clustered regression models, and graphical assessment of covariate balance, were performed using appropriate statistical packages when required.

All eligible women were analyzed according to their treatment group: calcium ionophore–assisted oocyte activation or standard ICSI. The patient was considered the main unit of recruitment and treatment allocation. However, oocyte-level and embryo-level outcomes were considered clustered observations within the same patient and were analyzed using methods that account for within-patient correlation where appropriate.

Numerical variables were assessed for normality using the Shapiro–Wilk test, supported by visual inspection of histograms and Q-Q plots when needed. Normally distributed continuous variables were presented as mean ± standard deviation, while non-normally distributed variables were presented as median and interquartile range. Categorical variables were summarized as frequencies and percentages. All tests were two-sided, and a p value <0.05 was considered statistically significant.

Analysis of main manuscript tables

For Table 1, baseline demographic, infertility, ovarian reserve, semen, and cycle characteristics were compared between the calcium ionophore and standard ICSI groups. Continuous variables were analyzed using the independent-samples t-test or Mann–Whitney U test according to data distribution. Categorical variables were compared using the chi-square test or Fisher's exact test, as appropriate. Effect estimates were reported as mean difference for continuous variables and risk difference for categorical variables with 95% confidence intervals when applicable. Standardized mean difference was used to assess the magnitude of baseline imbalance between groups; values below 0.10 were considered acceptable, while values above 0.20 were considered suggestive of meaningful imbalance.

For Table 2, the primary fertilization outcome and key embryological outcomes were analyzed at the patient level. Fertilization rate was calculated as the number of normally fertilized two-pronuclear oocytes divided by the number of injected metaphase II oocytes ×100. Cleavage rate was calculated as the number of cleaved embryos divided by the number of normally fertilized oocytes ×100. Blastocyst formation rate was calculated as the number of blastocysts

divided by the number of normally fertilized oocytes ×100. Between-group comparisons were performed using the independent-samples t-test or Mann–Whitney U test according to distribution. Effect sizes were reported as mean difference with 95% confidence interval and Cohen's d.

For Table 3, embryo transfer, implantation, and clinical pregnancy outcomes were analyzed as secondary clinical outcomes. Continuous outcomes, including number of embryos transferred, number of gestational sacs, implantation rate, and β-hCG level, were compared using the independent-samples t-test or Mann–Whitney U test, as appropriate. Clinical pregnancy was analyzed as a binary patient-level outcome using the chi-square test or Fisher's exact test, and the effect was reported as odds ratio and risk difference with 95% confidence intervals. Implantation rate was calculated as the number of gestational sacs divided by the number of embryos transferred ×100. Pregnancy-related outcomes were considered exploratory because the study was primarily designed to assess fertilization and embryological outcomes.

For Table 4, propensity score-weighted analyses were performed to reduce treatment-selection bias arising from the non-randomized observational design. The propensity score represented the probability of receiving calcium ionophore–assisted oocyte activation based on clinically relevant baseline and cycle variables. Candidate covariates included age, body mass index, anti-Müllerian hormone, antral follicle count, endometriosis stage, previous ICSI history or previous fertilization impairment, number of metaphase II oocytes, sperm concentration, progressive sperm motility, and sperm morphology. Inverse probability of treatment weighting was used as the main propensity score method, while overlap weighting was used as a complementary approach emphasizing patients with comparable probabilities of receiving either treatment. Covariate balance before and after weighting was assessed using standardized mean differences and graphically by a Love plot. Weighted treatment effects were reported as mean differences for continuous rate outcomes, incidence rate ratios for count outcomes, and odds ratios for binary outcomes, all with 95% confidence intervals. Robust standard errors were used to account for weighting.

For Table 5, adjusted multivariable treatment-effect analyses were performed for the primary and secondary outcomes. Normal fertilization per injected metaphase II oocyte, cleavage per normally fertilized oocyte, blastocyst formation per normally fertilized oocyte, and implantation per transferred embryo were analyzed using clustered logistic regression or generalized estimating equations with robust standard errors to account for clustering within patients. High-quality embryo production was analyzed as a count outcome using Poisson regression with a log-transformed metaphase II oocyte number as an offset; negative binomial regression was considered if overdispersion was detected. Clinical pregnancy was analyzed at the patient level using binary logistic regression. Adjusted models included clinically relevant confounders selected a priori, including age, body mass index, ovarian reserve markers, endometriosis stage, previous ICSI or previous fertilization impairment, number of metaphase II oocytes, and semen parameters. For implantation and pregnancy-related models, number of embryos transferred and embryo quality were considered when appropriate. Results were reported as adjusted odds ratios, adjusted incidence rate ratios, relative risks or rate ratios, and 95% confidence intervals.

Analysis of supplementary tables

Supplementary Table S1 included the full detailed baseline characteristics that were condensed in the main manuscript Table 1. These variables were summarized using the same descriptive methods as the main baseline table. Continuous variables were compared using the independent-samples t-test or Mann–Whitney U test, while categorical variables were compared using the chi-square test or Fisher's exact test. Standardized mean differences were reported to support assessment of baseline balance.

Supplementary Table S2 included the full patient-level secondary continuous and rate outcomes. These outcomes were analyzed using unadjusted patient-level comparisons. Mean differences with 95% confidence intervals were reported for continuous and percentage outcomes, together with p values and appropriate effect sizes.

Supplementary Table S3 included advanced regression analyses of secondary outcomes. Logistic regression was used for binary outcomes, Poisson or negative binomial regression for count outcomes, and linear regression for continuous outcomes when model assumptions were satisfied. These analyses were considered supportive and were used to confirm the direction and consistency of the main findings.

Supplementary Table S4 included generalized estimating equation sensitivity analyses. GEE models were used for oocyte-level, embryo-level, and transferred embryo-level outcomes to account for clustering within the same patient. Robust standard errors were applied, and results were expressed as odds ratios or incidence rate ratios with 95% confidence intervals.

Supplementary Table S5 included exploratory subgroup analyses for fertilization rate. Subgroups included endometriosis stage, previous ICSI trial or previous fertilization impairment, ovarian response according to metaphase II oocyte yield, and anti-Müllerian hormone category. Treatment-by-subgroup interaction terms were tested to explore whether the effect of calcium ionophore differed across subgroups. These analyses were considered exploratory and hypothesis-generating because the study was not powered for definitive subgroup effects.

Supplementary Table S6 included sensitivity analyses for the primary fertilization outcome. These analyses evaluated the robustness of the main fertilization findings using alternative analytical approaches, including patient-level analysis, multivariable-adjusted analysis, clustered oocyte-level analysis, inverse probability-weighted analysis, overlap-weighted analysis, propensity score–matched analysis when applicable, and exclusion of extreme responders.

Supplementary Table S7 included sensitivity analyses for clinical pregnancy. These analyses included unadjusted patient-level analysis, multivariable-adjusted logistic

regression, propensity score-weighted analysis, restriction to cycles with embryo transfer, and restriction to cycles with adequate transfer conditions when applicable. Because clinical pregnancy was a secondary and underpowered outcome, these results were interpreted cautiously and used only to support the main clinical interpretation.

Effect sizes were reported in addition to p values. Mean differences were used for continuous outcomes, risk differences and relative risks for categorical outcomes when clinically interpretable, odds ratios for binary regression outcomes, incidence rate ratios for count outcomes, and standardized mean differences for baseline balance. Missing data were handled by complete-case analysis for the relevant outcome, and the number of analyzed observations was reported when it differed from the total sample.

RESULTS

A total of 153 women were assessed for eligibility. Forty-five were excluded before study inclusion because they did not meet the eligibility criteria, declined participation, or withdrew before allocation. The remaining 108 women were included in the study, with 54 undergoing calcium ionophore-assisted oocyte activation and 54 undergoing standard ICSI. No included cycles were cancelled before retrieval, no participant had zero MII oocytes after retrieval, and no outcome data were missing. All included women were analyzed for fertilization, implantation, and clinical pregnancy outcomes (Figure 1).

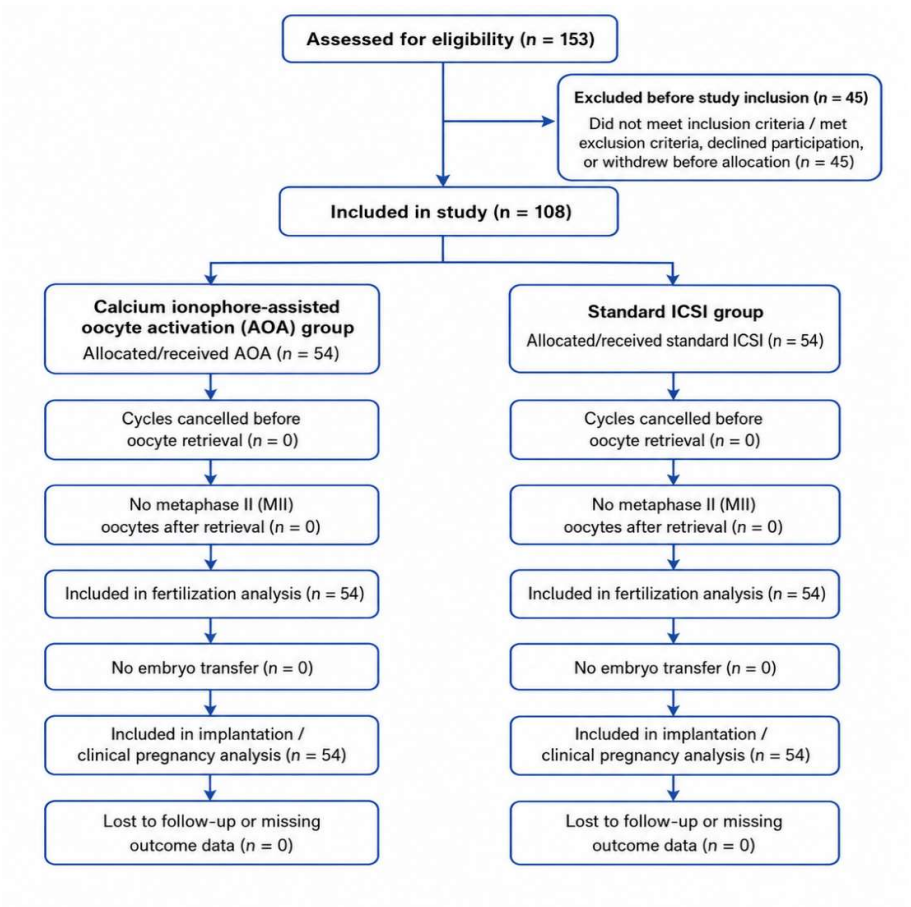

Figure 1. Flow chart of the participants.

Study population and baseline characteristics

Baseline demographic, infertility, ovarian reserve, semen, and cycle characteristics are summarized in Table 1. Age, AMH, AFC, semen concentration, progressive motility, sperm morphology, type of infertility, rASRM stage, previous ICSI history, and previous endometriosis surgery were broadly similar between groups. However, several clinically relevant baseline and cycle imbalances were identified. Compared with the calcium ionophore group, the standard ICSI group had a significantly higher BMI (27.74 ± 1.78 vs. 27.11 ± 1.63 kg/m²; p=0.036), longer duration of infertility (4.98 ± 1.57 vs. 4.26 ± 1.56 years; p=0.021), and a higher number of injected MII oocytes (4.93 ± 1.13 vs. 4.48 ± 1.00; p=0.025). Meaningful standardized mean differences were also observed for BMI (SMD 0.37), years of marriage (SMD 0.53), duration of infertility (SMD 0.46), previous ICSI trials (SMD 0.33), and MII oocytes (SMD 0.42). These imbalances were considered clinically important and supported the use of adjusted multivariable, propensity-weighted, and sensitivity analyses.

Primary fertilization and embryological outcomes

The primary and key embryological outcomes are shown in **Table 2**. The calcium ionophore group had a significantly higher number of normally fertilized oocytes compared

with the standard ICSI group (3.41 ± 0.92 vs. 2.87 ± 0.80; mean difference 0.54, 95% CI 0.21 to 0.87; p = 0.003). Fertilization rate was also markedly higher in the calcium ionophore group (75.99 ± 12.79% vs. 59.10 ± 15.94%; mean difference 16.90%, 95% CI 11.38 to 22.41; p < 0.001). This represented the main beneficial effect of calcium ionophore–assisted oocyte activation.

Regarding downstream embryological outcomes, the calcium ionophore group showed significantly higher numbers of cleaved embryos (3.19 ± 0.93 vs. 2.67 ± 0.73; p = 0.002), blastocysts (2.61 ± 0.81 vs. 2.02 ± 0.71; p < 0.001), and high-quality embryos (2.33 ± 0.75 vs. 1.80 ± 0.68; p < 0.001). Blastocyst formation rate was also significantly higher in the calcium ionophore group (77.38 ± 15.86% vs. 69.75 ± 17.98%; p = 0.017). In contrast, cleavage rate was comparable between both groups (93.64 ± 12.64% vs. 94.14 ± 11.85%; p = 0.880), suggesting that the main advantage of calcium ionophore occurred at the fertilization stage and improved embryo yield rather than altering cleavage once fertilization had occurred.

Embryo transfer, implantation and pregnancy outcomes

Embryo transfer and pregnancy-related outcomes are presented in **Table 3**. The mean number of embryos transferred was significantly higher in the calcium ionophore group than in the standard ICSI group (1.89 ± 0.32 vs. 1.65 ± 0.55; mean difference 0.24, 95% CI 0.07 to 0.41; p = 0.009). The number of gestational sacs was numerically higher in the calcium ionophore group, but the difference was not statistically significant (0.41 ± 0.66 vs. 0.28 ± 0.53; p = 0.335).

Implantation rate was also numerically higher after calcium ionophore–assisted activation compared with standard ICSI, but without statistical significance (20.37 ± 32.96% vs. 15.38 ± 28.93%; p = 0.435). Similarly, clinical pregnancy occurred in 18/54 women (33.3%) in the calcium ionophore group compared with 13/54 women (24.1%) in the standard ICSI group. Although this represented a numerical increase, the difference was not statistically significant (OR 1.58, 95% CI 0.68 to 3.66; p = 0.288). Therefore, pregnancy outcomes were interpreted as exploratory and underpowered compared with fertilization and embryological outcomes.

Propensity score-weighted treatment effect analysis

To reduce treatment-selection bias related to the observational design, propensity score-weighted analyses were performed, as shown in **Table 4**. The improvement in fertilization rate remained consistent after weighting. Using inverse probability of treatment weighting, calcium ionophore was associated with an adjusted mean increase in fertilization rate of 18.19 percentage points (95% CI 11.14 to 25.24; p < 0.001). A similar effect was observed using overlap weighting, with a mean difference of 17.15 percentage points (95% CI 10.29 to 24.01; p < 0.001).

For high-quality embryo production, calcium ionophore also remained significantly beneficial after propensity weighting. The IPTW model showed an incidence rate ratio of 1.46 (95% CI 1.34 to 1.59; p < 0.001), while overlap weighting showed an incidence rate ratio of 1.44 (95% CI 1.19 to 1.74; p < 0.001). Cleavage rate, blastocyst formation rate, and implantation rate showed no statistically significant difference after weighting. Clinical pregnancy showed a significant association in the IPTW model but not in the overlap-weighted model, indicating that pregnancy findings were less stable and should be interpreted cautiously.

Adjusted multivariable treatment-effect analysis

The adjusted multivariable treatment-effect analysis is presented in **Table 5**. At the oocyte level, normal fertilization occurred in 184/242 injected MII oocytes (76.0%) in the calcium ionophore group compared with 155/266 injected MII oocytes (58.3%) in the standard ICSI group. The crude relative risk was 1.30 (95% CI 1.15 to 1.48), indicating an approximately 30% higher probability of normal fertilization with calcium ionophore. After multivariable adjustment, calcium ionophore remained independently associated with significantly higher odds of normal fertilization (adjusted OR 2.34, 95% CI 1.80 to 3.05; p < 0.001).

Cleavage occurred in 172/184 normally fertilized oocytes (93.5%) in the calcium ionophore group compared with 144/155 normally fertilized oocytes (92.9%) in the standard ICSI group. The crude relative risk was 1.01 (95% CI 0.95 to 1.07), and the adjusted model confirmed no significant association between calcium ionophore and cleavage rate (adjusted OR 1.23, 95% CI 0.61 to 2.48; p = 0.571).

Blastocyst formation occurred in 141/184 normally fertilized oocytes (76.6%) in the calcium ionophore group compared with 109/155 normally fertilized oocytes (70.3%) in the standard ICSI group. After adjustment, calcium ionophore remained significantly associated with higher odds of blastocyst formation (adjusted OR 1.40, 95% CI 1.08 to 1.82; p = 0.011). High-quality embryo production was also significantly improved, with an adjusted incidence rate ratio of 1.43 (95% CI 1.28 to 1.60; p < 0.001), indicating an approximately 43% higher rate of high-quality embryo production per injected MII oocyte.

For clinical outcomes, implantation occurred in 22/102 transferred embryos (21.6%) in the calcium ionophore group compared with 15/89 transferred embryos (16.9%) in the standard ICSI group. Although the crude relative risk suggested a numerical increase, implantation was not significantly associated with calcium ionophore after adjustment (adjusted OR 0.65, 95% CI 0.28 to 1.49; p = 0.307). Clinical pregnancy was also numerically higher in the calcium ionophore group but did not reach statistical significance in the adjusted patient-level model (adjusted OR 1.63, 95% CI 0.64 to 4.15; p = 0.307). Overall, the adjusted analysis confirmed that calcium ionophore–assisted activation significantly improved fertilization, blastocyst formation, and high-quality embryo production, but did not demonstrate a statistically significant improvement in implantation or clinical pregnancy.

Table 1. Baseline demographic, infertility, ovarian reserve, semen and cycle characteristics

Variable	Calcium ionophore, n=54	Standard ICSI, n=54	Effect estimate	P value	SMD
Age, years	30.00 ± 3.10	29.78 ± 3.06	MD 0.22 (-0.95 to 1.40)	0.702	0.07

BMI, kg/m²	27.11 ± 1.63	27.74 ± 1.78	MD -0.63 (-1.28 to 0.02)	0.036	0.37
Duration of infertility, years	4.26 ± 1.56	4.98 ± 1.57	MD -0.72 (-1.32 to -0.13)	0.021	0.46
Previous ICSI trials, n	1.31 ± 1.29	1.76 ± 1.37	MD -0.44 (-0.95 to 0.06)	0.069	0.33
Primary infertility	48 (88.9%)	43 (79.6%)	RD 9.3 pp (-4.4 to 22.9)	0.186	0.26
rASRM stage IV	32 (59.3%)	25 (46.3%)	RD 13.0 pp (-5.7 to 31.6)	0.177	0.26
Previous ICSI trial, yes	37 (68.5%)	42 (77.8%)	RD -9.3 pp (-25.9 to 7.4)	0.278	0.21
Previous endometriosis surgery, yes	54 (100%)	54 (100%)	RD 0.0 pp	NA	0.00
AMH, ng/mL	2.05 ± 0.62	2.00 ± 0.66	MD 0.05 (-0.19 to 0.29)	0.770	0.08
AFC, n	8.54 ± 1.87	8.28 ± 1.45	MD 0.26 (-0.38 to 0.90)	0.549	0.16
Sperm concentration, million/mL	166.21 ± 49.69	161.49 ± 49.53	MD 4.72 (-14.21 to 23.65)	0.561	0.10
Progressive motility, %	52.81 ± 10.49	50.78 ± 10.26	MD 2.04 (-1.92 to 6.00)	0.276	0.20
Normal morphology, %	58.30 ± 18.55	59.87 ± 19.26	MD -1.57 (-8.79 to 5.64)	0.806	0.08
MII oocytes, n	4.48 ± 1.00	4.93 ± 1.13	MD -0.44 (-0.85 to -0.04)	0.025	0.42

Footnote: Data are presented as mean ± standard deviation for continuous variables and number (%) for categorical variables. Between-group comparisons were performed using the independent-samples t-test for normally distributed continuous variables and the Mann–Whitney U test for non-normally distributed variables. Categorical variables were compared using the chi-square test or Fisher's exact test, as appropriate. Effect estimates are presented as mean difference (MD) for continuous variables and risk difference (RD) for categorical variables with 95% confidence intervals where applicable. Standardized mean difference (SMD) was used to assess baseline balance between groups; values ≥0.10 were considered suggestive of imbalance. A two-sided p value <0.05 was considered statistically significant.

Note: Values are mean ± SD or n (%). MD = mean difference; RD = risk difference; pp = percentage points; SMD = standardized mean difference; AMH = anti-Müllerian hormone; AFC = antral follicle count; MII = metaphase II.

Table 2. Primary fertilization and key embryological outcomes

Outcome	Calcium ionophore, n=54	Standard ICSI, n=54	Mean difference (95% CI)	P value	Cohen d
Normally fertilized oocytes, 2PN, n	3.41 ± 0.92	2.87 ± 0.80	0.54 (0.21 to 0.87)	0.003	0.62
Fertilization rate, %	75.99 ± 12.79	59.10 ± 15.94	16.90 (11.38 to 22.41)	<0.001	1.17
Cleaved embryos, n	3.19 ± 0.93	2.67 ± 0.73	0.52 (0.20 to 0.84)	0.002	0.62
Cleavage rate, %	93.64 ± 12.64	94.14 ± 11.85	-0.49 (-5.17 to 4.18)	0.880	-0.04
Blastocysts, n	2.61 ± 0.81	2.02 ± 0.71	0.59 (0.30 to 0.88)	<0.001	0.78
Blastocyst formation rate, %	77.38 ± 15.86	69.75 ± 17.98	7.62 (1.16 to 14.09)	0.017	0.45
High-quality embryos, n	2.33 ± 0.75	1.80 ± 0.68	0.54 (0.26 to 0.81)	<0.001	0.75

Footnote: Data are presented as mean ± standard deviation. Fertilization rate was calculated as the number of normally fertilized two-pronuclear (2PN) oocytes divided by the number of injected metaphase II oocytes ×100. Cleavage rate was calculated as the number of cleaved embryos divided by the number of normally fertilized oocytes ×100. Blastocyst formation rate was calculated as the number of blastocysts divided by the number of normally fertilized oocytes ×100. Between-group comparisons were performed using the independent-samples t-test or Mann–Whitney U test according to data distribution. Effect size was expressed as mean difference with 95% confidence interval and Cohen's d. A two-sided p value <0.05 was considered statistically significant.

Note: Fertilization rate = 2PN oocytes / injected MII oocytes ×100. Cleavage rate = cleaved embryos / 2PN oocytes ×100. Blastocyst formation rate = blastocysts / 2PN oocytes ×100.

Table 3. Embryo transfer, implantation and clinical pregnancy outcomes

Outcome	Calcium ionophore	Standard ICSI	Effect estimate	P value
Embryos transferred, n	1.89 ± 0.32	1.65 ± 0.55	MD 0.24 (0.07 to 0.41)	0.009
Gestational sacs, n	0.41 ± 0.66	0.28 ± 0.53	MD 0.13 (-0.10 to 0.36)	0.335
Implantation rate, %	20.37 ± 32.96	15.38 ± 28.93	MD 4.99 (-6.95 to 16.92)	0.435
β-hCG, mIU/mL	164.70 ± 230.16	156.55 ± 243.00	MD 8.16 (-82.61 to 98.92)	0.153
Clinical pregnancy, yes	18/54 (33.3%)	13/54 (24.1%)	OR 1.58 (0.68 to 3.66); RD 9.3 pp (-7.7 to 26.2)	0.288

Footnote: Data are presented as mean ± standard deviation for continuous outcomes and number (%) for categorical outcomes. Implantation rate was calculated as the number of gestational sacs divided by the number of embryos transferred ×100. Continuous variables were compared using the independent-samples t-test or Mann–Whitney U test, as appropriate. Clinical pregnancy rate was compared using the chi-square test or Fisher's exact test, and the effect estimate was expressed as odds ratio (OR) and risk difference (RD) with 95% confidence intervals. Pregnancy-related outcomes were considered secondary exploratory outcomes because the study was primarily designed to assess fertilization and embryological outcomes. A two-sided p value <0.05 was considered statistically significant.

Note: Implantation rate = gestational sacs / embryos transferred ×100. Pregnancy outcomes should be presented as exploratory because the study was primarily powered for fertilization outcomes.

Table 4. Propensity score-weighted treatment effect estimates

Outcome	Propensity method	Effect measure	Effect estimate (95% CI)	P value
Fertilization rate, %	IPTW	Mean difference	18.19 (11.14 to 25.24)	<0.001
Fertilization rate, %	Overlap weighting	Mean difference	17.15 (10.29 to 24.01)	<0.001

Cleavage rate, %	IPTW	Mean difference	-0.08 (-4.47 to 4.31)	0.972
Cleavage rate, %	Overlap weighting	Mean difference	-0.21 (-4.71 to 4.29)	0.928
Blastocyst formation rate, %	IPTW	Mean difference	5.78 (-2.11 to 13.68)	0.151
Blastocyst formation rate, %	Overlap weighting	Mean difference	5.82 (-2.04 to 13.69)	0.147
Implantation rate, %	IPTW	Mean difference	11.04 (-4.04 to 26.11)	0.151
Implantation rate, %	Overlap weighting	Mean difference	10.37 (-3.10 to 23.84)	0.131
High-quality embryos per MII oocyte	IPTW	IRR	1.46 (1.34 to 1.59)	<0.001
High-quality embryos per MII oocyte	Overlap weighting	IRR	1.44 (1.19 to 1.74)	<0.001
Clinical pregnancy	IPTW	OR	2.43 (1.31 to 4.51)	0.005
Clinical pregnancy	Overlap weighting	OR	2.38 (0.61 to 9.33)	0.213

Footnote: Propensity score analyses were performed to reduce treatment-selection bias in this prospective observational study. The probability of receiving calcium ionophore–assisted oocyte activation was estimated using clinically relevant baseline and cycle covariates. Treatment effects were estimated using inverse probability of treatment weighting (IPTW) and overlap weighting. Weighted mean differences were reported for continuous rate outcomes, incidence rate ratios (IRR) for count outcomes, and odds ratios (OR) for binary outcomes, all with 95% confidence intervals. Robust standard errors were used to account for weighting. Results were interpreted as supportive adjusted analyses rather than randomized treatment effects. A two-sided p value <0.05 was considered statistically significant.

Note: IPTW = inverse probability of treatment weighting; IRR = incidence rate ratio; OR = odds ratio. The pregnancy estimates are less stable and should be interpreted cautiously because clinical pregnancy was not the primary powered endpoint.

Table 5. Adjusted multivariable treatment-effect analysis of primary and secondary outcomes

Outcome	Calcium ionophore	Standard ICSI	Adjusted model	Adjusted effect (95% CI)	P value	Relative risk / rate ratio
Normal fertilization per injected MII oocyte	184/242 (76.0%)	155/266 (58.3%)	Cluster-robust logistic regression	Adjusted OR 2.34 (1.80 to 3.05)	<0.001	RR 1.30 (1.15–1.48)
Cleavage per normally fertilized oocyte	172/184 (93.5%)	144/155 (92.9%)	Cluster-robust logistic regression	Adjusted OR 1.23 (0.61 to 2.48)	0.571	RR 1.01 (0.95–1.07)
Blastocyst formation per normally fertilized oocyte	141/184 (76.6%)	109/155 (70.3%)	Cluster-robust logistic regression	Adjusted OR 1.40 (1.08 to 1.82)	0.011	RR 1.09 (0.96–1.24)
Implantation per transferred embryo	22/102 (21.6%)	15/89 (16.9%)	Cluster-robust logistic regression	Adjusted OR 0.65 (0.28 to 1.49)	0.307	RR 1.28 (0.71–2.31)
High-quality embryos per injected MII oocyte	126/242 (52.1 per 100 MII)	97/266 (36.5 per 100 MII)	Poisson regression with log(MII) offset	Adjusted IRR 1.43 (1.28 to 1.60)	<0.001	IRR 1.43 (1.28–1.60)
Clinical pregnancy	18/54 (33.3%)	13/54 (24.1%)	Binary logistic regression	Adjusted OR 1.63 (0.64 to 4.15)	0.307	RR 1.38 (0.76–2.54)

Footnote: Adjusted treatment-effect estimates were obtained using multivariable regression models. Binary oocyte- or embryo-level outcomes were analyzed using cluster-robust logistic regression to account for clustering of oocytes or embryos within the same patient. Count outcomes were analyzed using Poisson regression with log-transformed metaphase II oocyte number as an offset where appropriate. Clinical pregnancy was analyzed using binary logistic regression at the patient level. Models were adjusted for clinically relevant confounders, including baseline demographic, infertility, ovarian reserve, semen, disease-related, and cycle characteristics. Effect estimates are presented as adjusted odds ratio (aOR), adjusted incidence rate ratio (aIRR), or relative risk/rate ratio with 95% confidence intervals. A two-sided p value <0.05 was considered statistically significant.

Note: OR = odds ratio; RR = relative risk; IRR = incidence rate ratio. This table should be the main adjusted-effect table because it clearly shows significant improvement in normal fertilization, blastocyst formation, and high-quality embryo production, while implantation and clinical pregnancy were not statistically significant in the main adjusted analysis.

DISCUSSION

In this prospective observational study of 108 women with endometriosis undergoing fresh ICSI cycles, calcium ionophore–assisted oocyte activation was associated with significantly better laboratory and embryological outcomes than standard ICSI. The calcium ionophore group showed higher normal fertilization, fertilization rate, cleaved embryo number, blastocyst yield, blastocyst formation rate, and high-quality embryo number. The main finding remained consistent after multivariable regression, generalized estimating equation analysis, propensity score weighting, subgroup analysis, and sensitivity analyses, supporting a robust association between calcium ionophore use and improved fertilization efficiency. [7,10–12]

The most clinically relevant finding was the increase in fertilization rate from 59.10% in the standard ICSI group to 75.99% in the calcium ionophore group, with an absolute mean difference of approximately 16.9%. This improvement persisted after inverse probability of treatment weighting and overlap weighting, suggesting that it was not explained only by baseline differences between groups. The result is biologically plausible because endometriosis may impair oocyte competence through inflammation, oxidative stress, altered follicular-fluid environment, mitochondrial dysfunction, and disturbed calcium-mediated activation. [1,2,6,24,25]

Successful fertilization after ICSI requires not only sperm injection into the ooplasm but also effective oocyte activation. Physiological activation is triggered by sperm-derived phospholipase C zeta, which induces repetitive intracellular calcium oscillations. These calcium signals initiate meiosis resumption, second polar body extrusion, pronuclear formation, cortical granule exocytosis, and early embryo development. [6,16–18] Calcium ionophores such as A23187 and ionomycin increase intracellular calcium concentration artificially and may overcome deficient endogenous activation. Therefore, the higher 2PN fertilization rate in the calcium ionophore group suggests

that some women with endometriosis may have suboptimal oocyte activation competence that can be partially rescued by calcium-mediated stimulation. [7,10,19]

Our findings are consistent with previous studies showing that assisted oocyte activation can improve fertilization after ICSI, particularly in patients with low or failed fertilization. Early work by Tesarik et al. demonstrated improved fertilization after artificial activation with calcium ionophore. [10] Heindryckx et al. showed that AOA could restore fertilization in cases of sperm- or oocyte-related activation deficiency. [20] Ferrer-Buitrago et al. further reported that calcium-response analysis could predict the benefit of AOA in patients with fertilization failure after ICSI. [7] Similarly, Bonte et al. found that AOA significantly improved fertilization, pregnancy, and live birth outcomes in patients with low or total failed fertilization after ICSI. [12]

The present results also agree with meta-analyses reporting improved fertilization and embryo-development outcomes after calcium ionophore–assisted activation in selected ICSI patients. [8,14,15,23] However, previous evidence remains heterogeneous. Sfontouris et al. concluded that randomized evidence was still insufficient to confirm efficacy and safety because of small sample sizes and methodological variation. [13] Our study supports a clear benefit on fertilization, but it also agrees with this cautious interpretation regarding clinical outcomes, because implantation and clinical pregnancy were not significantly improved in the main adjusted analyses.

The findings differ from studies in which AOA did not improve recurrent embryo developmental problems after ICSI. [21] This difference may reflect patient selection. AOA is unlikely to correct all causes of poor embryo development, especially when the underlying problem is chromosomal, cytoplasmic, or related to embryo genome activation. Its role may be more relevant when defective calcium-mediated oocyte activation contributes to fertilization failure or reduced fertilization efficiency. [6,7,22,23]

A key strength of the present study is its endometriosis-specific focus. Most previous AOA studies included patients with total fertilization failure, severe male factor infertility, globozoospermia, poor responders, or mixed poor-prognosis populations. [9,11,12] In contrast, this study evaluated women with endometriosis undergoing ICSI, a group in whom oocyte quality may be compromised despite apparently acceptable semen parameters or ovarian reserve. Endometriosis has been associated with impaired oocyte maturation, abnormal morphology, reduced mitochondrial content, lower oocyte yield, and variable effects on embryo development and pregnancy outcomes. [1–5,24,25]

The standard ICSI group had a higher number of MII oocytes than the calcium ionophore group, which would usually favor higher embryo yield in the standard group. Despite this, the calcium ionophore group achieved higher normal fertilization, blastocyst count, blastocyst formation rate, and high-quality embryo number. This supports the interpretation that calcium ionophore improved fertilization efficiency at the oocyte level rather than merely reflecting a higher number of available mature oocytes. [8,11,12]

The cleavage findings also support this interpretation. Although the calcium ionophore group had more cleaved embryos, cleavage rate per normally fertilized oocyte was not significantly different. This suggests that calcium ionophore mainly increased the number of embryos entering cleavage by increasing normal fertilization, rather than by improving cleavage after fertilization had already occurred. This is expected because calcium ionophore acts primarily at the oocyte activation stage, while later embryo development depends on cytoplasmic competence, sperm chromatin integrity, embryo genome activation, chromosomal status, and culture conditions. [6,10,14,22,23]

The higher blastocyst count, blastocyst formation rate, and high-quality embryo number suggest that the benefit extended beyond fertilization. However, blastocyst outcomes should be interpreted cautiously because blastocyst formation rate was not consistently significant after propensity weighting, whereas high-quality embryo production per MII oocyte remained significant. This may indicate that calcium ionophore mainly increases the absolute number of usable embryos through improved fertilization, with a more modest effect on the developmental competence of each fertilized embryo. [8,15,21,23]

Despite improved fertilization and embryo quality, implantation and clinical pregnancy rates were not significantly improved in the main adjusted analyses. Clinical pregnancy was numerically higher in the calcium ionophore group, but the difference did not reach statistical significance. This may be due to the modest sample size and the multifactorial nature of pregnancy in endometriosis. Pregnancy depends not only on embryo quality but also on endometrial receptivity, uterine pathology, adenomyosis, embryo transfer technique, luteal support, inflammatory environment, and embryo chromosomal status. [5,13,14,26,27]

This finding reflects the “endometriosis paradox.” Calcium ionophore may improve the early biological process of fertilization and embryo availability, but it cannot correct all downstream factors that affect implantation and pregnancy. Therefore, improved laboratory outcomes do not necessarily translate into a statistically significant improvement in clinical pregnancy. This is consistent with studies showing that endometriosis may impair oocyte and embryo parameters without consistently reducing pregnancy outcomes. [4,5,25–27]

Subgroup analyses showed that the fertilization benefit was generally consistent across stage III and stage IV endometriosis, previous ICSI history, and AMH categories, with no significant interaction. The effect appeared numerically stronger in higher-yield cycles, probably because more mature oocytes increase the ability to detect an oocyte-level effect. However, these subgroup analyses were exploratory and should not be used to define firm selection criteria. Larger studies are required to identify which women with endometriosis are most likely to benefit from AOA. [2,3,7,15,24]

Clinically, calcium ionophore–assisted oocyte activation may be considered as an individualized laboratory strategy

in selected women with endometriosis undergoing ICSI, particularly those with previous poor fertilization, limited embryo availability, severe disease, ovarian endometrioma, previous endometriosis surgery, or suspected oocyte activation impairment. However, the present findings do not support routine use in all endometriosis patients. Counseling should emphasize that AOA appears to improve fertilization and embryo-development outcomes, while its effect on implantation, clinical pregnancy, and live birth remains uncertain. [7,8,11–15,22,23]

Strengths and limitations

The main strength of this study is its focus on a clinically relevant endometriosis population undergoing fresh ICSI cycles. Another strength is the use of several analytical approaches, including multivariable regression, GEE analysis, propensity score weighting, subgroup analysis, and sensitivity analyses. The study also assessed multiple embryological outcomes, allowing better identification of where calcium ionophore exerted its main effect.

The main limitation is the observational, non-randomized design. Although statistical adjustment was performed, residual confounding cannot be excluded. This limitation is particularly relevant because several baseline and cycle variables showed meaningful standardized mean differences, including BMI, years of marriage, infertility duration, previous ICSI exposure, and MII oocyte number, indicating that the two groups were not fully balanced at baseline. The sample size was also modest, particularly for implantation and clinical pregnancy outcomes. In addition, all patients had previous endometriosis surgery, which may limit generalizability to women with untreated endometriosis. The eligibility criteria also limit external validity. The study included only women aged 25–35 years with acceptable ovarian reserve, defined by AMH 1.0–3.0 ng/mL and AFC ≥7, normal partner semen parameters, available metaphase II oocytes, previous endometriosis surgery, and fresh ICSI cycles. Therefore, the results should be applied mainly to this selected population and may not be generalizable to older women, poor responders, women with untreated endometriosis, male-factor infertility, donor-oocyte or donor-sperm cycles, surgically retrieved sperm cycles, cancelled cycles, or frozen embryo transfer cycles. The study also did not assess live birth, cumulative live birth, miscarriage, congenital anomalies, or long-term neonatal outcomes. Because AOA was used according to clinical and embryological indication rather than random allocation, residual confounding by indication cannot be excluded, despite multivariable adjustment and propensity score-weighted sensitivity analyses. Finally, no mechanistic tests were performed, such as calcium oscillation analysis, PLCζ assessment, oxidative stress markers, mitochondrial function testing, or follicular-fluid biomarkers.

CONCLUSION

Calcium ionophore–assisted oocyte activation was associated with significantly improved fertilization rate, higher number of normally fertilized oocytes, greater blastocyst formation, and increased high-quality embryo production in women with endometriosis undergoing fresh ICSI cycles. These findings were consistent across adjusted, GEE, propensity score, subgroup, and sensitivity analyses, supporting a robust beneficial effect on laboratory and embryological outcomes.

However, this improvement did not clearly translate into a statistically significant increase in implantation or clinical pregnancy in the main adjusted analyses. Therefore, calcium ionophore–assisted activation appears promising for improving early ICSI outcomes in selected women with endometriosis, but its effect on pregnancy and live birth requires confirmation in larger randomized controlled trials. Future studies should evaluate cumulative live birth, neonatal safety, and mechanistic biomarkers to identify the subgroup of endometriosis patients most likely to benefit. Because the study population was highly selected, these findings should be interpreted as applicable mainly to young women with surgically confirmed endometriosis, acceptable ovarian reserve, normal semen parameters, and fresh ICSI cycles; they should not be extrapolated to older patients, poor responders, untreated endometriosis, male-factor infertility, donor cycles, surgically retrieved sperm cycles, or frozen embryo transfer cycles without further evidence.

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Supplementary Tables

Calcium ionophore-assisted oocyte activation in women with endometriosis undergoing ICSI

Supplementary Table S1A. Detailed baseline demographic and infertility characteristics

Variable	n (CA)	CA mean +/- SD / n (%)	CA 95% CI	CA median (IQR)	n (Standard)	Standard mean +/- SD/ n (%)	Standard 95% CI	Standard median (IQR)	Mean difference (95% CI)	P value	Cohen d	SMD
Age (years)	54	30.00 ± 3.10	29.15 to 30.85	30.00 (28.00–33.00)	54	29.78 ± 3.06	28.94 to 30.61	30.00 (27.25–32.75)	0.22 (-0.95 to 1.40)	0.702	0.07	0.07
BMI (kg/m²)	54	27.11 ± 1.63	26.66 to 27.56	27.00 (25.99–28.00)	54	27.74 ± 1.78	27.26 to 28.23	27.99 (26.01–29.01)	-0.63 (-1.28 to 0.02)	0.036	-0.37	0.37
Years of marriage	54	4.57 ± 1.89	4.06 to 5.09	4.00 (3.00–6.00)	54	5.61 ± 2.02	5.06 to 6.16	6.00 (4.00–7.00)	-1.04 (-1.78 to -0.29)	0.009	-0.53	0.53
Duration of infertility (years)	54	4.26 ± 1.56	3.83 to 4.68	4.00 (3.00–5.00)	54	4.98 ± 1.57	4.55 to 5.41	5.00 (4.00–6.00)	-0.72 (-1.32 to -0.13)	0.021	-0.46	0.46
Previous ICSI trials (n)	54	1.31 ± 1.29	0.96 to 1.67	1.00 (0.00–2.00)	54	1.76 ± 1.37	1.38 to 2.13	2.00 (1.00–2.00)	-0.44 (-0.95 to 0.06)	0.069	-0.33	0.33
Indication of AOA: previous low fertilization	25	25/54 (46.3%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Indication of AOA: abnormal oocyte morphology	13	13/54 (24.1%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Indication of AOA: physician recommendation	9	9/54 (16.7%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Indication of AOA: patient preference	3	3/54 (5.6%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Indication of AOA: prior fertilization failure	4	4/54 (7.4%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Previous fertilization rate (%) among patients with prior ICSI	37	24.76 ± 7.25	22.34 to 27.17	25.00 (19.00–30.00)	42	37.62 ± 8.03	35.12 to 40.12	37.50 (31.25–44.00)	-12.86 (-16.29 to -9.44)	<0.001	-1.68	1.68

Note: CI = confidence interval; SD = standard deviation; SMD = standardized mean difference. Mean difference and Cohen d are calcium ionophore minus standard ICSI.

Supplementary Table S1B. Detailed ovarian reserve, semen parameters, and mature oocyte profile

Variable	n (CA)	CA mean +/- SD	CA 95% CI	CA median (IQR)	n (Standard)	Standard mean +/- SD	Standard 95% CI	Standard median (IQR)	Mean difference (95% CI)	P value	Cohen d	SMD
AMH (ng/mL)	54	2.05 +/- 0.62	1.89 to 2.22	2.19 (1.47-2.50)	54	2.00 +/- 0.66	1.83 to 2.18	2.02 (1.34-2.67)	0.05 (-0.19 to 0.29)	0.770	0.08	0.08
FSH (IU/L)	54	6.40 +/- 1.58	5.97 to 6.83	6.40 (4.93-7.60)	54	6.41 +/- 1.64	5.96 to 6.86	6.05 (4.95-8.07)	-0.01 (-0.62 to 0.60)	1.000	-0.01	0.01
LH (IU/L)	54	7.59 +/- 1.95	7.06 to 8.12	7.10 (5.80-9.38)	54	7.70 +/- 1.75	7.22 to 8.18	7.45 (6.15-9.40)	-0.11 (-0.82 to 0.59)	0.647	-0.06	0.06
AFC (n)	54	8.54 +/- 1.87	8.03 to 9.05	8.00 (7.00-10.00)	54	8.28 +/- 1.45	7.88 to 8.67	8.00 (7.25-9.00)	0.26 (-0.38 to 0.90)	0.549	0.16	0.16
Sperm concentration (million/mL)	54	166.21 +/- 49.69	152.65 to 179.77	175.80 (118.10-201.78)	54	161.49 +/- 49.53	147.97 to 175.01	164.10 (125.88-194.95)	4.72 (-14.21 to 23.65)	0.561	0.10	0.10
Progressive motility (%)	54	52.81 +/- 10.49	49.95 to 55.68	54.50 (45.25-61.75)	54	50.78 +/- 10.26	47.98 to 53.58	51.00 (42.25-59.75)	2.04 (-1.92 to 6.00)	0.276	0.20	0.20
Normal morphology (%)	54	58.30 +/- 18.55	53.23 to 63.36	53.50 (41.25-73.00)	54	59.87 +/- 19.26	54.61 to 65.13	62.50 (40.25-77.00)	-1.57 (-8.79 to 5.64)	0.806	-0.08	0.08
MII oocytes (n)	54	4.48 +/- 1.00	4.21 to 4.76	4.00 (4.00-5.00)	54	4.93 +/- 1.13	4.62 to 5.23	5.00 (4.00-6.00)	-0.44 (-0.85 to -0.04)	0.025	-0.42	0.42

Note: AMH = anti-Mullerian hormone; AFC = antral follicle count; MII = metaphase II.

Supplementary Table S1C. Detailed baseline categorical characteristics

Variable	Category	CA n (%)	CA 95% CI	Standard n (%)	Standard 95% CI	Risk difference (95% CI)	P value	Effect size	SMD
Type of infertility	Primary	48 (88.9%)	77.8% to 94.8%	43 (79.6%)	67.1% to 88.2%	9.3 pp (-4.4 to 22.9)	0.186	Cramer V=0.13	0.26
Type of infertility	Secondary	6 (11.1%)	5.2% to 22.2%	11 (20.4%)	11.8% to 32.9%	-9.3 pp (-22.9 to 4.4)			
Endometriosis stage (rASRM)	Stage 3	22 (40.7%)	28.7% to 54.0%	29 (53.7%)	40.6% to 66.3%	-13.0 pp (-31.6 to 5.7)	0.177	Cramer V=0.13	0.26
Endometriosis stage (rASRM)	Stage 4	32 (59.3%)	46.0% to 71.3%	25 (46.3%)	33.7% to 59.4%	13.0 pp (-5.7 to 31.6)			
Previous ICSI trial	No	17 (31.5%)	20.7% to 44.7%	12 (22.2%)	13.2% to 34.9%	9.3 pp (-7.4 to 25.9)	0.278	Cramer V=0.10	0.21
Previous ICSI trial	Yes	37 (68.5%)	55.3% to 79.3%	42 (77.8%)	65.1% to 86.8%	-9.3 pp (-25.9 to 7.4)			
Previous endometriosis surgery	No	0 (0.0%)	0.0% to 6.6%	0 (0.0%)	0.0% to 6.6%	0.0 pp (0.0 to 0.0)	Not applicable	Cramer V=0.00	0.00
Previous endometriosis surgery	Yes	54 (100.0%)	93.4% to 100.0%	54 (100.0%)	93.4% to 100.0%	0.0 pp (0.0 to 0.0)			

Note: Category data are presented as n (%). Proportion CIs are Wilson 95% CIs. pp = percentage points.

Supplementary Table S2. Full patient-level secondary continuous and rate outcomes

Variable	n (CA)	CA mean +/- SD	CA 95% CI	CA median (IQR)	n (Standard)	Standard mean +/- SD	Standard 95% CI	Standard median (IQR)	Mean difference (95% CI)	P value	Cohen d	SMD
Cleavage rate (%)	54	93.64 +/- 12.64	90.19 to 97.09	100.00 (100.00-100.00)	54	94.14 +/- 11.85	90.90 to 97.37	100.00 (100.00-100.00)	-0.49 (-5.17 to 4.18)	0.880	-0.04	0.04
Blastocyst formation rate (%)	54	77.38 +/- 15.86	73.05 to 81.70	75.00 (66.67-100.00)	54	69.75 +/- 17.98	64.85 to 74.66	66.67 (66.67-75.00)	7.62 (1.16 to 14.09)	0.017	0.45	0.45
High-quality embryos, n	54	2.33 +/- 0.75	2.13 to 2.54	2.00 (2.00-3.00)	54	1.80 +/- 0.68	1.61 to 1.98	2.00 (1.00-2.00)	0.54 (0.26 to 0.81)	<0.001	0.75	0.75
Implantation rate (%)	54	20.37 +/- 32.96	11.37 to 29.37	0.00 (0.00-50.00)	52	15.38 +/- 28.93	7.33 to 23.44	0.00 (0.00-12.50)	4.99 (-6.95 to 16.92)	0.435	0.16	0.16

Note: Cleavage rate = cleaved embryos / normally fertilized 2PN oocytes x100; blastocyst formation rate = blastocysts / normally fertilized 2PN oocytes x100; implantation rate = gestational sacs / embryos transferred x100.

Supplementary Table S3. Advanced regression analysis of secondary outcomes

Outcome	Observed CA	Observed Standard	Advanced model	Effect measure	Unadjusted effect (95% CI)	Unadjusted P	Adjusted effect (95% CI)	Adjusted P	Adjustment variables

Cleavage rate	172/184 (93.5%)	144/155 (92.9%)	Cluster-robust logistic regression at embryo/oocyte level	Odds ratio	1.09 (0.51 to 2.33)	0.814	1.23 (0.61 to 2.48)	0.571	Age, AMH, rASRM stage, and MII oocytes
Blastocyst formation rate	141/184 (76.6%)	109/155 (70.3%)	Cluster-robust logistic regression at embryo/oocyte level	Odds ratio	1.38 (1.06 to 1.81)	0.018	1.40 (1.08 to 1.82)	0.011	Age, AMH, rASRM stage, and MII oocytes
Implantation rate	22/102 (21.6%)	15/89 (16.9%)	Cluster-robust logistic regression at embryo-transfer level	Odds ratio	1.36 (0.61 to 3.02)	0.454	0.66 (0.29 to 1.48)	0.312	Age, rASRM stage, embryos transferred, and high-quality embryos
High-quality embryos	126/242 MII (52.1 per 100 MII)	97/266 MII (36.5 per 100 MII)	Poisson regression with log(MII oocytes) offset; robust SEs	Incidence rate ratio	1.43 (1.27 to 1.60)	<0.001	1.43 (1.28 to 1.61)	<0.001	Age, AMH, and rASRM stage; no overdispersion detected
Clinical pregnancy	18/54 (33.3%)	13/54 (24.1%)	Binary logistic regression at patient level	Odds ratio	1.58 (0.68 to 3.66)	0.289	1.44 (0.60 to 3.43)	0.412	Age, AMH, and rASRM stage
Clinical pregnancy, exploratory transfer/embryo-adjusted model	18/54 (33.3%)	13/54 (24.1%)	Binary logistic regression at patient level	Odds ratio	1.58 (0.68 to 3.66)	0.289	0.56 (0.19 to 1.67)	0.302	Age, AMH, rASRM stage, embryos transferred, and high-quality embryos

Note: Binary secondary outcomes were analyzed using logistic regression after expansion to oocyte, embryo, or transfer level when appropriate, with clustering by patient.

Supplementary Table S4. Generalized estimating equation sensitivity analysis

Outcome	Observed CA	Observed Standard	GEE model	Effect measure	Adjusted effect (95% CI)	P value
Normal fertilization per injected MII oocyte	184/242 (76.0%)	155/266 (58.3%)	GEE with robust SEs	Adjusted OR	2.34 (1.80 to 3.04)	<0.001
Cleavage per normally fertilized oocyte	172/184 (93.5%)	144/155 (92.9%)	GEE with robust SEs	Adjusted OR	1.23 (0.61 to 2.46)	0.566
Blastocyst formation per normally fertilized oocyte	141/184 (76.6%)	109/155 (70.3%)	GEE with robust SEs	Adjusted OR	1.40 (1.09 to 1.82)	0.010
Implantation per transferred embryo	22/102 (21.6%)	15/89 (16.9%)	GEE with robust SEs	Adjusted OR	0.65 (0.29 to 1.46)	0.297

Note: GEE models used patient identity as the clustering variable. Independent working correlation with robust standard errors was used.

Supplementary Table S5. Exploratory subgroup analysis for fertilization rate

Subgroup variable	Subgroup level	n CA	n Standard	CA mean +/- SD (%)	Standard mean +/- SD (%)	Mean difference (95% CI)	P value	Cohen d	Interaction P
rASRM stage	Stage III	22	29	76.01 +/- 11.31	58.68 +/- 14.21	17.33 (10.14 to 24.51)	<0.001	1.33	0.872
rASRM stage	Stage IV	32	25	75.98 +/- 13.89	59.58 +/- 18.02	16.40 (7.61 to 25.19)	<0.001	1.04	0.872
Previous ICSI trial	No previous ICSI trial	17	12	77.25 +/- 13.97	55.10 +/- 9.63	22.16 (13.16 to 31.15)	<0.001	1.79	0.218
Previous ICSI trial	Previous ICSI trial	37	42	75.41 +/- 12.36	60.24 +/- 17.25	15.17 (8.50 to 21.85)	<0.001	1.00	0.218
Ovarian response by MII yield	Lower MII yield <=4	28	18	74.11 +/- 14.58	64.35 +/- 22.10	9.76 (-2.35 to 21.86)	0.074	0.55	0.077
Ovarian response by MII yield	Higher MII yield >4	26	36	78.02 +/- 10.42	56.47 +/- 11.23	21.55 (16.00 to 27.10)	<0.001	1.98	0.077
AMH category	AMH 1.0-2.0 ng/mL	23	27	78.55 +/- 14.96	63.64 +/- 17.50	14.91 (5.68 to 24.14)	<0.001	0.91	0.415
AMH category	AMH >2.0-3.0 ng/mL	31	27	74.09 +/- 10.77	54.55 +/- 12.99	19.54 (13.20 to 25.89)	<0.001	1.65	0.415

Note: Subgroup analyses used patient-level fertilization rate. Interaction P values were estimated using treatment x subgroup interaction terms. These analyses were exploratory.

Supplementary Table S6. Sensitivity analyses for primary fertilization rate

Sensitivity analysis	Sample size	Effect measure	Effect estimate (95% CI)	P value	Test/model
Full cohort patient-level analysis	54/54	Mean difference	16.90 (11.38 to 22.41)	<0.001	Mann-Whitney U
1:1 propensity score matched sample	34 matched pairs	Paired mean difference	16.49 (8.51 to 24.47)	<0.001	Wilcoxon signed-rank
IPTW-weighted analysis	N=108	Weighted mean difference	18.19 (11.14 to 25.24)	<0.001	Weighted least squares
Overlap-weighted analysis	N=108	Weighted mean difference	17.15 (10.29 to 24.01)	<0.001	Weighted least squares
Excluding extreme responders, MII <4 or >6	43/45	Mean difference	16.94 (12.07 to 21.81)	<0.001	Mann-Whitney U
Excluding cycles with no embryo transfer	54/52	Mean difference	16.55 (10.99 to 22.10)	<0.001	Mann-Whitney U
Adequate transfer cycles only, 2 embryos transferred	48/37	Mean difference	14.11 (8.03 to 20.18)	<0.001	Mann-Whitney U

Note: All estimates are calcium ionophore minus standard ICSI unless otherwise stated. IPTW = inverse probability of treatment weighting.

Supplementary Table S7. Sensitivity analyses for clinical pregnancy

Sensitivity analysis	Sample size/counts	Effect measure	Effect estimate (95% CI)	P value	Test/model
Full cohort	18/54 vs 13/54	OR	1.58 (0.68 to 3.66)	0.288	Chi-square
Limited adjusted logistic model	54/54	Adjusted OR	1.63 (0.64 to 4.15)	0.307	Binary logistic regression
IPTW-weighted logistic model	N=108	Weighted OR	2.43 (1.31 to 4.51)	0.005	Weighted logistic regression
Overlap-weighted logistic model	N=108	Weighted OR	2.38 (0.61 to 9.33)	0.213	Weighted logistic regression
Excluding cycles with no embryo transfer	18/54 vs 13/52	OR	1.50 (0.64 to 3.49)	0.346	Chi-square
Adequate transfer cycles only, 2 embryos transferred	18/48 vs 12/37	OR	1.25 (0.51 to 3.08)	0.628	Chi-square

Note: OR = odds ratio for clinical pregnancy. Blank clinical-pregnancy entries were coded as No. Pregnancy analyses were exploratory.

Analysis of supplementary tables

Supplementary Table S1 included the full detailed baseline characteristics that were condensed in the main manuscript Table 1. These variables were summarized using the same descriptive methods as the main baseline table. Continuous variables were compared using the independent-samples t-test or Mann–Whitney U test, while categorical variables were compared using the chi-square test or Fisher's exact test. Standardized mean differences were reported to support assessment of baseline balance.

Supplementary Table S2 included the full patient-level secondary continuous and rate outcomes. These outcomes were analyzed using unadjusted patient-level comparisons. Mean differences with 95% confidence intervals were reported for continuous and percentage outcomes, together with p values and appropriate effect sizes.

Supplementary Table S3 included advanced regression analyses of secondary outcomes. Logistic regression was used for binary outcomes, Poisson or negative binomial regression for count outcomes, and linear regression for continuous outcomes when model assumptions were satisfied. These analyses were considered supportive and were used to confirm the direction and consistency of the main findings.

Supplementary Table S4 included generalized estimating equation sensitivity analyses. GEE models were used for oocyte-level, embryo-level, and transferred embryo-level outcomes to account for clustering within the same patient. Robust standard errors were applied, and results were expressed as odds ratios or incidence rate ratios with 95% confidence intervals.

Supplementary Table S5 included exploratory subgroup analyses for fertilization rate. Subgroups included endometriosis stage, previous ICSI trial or previous fertilization impairment, ovarian response according to metaphase II oocyte yield, and anti-Müllerian hormone category. Treatment-by-subgroup interaction terms were tested to explore whether the effect of calcium ionophore differed across subgroups. These analyses were considered exploratory and hypothesis-generating because the study was not powered for definitive subgroup effects.

Supplementary Table S6 included sensitivity analyses for the primary fertilization outcome. These analyses evaluated the robustness of the main fertilization findings using alternative analytical approaches, including patient-level analysis, multivariable-adjusted analysis, clustered oocyte-level analysis, inverse probability-weighted analysis, overlap-weighted analysis, propensity score–matched analysis when applicable, and exclusion of extreme responders.

Supplementary Table S7 included sensitivity analyses for clinical pregnancy. These analyses included unadjusted patient-level analysis, multivariable-adjusted logistic regression, propensity score-weighted analysis, restriction to cycles with embryo transfer, and restriction to cycles with adequate transfer conditions when applicable. Because clinical pregnancy was a secondary and underpowered outcome, these results were interpreted cautiously and used only to support the main clinical interpretation.

Effect sizes were reported in addition to p values. Mean differences were used for continuous outcomes, risk differences and relative risks for categorical outcomes when clinically interpretable, odds ratios for binary regression outcomes, incidence rate ratios for count outcomes, and standardized mean differences for baseline balance. Missing data were handled by complete-case analysis for the relevant outcome, and the number of analyzed observations was reported when it differed from the total sample.