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**Original Research Article**

# Comparison of Calcimycin and Ionomycin-induced Assisted Oocyte Activation in Cases with Male Infertility: A Study of Control Sibling Oocytes

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## ABSTRACT

Using control sibling oocytes, the prospective study on the treatment of male infertility factors was conducted in a private IVF center located in Cairo, Egypt. Each case of male factor infertility was treated with both calcimycin and ionomycin, and then the rate of fertilization, cleavage, and quality of embryo were statistically compared. The total number of included cases in the study was n=112. Pregnancy and postpartum follow-up were not investigated. Calcimycin and ionomycin showed nonsignificant T-value in rates of fertilization, cleavage, and good-quality embryos than non-treated embryos. Group I (n=59) treated versus control show fertilization rate of 76.6%, 48.9%, cleavage rate of 95.4%, 85.3%, the good quality embryos 47.1%, 18.5%, respectively. Group II (n=53) treated versus control show fertilization rate of 81.1 %, 50.8%, cleavage rate of 92.5%, 81.4%, the good quality embryos 42%, 29.9%, respectively. The utilization of assisted oocyte activation, ionomycin, and calcimycin has been proposed to possibly increase the number of zygotes, improve cleavage, and create high-quality embryos, boosting the probability of embryo transfer trials.

**KEY WORDS**: Calcimycin, Ionomycin, Male factor infertility, Sibling oocytes.

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## INTRODUCTION

The population of infertile couples has increased, and new techniques have been introduced into assisted reproductive technology (ART) [1]. Intracytoplasmic sperm injection (ICSI) is one of the techniques that has increased the outcome in cases with or without male factor infertility; the technique bypasses the barriers to fertilization [2].

Moreover, it minimizes the number of infertile couples diagnosed as failed in vitro fertilization (IVF), severe male factor infertility, or unexplained infertility [3].

However, there is still 1-3 % of couples still suffer from total fertilization failure (TFF) or low fertilization rate (LFR), even in cases with morphologically normal gametes [4]. In order to achieve successful activation of the human oocyte, both oocytes and sperm factors were mediated by various oscillations of $Ca^{2+}$ concentration in the cytoplasm, which was triggered by sperm factor called phospholipase C zeta PLCz [5, 6]. After ICSI, PLCz hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) in plasma membrane sources to produces inositol 1,4,5-trisphosphate (InsP3) and

diacylglycerol (DAG) [7]. IP3 in turn binds to its receptor (InsP3R) on the membrane of the endoplasmic reticulum (ER).

Accordingly, $Ca^{2+}$ release from the internal ER stores while DAG still remains linked to the plasma membrane. Both DAG and released $Ca^{2+}$ activate protein kinase C (PKC) [8, 9].

Movement of $Ca^{2+}$ in the ooplasm shortly ends after the time of pronuclear formation [6]. PLCz were remarkably associated with oocyte activation deficiency (OAD) [10, 11]. Therefore, for such cases, assisted oocyte activation (AOA) is applied to couples who have a history of fertilization failure after ICSI after proper counseling. Chemical, electrical, and mechanical stimuli can be introduced to improve oocyte activation after ICSI [12].

This study aims to investigate the role of calcimycin and ionomycin in improving the rates of fertilization, cleavage, and good-quality embryos in cases of male factor infertility after ICSI using sibling oocytes.

## METHODS

**Study design, setting, and participants:** Individuals who were registered with clinical diagnoses of male factor infertility and subsequently qualified as having a poor prognosis following ICSI. So, only patients with Oligo-Astheno-Teratozoospermia (OAT), asthenozoospermia, and teratozoospermia were included. The included oocytes were divided into 2 groups. Each group had siblings as a negative control. The study ran out from March 2021 to December 2022 at Queens fertility center, Cairo, Egypt, on the enrolled couples who signed a written consent after proper counseling regarding the whole process. Only females with clear reproduction and endocrinology were included in the study, with an age range of 20-35 years and shared their fresh oocytes and fresh husband's sperm. All participants have consented to be enrolled in the study and have the right to decline participation at any time without affecting medical care. The study's procedure follows the Helsinki Declaration's guidelines for conducting ethical medical research, revised in 2013.

Participants were classified as the following: Group I: sibling oocytes were split; some treated with Calcimycin A23187 (GM508 CultActive, Gynemed, Germany) while others non-treated, negative control. Group II: sibling oocytes were split; some treated with Ionomycin (Sigma-Aldrich) while others non-treated, negative control. Fertilization rate was the primary outcome, while cleavage rate, lysis rate, arrest rate, and embryo quality on day three were secondary outcomes.

**Semen analysis and preparation:** Fresh ejaculated semen samples were collected in special sterilized plastic wide-mouthed containers on the day of oocytes retrieval by masturbation in a private room near the laboratory. The sample was kept for half an hour at 35°C for liquefaction. Analysis was done based on the guidelines of the World Health Organization (WHO), 5th edition manual published in 2010, based on a large group of fertile men [13]. Only fresh semen samples were included. Density gradient media (Isolate, Irvine Scientific) was used for the candidates of isolation method, then washed by sperm wash media (Sperm Wash, Irvin Scientific). On the other hand, the same sperm wash media was used for oligo-Asthenozoospermia.

**Ovarian stimulation, ovum pick-up, and denudation:** Agonist protocol (Decapeptyl, Ferring) or antagonist protocol (Cetrotide; Merck Serono) was used based on women's age, response, and ovarian reserve. Ultrasonographic and blood monitoring was used to track the progress of the stimulation, which was carried out using recombinant FSH (Gonal F, Merck Serono) or hMG (Menopur, Ferring) at a dosage of 150-300 IU daily. For ovulation induction, hCG (Choriomon, IBSA International) 5,000 IU or (Decapeptyl, Ferring) were used. After 35-36 hours, ovum pick-up was done at a pressure 100 mmgh. Harvested oocyte cumulus complexes were collected in buffer media (multipurpose handling media MHM, Irvin Scientific), then moved to culture media (Global for fertilization, life global group). 2 hours later, the oocytes were biochemically denuded at a buffered hyaluronidase solution 80 IU (90101, Irvin Scientific) followed by

mechanical denudation. The mature oocytes were marked by the presence of primary polar body.

**Procedure (ICSI and AOA):** ICSI was done as described by [14]. Briefly, a single spermatozoa was immobilized in polyvinylpyrrolidone media, aspirated from the tail, and directly injected into the cytoplasm after single cytoplasm suction. In Group I, the siblings were split; about half oocytes were chemically activated, while others non-treated. $Ca^{2+}$ ionophore ready-to-use A23187 solution (GM508 CultActive, Gynemed, Germany) was used directly after ICSI for 15 minutes. Then twice washed in the same culture media. Non-treated oocytes were cultured as a negative control in single-step media (Global® Total, life global group). In Group II, spermatozoa were injected along with 0.1 mol/L $CaCl_2$ (Sigma-Aldrich). Then they were cultured in 10 micro mol/L ionomycin (Sigma-Aldrich) dissolved in CC culture media, then twice cultured for 5 min each (with a 30 min interval), then twice washed to get cultured in the same culture media. All oocytes were cultured at $CO_2$ 6.2%, $O_2$ 5%, and 37 ! in the dry incubator (Miri esco, Denmark).

**Fertilization check and embryos assessment:** After 16-18 hours, pronucleus PN fertilization was checked using (SZ61 Olympus, Japan). On day two, cleavage was checked, and on day three, embryos were scored as described in Society for Assisted Reproductive Technologies (SART) grading system **[15].**

**Data analysis:** SPSS 25.0 (IBM, USA) was used for the statistical analysis. Statistical significance was $P < 0.05$ using the T-test for comparing means and the multivariate Anova test for comparing group differences.

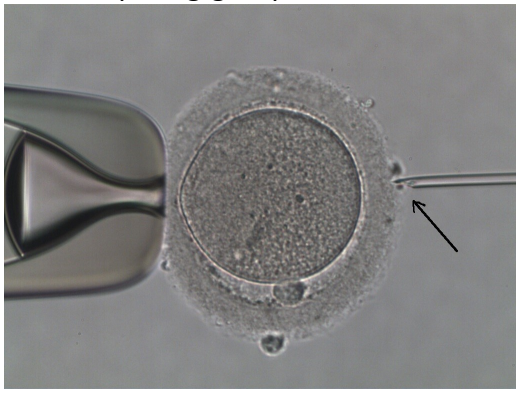

**Fig. 1:** shows ICSI start, arrow points to spermatozoon.

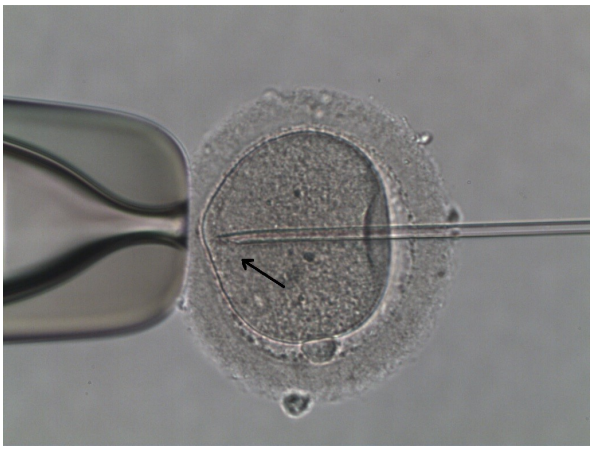

**Fig. 2:** shows spermatozoon is injected into the cytoplasm, arrow points to spermatozoon.

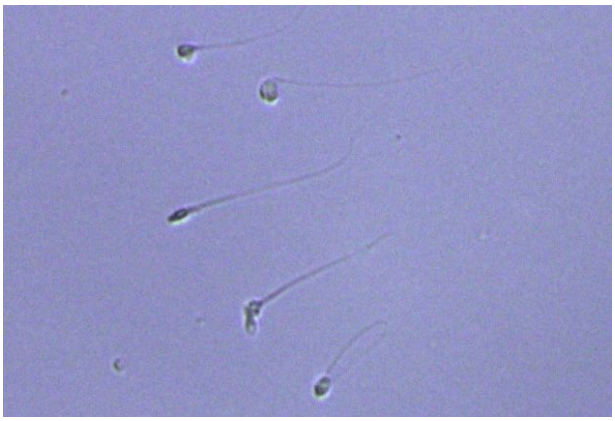

**Fig. 3:** Shows absolute teratozoospermia.

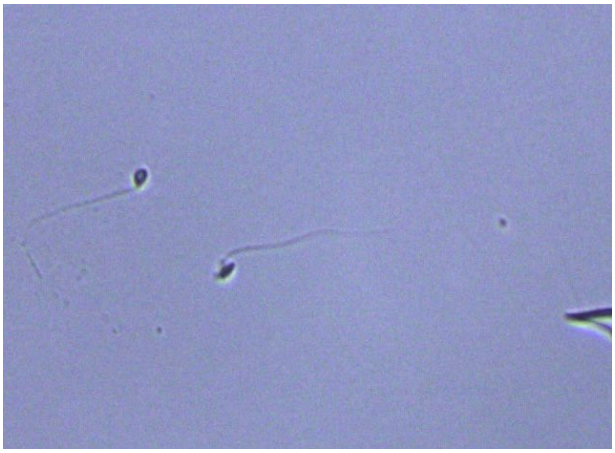

**Fig. 4:** shows absolute teratozoospermia.

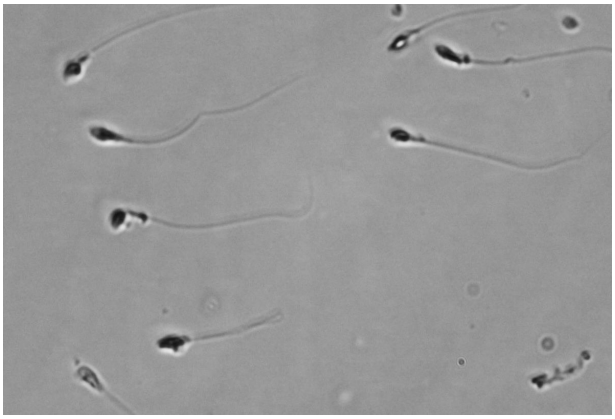

**Fig. 5:** shows absolute teratozoospermia.

In the present study, 112 participating couples had male factor infertility, randomly assigned and portioned into two groups for intervention. Group I calcimycin had 59 cases, and group II ionomycin had 53 cases. The total included 1196 oocytes of which 573 of them were treated. Fertilization, degeneration, arrest, cleavage and quality of embryos on day 3 were estimated after each treatment and compared with non-treated sibling oocytes.

**Table 1:** Group I calcimycin treated and non-treated male factor patients' oocytes descriptive statistics.

| S no. | Group I | Mean | Std. Dev. |
|---|---|---|---|
| 1 | Age | 28 | 5.07 |
| 2 | Calcimycin treated oocytes | 9 | 33.7 |
| 3 | T. Fertilized | 6.7 | 25.3 |
| 4 | T. Fertilization Rate | 76.6 | 22.1 |
| 5 | T. Degenerate | 0.3 | 1.4 |
| 6 | T. Degeneration Rate | 2.9 | 7.3 |
| 7 | T. Arrest | 0.3 | 1.2 |
| 8 | T. Arrest. Rate | 4.8 | 15.6 |
| 9 | T. Cleavage | 6.4 | 24.2 |
| 10 | T. C.leavage Rate | 71.7 | 24.5 |
| 11 | T. Good Quality | 4.3 | 16.3 |
| 12 | T. Good Quality Rate | 47.1 | 26.2 |
| 13 | Non-treated oocytes | 9.4 | 35.3 |
| 14 | Non-T. Fertilized | 4.3 | 16.4 |
| 15 | Non-T. Fertilization Rate | 48.9 | 35.8 |
| 16 | Non-T. Degenerate | 0.3 | 1.3 |
| 17 | Non-T. Degeneration rate | 2.8 | 8.7 |
| 18 | Non-T. Arrest | 0.6 | 2.5 |
| 19 | Non-T. Arrest Rate | 7 | 12 |
| 20 | Non-T. Cleavage | 3.7 | 14 |
| 21 | Non-T. Cleavage Rate | 41.9 | 34.4 |
| 22 | Non-T. Good Quality | 1.5 | 5.7 |
| 23 | Non-T. Good Quality Rate | 18.5 | 23.7 |

(T) Treated

**Group I:** (n=59) results showed the mean of calcimycin-treated fertilized oocytes was 6.7 with a fertilization rate of 76.6%, while degenerated oocytes mean was 0.3 with a degeneration rate of 2.9%. On the other hand, the arrested oocytes mean was 0.3, with an arrest rate of 4.8%. The mean of calcimycin-treated oocytes cleavage was 6.4, with a cleavage rate of 95.4%. The good quality embryos were estimated with 4.3 and 47.1% mean and rate, respectively. Furthermore, the results of Group I revealed that the mean of non-treated fertilized oocytes was 4.3 with a fertilization rate of 48.9 %, while degenerated oocytes mean was 0.3 with a degeneration rate of 2.8%. While the arrested oocytes mean was0.6 with an arrest rate of 7 %. The mean of non-treated oocytes cleavage was 3.7, with a cleavage rate of 85.3 %. The good quality embryos were estimated with 1.5 and 18.5 % mean and rate, respectively (**Table 1**).

The results of the paired T-test revealed that calcimycin-treated male factor patients' oocytes had nonsignificant T- value of fertilization rate than non-treated, and calcimycin-treated oocytes had nonsignificant T- value of later embryogenesis, cleavage rate and good quality on day three **(Table 2).**

**Table 2:** Calcimycin treated and non-treated male factor patients' oocytes fertilization, degeneration, arrest, cleavage, and quality rate comparison

| S no. | Siblings Treated vs. Non-treated | Paired Differences Mean | Std. Dev. | Std. Error Mean | T Value | Sig. (2-tailed) |
|---|---|---|---|---|---|---|
| 1 | Calcimycin treated - Non- treated | -0.4 | 2.3 | 0.3 | -1.3 | 0.1 |
| 2 | Fertilization | 2.3 | 8.9 | 1.1 | 2 | 0.04* |
| 3 | Fertilization Rate | 27.6 | 30.6 | 4 | 6.8 | 000* |
| 4 | Degeneration after ICSI | 0.03 | 0.5 | 0 | 0.4 | 0.6 |
| 5 | Degeneration Rate | 0.1 | 11.1 | 1.4 | 0 | 0.9 |
| 6 | Arrest | -0.3 | 1.4 | 0.1 | -1.8 | 0.07 |
| 7 | Arrest Rate | -2.1 | 20.7 | 2.7 | -0.7 | 0.4 |
| 8 | Cleavage | 2.7 | 10.2 | 1.3 | 2 | 0.04* |
| 9 | Cleavage Rate | 29.8 | 29.3 | 3.8 | 7.6 | 000* |
| 10 | Good quality | 2.8 | 10.7 | 1.4 | 2 | 0.04* |
| 11 | Good quality Rate | 28.6 | 30.3 | 4 | 7.1 | 000* |

**Table 3:** Ionomycin treated and non-treated male factor patients' oocytes descriptive statistics.

| Sno. | Group II | Mean | Std. Dev. |
|---|---|---|---|
| 1 | Age | 27.5 | 4.4 |
| 2 | Ionomycin treated oocytes | 6.1 | 3.4 |
| 3 | T. Fertilized | 5 | 3 |
| 4 | T. Fertilization Rate | 81.1 | 21.8 |
| 5 | T. Degenerate | 0.2 | 0.6 |
| 6 | T. Degeneration Rate | 3.2 | 7.5 |
| 7 | T. Arrest | 0.3 | 0.6 |
| 8 | T. Arrest Rate | 8.8 | 19.2 |
| 9 | T. Cleavage | 4.6 | 2.9 |
| 10 | T. Cleavage Rate | 72.2 | 27.2 |
| 11 | T. Good Quality | 2.3 | 1.8 |
| 12 | T. Good Quality Rate | 42 | 28.5 |
| 13 | Non-T. oocytes | 6.8 | 3.1 |
| 14 | Non-T. Fertilized | 3.4 | 2 |
| 15 | Non-T. Fertilization Rate | 71.2 | 52.7 |
| 16 | Non-T. Degenerate | 0.1 | 0.4 |
| 17 | Non-T. Degeneration Rate | 2.8 | 7.2 |
| 18 | Non-T. Arrest | 0.6 | 0.9 |
| 19 | Non-T. Arrest Rate | 11.5 | 19.7 |
| 20 | Non-T. Cleavage | 2.8 | 1.8 |
| 21 | Non-T. Cleavage Rate | 59.7 | 50.1 |
| 22 | Non-T. Good Quality | 1 | 0.9 |
| 23 | Non-T. Good Quality Rate | 29.9 | 47.7 |

(T) Treated

In Group II total of 53 cases were included. The results of the second study, ionomycin-treated siblings achieved a fertilization rate of 81.1 %, the mean of ionomycin-treated oocytes was 5, while degenerated oocytes mean was 0.2 with a degeneration rate of 3.2%. On the other hand, the arrested oocytes mean was 0.3, with an arrest rate of 8.8%. The mean of ionomycin-treated oocytes cleavage was 4.6, with a cleavage rate of 92.5%. The good quality embryos were estimated with 2.3 and 42 % mean and rate, respectively. Moreover, the results of Group II revealed that the mean of non-treated fertilized with affected sperm morphology was 3.4 with a fertilization rate of 50.8%, while degenerated oocytes mean was 0.1 with a degeneration rate of 2.8%. While the arrested oocytes mean was 0.6 with an arrest rate of 11.5 %. The mean of non-treated oocytes cleavage was 2.8 with a cleavage rate of 59.7 %. The good quality embryos were estimated with 1 and 29.9 % mean and rate, respectively (**Table 3**).

**Table 4:** Ionomycin treated and non-treated male factor patients' oocytes fertilization, degeneration, arrest, cleavage, and quality rate comparison.

| S no. | Siblings Treated vs. non-treated | Paired Differences Mean | Std. Dev. | Std. Error Mean | T | Sig. (2-tailed) |
|---|---|---|---|---|---|---|
| 1 | Ionomycin treated – non-treated | -0.7 | 2.6 | 0.3 | -2 | 0.05* |
| 2 | Fertilization | 1.5 | 2.6 | 0.3 | 4 | 000* |
| 3 | Fertilization Rate | 9.9 | 55.4 | 7.7 | 1.2 | 0.2 |
| 4 | Degeneration after ICSI | 0.09 | 0.7 | 0.1 | 0.9 | 0.3 |
| 5 | Degeneration Rate | 0.3 | 9.9 | 1.3 | 0.2 | 0.7 |
| 6 | Arrest | -0.2 | 1 | 0.1 | -1.9 | 0.06 |
| 7 | Arrest Rate | -2.6 | 26 | 3.6 | -0.7 | 0.4 |
| 8 | Cleavage | 1.7 | 2.8 | 0.3 | 4.5 | 000* |
| 9 | Cleavage Rate | 12.5 | 57.3 | 8 | 1.5 | 0.1 |
| 10 | Good Quality | 1.3 | 1.8 | 0.2 | 5.1 | 000* |
| 11 | Good Quality Rate | 12 | 50 | 7 | 1.7 | 0.09 |

Paired T-test. *= Significant at (P-value < 0.05)

The results of the paired T-test revealed that ionomycin-treated male factor patients' oocytes had nonsignificant T- value of a fertilization rate than non-treated oocytes, and ionomycin-treated oocytes had nonsignificant T- value of cleavage rate and good quality than non-treated embryos **(Table4).**

## DISCUSSION

Fertilization, cleavage, and embryo quality may vary from cycle to cycle, despite the fact that oocytes may be collected from the same ovary [16]. Furthermore, using siblings as a control group in a specific cohort of patients might be better to express the clear efficacy of AOA [17]. Unlike most previous studies which compared AOA ICSI with their non-AOA ICSI in previous cycle outcomes, that might have biased the result [18-20]. Therefore, the current study was set up to verify the efficacy of AOA on the cycles that might face a risk of fertilization failure after non-AOA ICSI. However, the

fertilization failure after ICSI could be maternal, paternal or due to iatrogenic factors [17]. So, cases with a poor prognosis for fertilization due to male factor infertility were recruited, and malformed oocytes were excluded. In addition to that, patients with less than five injected oocytes were omitted in order to exclude technical issues affect and possible ovarian dysfunction. What is more, the control group of siblings made it clear to express the efficacy of AOA.

Although the ICSI fertilization rate is considered the highest ever among all available IVF techniques, fertilization failure is a threat to a low percentage of couples [4].

The common cause was mechanism deficiency of oocyte activation in spite of spermatozoa presence, the analysis post-ICSI postulated [21]. Studies showed the successful link of activation of the human oocyte with PLCz that was remarkably associated with oocyte activation deficiency [5, 6, 11, 22].

Many techniques have been postulated to overcome fertilization limitations and subsequently have enough good-quality embryos to reach healthy babies. Among these techniques is chemically AOA using puromycin, ethanol, phorbol, ester, and thimerosal which used in non-human oocytes. Strontium chloride SrCl is increasingly used in human oocytes. It improved the fertilization and embryo quality of those with previous fertilization failures. However, patients must be notified of the possible risks and benefits [23, 24]. Although improvement in clinical and embryological outcomes, safety, transgenerational effects, and lack of knowledge regarding SrCl2 were warned [25].

In this study, calcimycin was used as ready to use fertilization agent. It was first reported through a multicenter study including 75 severe male infertility cycles; they had statistically significant fertilization rates as compared with their previous cycles [18]. PLCz has been linked with abnormal sperm morphology [26], sperm DNA fragmentation, sperm parameters, potentially linked with abnormal embryogenesis [27], and that deficiency can be amended using AOA to get higher fertilization [28].

In this study, AOA was used in male factor cases to evoke calcium ions just after ICSI, which can mimic the natural oscillation in normal spermatozoa. The current study demonstrated that the both calcimycin and ionomycin can improve the fertilization significantly compared with negative control but couldn't reach to normal fertilization rate. Most IVF laboratories use ready to use calcimycin because it's easy, no need to double exposure, nor $CaCl_2$ injection, which might be difficult to control by manipulators except for highly trained embryologists. In addition, the safety of production because it follows high standard and sterile conditions [18].

Ionomycin showed a higher fertilization rate compared with calcimycin in the same patient's category, although both were highly significant than ICSI alone in the same siblings. To assist the differences between the included activators, the epifluorescence microscope was utilized to capture pictures of Ca patterns in human and mouse oocytes injected with globozoospermic sperm, which is unable to release Ca. The study showed that ionomycin protocol provoked three rises of Ca, whereas the calcimycin protocol caused only two Ca rises [29]. Ionomycin itself, $CaCl_2$ injected, or twice-incubated oocytes are the three potential contributors to the improvement of ionomycin against calcimycin. In the case of calcimycin, no $CaCl_2$ was injected, and only single incubated in AOA. The applied technique or protocol improved fertilization, pregnancy, and live birth rates, as reported in 17-year retrospective research [16]. The reported outcome was achieved with twofold ionomycin cultivation for 10 minutes at 30-minute intervals.

Similar results were reported that ionomycin showed significant fertilization rate than commercially available calcimycin in cases of sperm-related abnormalities in a retrospective study by Jia et al. [30]. The current findings also concur with the meta-analysis of 22 studies that showed the effectiveness and safety of ICSI-AOA compared to ICSI, but still, more confirmation is needed [31, 32].

Some publications grouped male factor infertility patients to assess the efficacy on certain

indication [10, 11]. However, other publications grouped previous ICSI failure and severe teratozoospermia patients [17]. However, the adverse effect of AOA was reported, whereas the number of good-quality embryos decreased; however, the fertilization rate in both treated and non-treated siblings was high [33]. Literature showed the use of IMSI instead of ICSI for a low sample size.

Meanwhile, insignificance was reported regarding fertilization, cleavage, implantation, and pregnancy [34]. However, the insignificance may be due to the used protocol, whereas the manufacturer has recommended the exposure to calcimycin for 15 minutes, but they incubated for only five minutes which may be no enough to provoke Ca rises. Some studies reported no difference from the previous ICSI cycles for couples who actually had recurrent embryo developmental defects. However, no major teratology has been observed in AOA-ICSI children [35]. Still, it can harvest more fertilized oocytes which increase the chance for more embryo transfer. When calcimycin compared with other activators in male factor infertility it showed better outcome than $SrCl_2$ that achieved better result in abnormal sperm morphology [25, 36].

It may increase Ca inflow into the oocyte from the outside, enhancing oscillation start. The latter research indicated that $SrCl_2$ causes sperm-mediated oocyte activation waves. Therefore, disability of $SrCl_2$ to mimic the natural $Ca_{2+}$ to induce fertilization in samples with altered activity, reduced expression, or total absence of PLCz. In general, $SrCl_2$ may have less impact due to the defect is not with the machinery itself; it's just the initiation of the oscillation [37].

Therefore, calcimycin AOA may be promising for ICSI cycles with OAT. The negative impact of gametes management during AOA has never been investigated. Moving the treated oocytes two or three times in treatment plates outside the incubator with further washing steps, along with $CaCl_2$ injection before ionomycin, these actions might alter the outcome. Since, mishandling may impact deterioration and arrest rates. Degeneration following ICSI and arrest rates were non-significant in treated and untreated patients.

This study had some limitations, such as follow-up of newborns; though, it was assessed in many studies [10, 16-18, 35, 38-40]. It was reported that there was no adverse effect on AOA children compared to ICSI alone. However, more studies were recommended on a large scale. Another limitation regarding the pre-ICSI test that this study didn't apply to any heterologous ICSI mouse oocytes activation test MOAT, nor human oocytes calcium activation test HOCA due to lacking of an animal facility in the lab, nor donor regulations were established. MOAT and HOCA were reported with effectiveness whether sperm-related activation deficiency or OAD [41]. It is worth mentioning that only calcium-related failing patients benefited from AOA therapy.

## CONCLUSION

The utilization of assisted oocyte activation, ionomycin, and calcimycin have been proposed to possibly increase the number of zygotes, improve cleavage, and create high-quality embryos, boosting the probability of embryo transfer trials. Additionally, this method also permits couples to use homologous gametes without the need for sperm donation. Patients affected by male factor infertility, and those with low chances of fertilization in particular, may benefit from both treatments, with ionomycin possessing a slight advantage in this regard.

## Declaration and statements

**Funding:** Unfunded project.

**Ethical Approval:** The study's procedure follows the Helsinki declaration's guidelines for conducting ethical medical research, revised in 2013.

**Conflicts of Interests:** The authors declare that they have no competing interests'.

**Author Contributions:** The authors have contributed to writing, designing, compiling and editing the final manuscript.

**Data availability statement:** Data is available within the manuscript and will be provided at the editor's request.

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