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Is There Any Role for Calcium Ionophore in ICSI Cycles for Cases of Non Obstructive Azoospermia?

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Abstract

Objectives: Evaluate the effect of artificial oocyte activation (AOA) using calcium ionophore (A23187) on the rate of fertilization and cleavage of embryos in surgically retrieved sperm of patients with non-obstructive azoospermia undergoing intracytoplasmic sperm injection (ICSI). **Study design:** This study was conducted on 60 infertile couples undergoing ICSI cycles as a randomized controlled parallel group's experimental study in a private IVF center in Egypt from January 2018 to July 2019. ICSI cycles were divided into two groups: Group A: includes 30 ICSI patients with surgically retrieved sperms of non-obstructive azoospermia treated with calcium ionophore (A23187). Group C/Control: includes 30 ICSI patients with surgically retrieved sperms of non-obstructive azoospermia non-treated with calcium ionophore (A23187). **Results:** There was no statistical difference between both groups regarding the fertilization rate (p = 0.853). There was no statistical difference between them regarding implantation rate (p = 0.237). The percentage of Class A embryos in the ca ionophore group was 81.7%, while it was 82.8% in the control group. There was insignificant difference between them (p = 0.782). There was no statistical significant difference between the two groups regarding the clinical pregnancy rate, it was (56.7%) in the ca ionophore group while it was (53.3%) in the control group. **Conclusion:** AOA by Ca^{2++} ionophore didn't improve the outcome of ICSI cycle in cases of non obstructive azoospermia in terms of fertilization, implantation and pregnancy rate.

Keywords

Fertilization Failure, ICSI, Azosperemia, Ca Ionophore

1. Introduction

Fertilization is the process resulting from the fusion of the two parental gametes,

the oocyte and the sperm. When oocytes and spermatozoa meet in the oviduct, a series of steps are set in motion that leads to fertilization and to the development of a zygote. Fertilization induces a cascade of events that result in the development of the zygote [1].

Fertilization failure after ICSI may occur because of the following conditions. First, the injected oocyte may fail to initiate the biochemical processes necessary for oocyte activation [2]. Second, the biochemical processes are initiated, but they may not occur normally, thus leading to incomplete activation. Third, the sperm can be not accessible for chromatin decondensation and formation of the male pronucleus in the oocyte [3]. Both sperm and oocyte factors are assumed to be involved in failed oocyte activation after ICSI [4] [5]. The Incidence of total fertilization failure (TFF) after ICSI is 1% - 5% [6] [7] [8].

Although some suggest no difference [9], the majority of reports show significantly impaired fertilization or pregnancy outcome in cycles using testicular sperm from non obstructive azoospermia (NOA) patients compared with testicular sperm from obstructive azoospermia (OA) patients [10] [11] [12].

Oocyte activation results in the release of the oocyte from its metaphase II (MII) arrested state and leads to further embryo development [13]. It was known that intracellular concentration of calcium (Ca^{2+}) levels dramatically change after fertilization [14], which focused attention on the role of this ion [15]. Oocyte activation is characterized by a dramatic rise in intracellular calcium concentration, which in mammals takes the form of calcium oscillations [16] [17], driven by an elevation in inositol triphosphate (IP3) concentrations [18]. The causative agent of these oscillations is proposed to be a recently described phosphoinositide-specific phospholipase C, which is a soluble sperm factor delivered to the egg following membrane fusion [19] [20] [21] [22] [23].

It has been discussed that the calcium oscillation pattern during oocyte activation may influence not only fertilization but also embryo development and therefore the implantation [24] [25]. The most commonly studied AOA model uses the calcium ionophore A23187 [26] [27] [28] [29] [30], to increase the concentration of free Ca^{2+} in the cytosol, thereby mimicking the physiological cell-signaling mechanism [31].

The aim of this study is to evaluate the effect of artificial oocyte activation (AOA) using calcium ionophore (A23187) on the rate of fertilization and cleavage of embryos in surgically retrieved sperm of patients with non-obstructive azoospermia undergoing intracytoplasmic sperm injection (ICSI).

2. Materials and Methods

Study design: randomized controlled.

Study settings: this study was conducted in a private IVF center in Egypt (Repro IVF center) from January 2018 to July 2019.

Sample size: This study was conducted on 60 infertile couples undergoing ICSI cycles as a randomized controlled parallel group's experimental study. ICSI

cycles were divided into two groups: Group A: includes 30 ICSI patients with surgically retrieved sperms of non-obstructive azoospermia treated with calcium ionophore (A23187). Group C/Control: includes 30 ICSI patients with surgically retrieved sperms of non-obstructive azoospermia non-treated with calcium ionophore (A23187) by closed envelopes methods. Sample size was calculated to include all patients fulfilling the inclusion criteria visiting the center in the study period.

Participants: Inclusion criteria were Couples aged between 20 - 38 years (for the female) with Infertility more than 2 years due to; non-obstructive azoospermic male with normal female. Exclusion criteria: Patient refusal, Poor responders, Endometriosis, Polycystic ovarian syndrome (PCOS), Diabetes and Female with any infertility factor.

Methods: Approval of Ethics Committee in Faculty of Medicine; Alexandria University was taken before conduction of the study. Informed consent was obtained from all participating subjects prior to their inclusion.

All the couples were subjected to the following: full history taking, Hormonal assessment for FSH, LH, AMH, TSH and serum prolactine and Transvaginal ultrasound.

Sperm retrieval was done through microscopic testicular biopsy. The recovered material was examined for the presence of sperms and then was processed by sperm double wash technique.

Ovarian stimulation was through the long agonist protocol. The oocytes were collected after 36 hours from HCG administration. After that, the oocytes were randomly divided into two groups: Experimental group: immediately after injection, the injected oocytes were incubated in culture medium containing 5 μmol/l of the calcium ionophore A23187 at 37˚C and 6% CO_2 for 30 min. The oocytes were then washed and incubated in culture medium at 37˚C. After three washing steps, injected eggs will be transferred to microdroplets (30 ml) of sequential culture media until the fertilization check. Control group: after injection, the injected oocytes were subjected to routine ICSI.

Primary outcome: fertilized oocytes and fertilization percentage; around 16 - 18 hours after ICSI cycle, percent of fertilization will be assessed by the presence of 2 pro nuclei. Secondary outcome: Number of embryos, the percentage of high quality embryos, implantation rate and pregnancy rates.

Embryo transfer was performed on the second or the third or the fifth day of development. High-quality embryos were defined as those having all the following characteristics: either 4 - 6 cells on the second day or 8 - 10 cells on the third day of development, less than 10% fragmentation, symmetric blastomeres, absence of multinucleation, colourless cytoplasm with moderate granulation and no inclusions, absence of perivitelline space granularity, and absence of zona pellucida dysmorphism.

For each couple, one to four embryos were transferred depending on the quality of embryos and the woman age.

Statistical analysis of the data [32]

Data were fed to the computer and analyzed using IBM SPSS software package version 21.0. [33] Qualitative data were described using numbers and percentages. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level.

The used tests were:

1) Chi-square test

For categorical variables, to compare between different groups.

2) Fisher's Exact or Monte Carlo correction

Correction for chi-square when more than 20% of the cells have expected count less than 5.

3) F-test (ANOVA)

For normally quantitative variables, to compare between more than two studied groups, and Post Hoc test (LSD) for pairwise comparisons.

4) Mann Whitney test

For abnormally quantitative variables, to compare between two studied groups.

3. Results

There was no statistical significant difference between the two groups regarding male and female age. Male age ranged from 26 years to 65 years with a mean of 39.83 ± 8.25 years, and female age ranged from 23 years to 38 years with a mean of 31.74 ± 4.62 years in the treated group, while in the control group, male age ranged from 26 years to 58 years with a mean of 37.13 ± 8.31 and female age ranged from 18 years to 38 years with a mean of 29.0 ± 7.83 ($p = 0.212$, $p = 0.143$ consecutively). The total number of mature oocytes in the Ca ionophore treated group was 373 oocytes, with a mean of 12.43 ± 5.82 oocytes, while in control group it was 347 oocytes with a mean of 11.57 ± 7.65 oocytes. On comparison between the two groups, there was no statistical significant difference between them ($p = 0.332$). Regarding the fertilized oocytes, the total number in the Ca ionophore treated group was 208 fertilized oocytes, the mean was 6.93 ± 3.85 oocytes, while in the control group it was 186 fertilized oocytes with a mean of 6.20 ± 3.95 oocytes. On comparison between the two groups, there was insignificant difference between them ($p = 0.494$). The fertilization rate, in the ca ionophore treated group ranged from 20 to 100 with a median of 56.70, while in control group, it ranged from zero to 100 with a median of 60.66. On comparing between the two groups, there was no statistical difference between them ($p = 0.853$) (Table 1). Regarding the implantation rate, in the ca ionophore group it ranged from 25 to 100 with a median of 66.67, while in the control group, it ranged from 16.76 to 100 with a median of 50.0. On comparison between the two groups, there was no statistical difference between them (Table 1). In the ca ionophore group, the number of embryos was 208 embryos, with a mean of 6.93 ± 3.85 embryos, while in the control group, it was 186 embryos ranged with a

Table 1. Comparison between the two studied groups according to fertilization and implantation rate.

	Treated	Control	U	*p*
Fertilization rate	**(n = 30)**	**(n = 30)**		
Min. - Max.	20.0 - 100.0	0.0 - 100.0		
Mean ± SD.	57.69 ± 22.77	57.52 ± 27.01	437.50	0.853
Median	56.70	60.66		
Implantation rate	**(n = 17)**	**(n = 16)**		
Min. - Max.	25.0 - 100.0	16.67 - 100.0		
Mean ± SD.	61.77 ± 23.58	54.06 ± 31.99	103.50	0.237
Median	66.67	50.0		

U, *p*: U and *p* values for **Mann Whitney test** for comparing between the two groups.

mean of 6.20 ± 3.95 embryos. On comparing between the two groups there was no statistical significant difference between them ($p = 0.494$) (Table 2). In the ca ionophore treated group, the number of Class A embryos was 170, with a median of 6 embryos, while in the control group it was 154, with a median of 4 embryos. The percentage of Class A embryos in the ca ionophore group was 81.7% from total embryos number (208), while it 82.8% from total embryos number (186). On comparing between the two groups there was insignificant difference between them ($p = 0.494$, $p = 0.782$ consecutively) (Table 2). Regarding number of embryos transferred, there was significant difference between the two groups ($p = 0.034$). In the ca ionophore group there was 90 (43.3%) embryos transferred with a mean of 3.0 ± 1.08 embryos, while in the control group it was 109 (58.6%) embryos transfered with a mean of 3.63 ± 1.47 embryos (Table 2). In the ca ionophore treated group, there was 17 (56.7%) woman become pregnant and 13 (43.3%) was non pregnant, while in the control group the women that become pregnant was 16 (53.3%) and 14 (46.7%) was non pregnant. On comparing between the two groups, there was no statistical significant difference between them (Table 3).

4. Discussion

Over the past 20 years, Ca ionophore have been successfully used in cases of complete globozoospermia [34] [35] [36], or other severe forms of isolated teratozoospermia [37] [38]. Further evidence that male factor infertility is the main indication for Ca ionophore treatment comes from studies dealing with crypto- or azoospermia [39] [40] [41].

Case reports suggesting the presence of a 50% previous ICSI fertilization rate threshold below which artificial oocyte activation with an ionophore is likely to improve outcome [28] [42] [43].

There is significant concern because calcium release patterns following ionophore treatment do not mimic those observed during normal fertilization which

Table 2. Comparison between the two studied groups according to embryo parameters.

	Treated (n = 30)	Control (n = 30)	Test of sig.	*p*
Number of embryos	**208**	**186**		
Min. - Max.	1.0 - 15.0	0.0 - 16.0		
Mean ± SD.	6.93 ± 3.85	6.20 ± 3.95	U = 404.00	0.494
Median	6.50	5.0		
Class A embryos	**170 (81.7%)**	**154 (82.8%)**	χ^2 = 0.076	0.782
Min. - Max.	1.0 - 14.0	0.0 - 14.0		
Mean ± SD.	5.67 ± 3.29	5.13 ± 3.45	U = 401.00	0.466
Median	6.0	4.0		
Number of embryos transferred	**90 (43.3%)**	**109 (58.6%)**	χ^2 = 9.235*	0.002*
Min. - Max.	1.0 - 5.0	0.0 - 6.0		
Mean ± SD.	3.0 ± 1.08	3.63 ± 1.47	U = 310.50*	0.034*
Median	3.0	4.0		

χ^2, *p*: χ^2 and *p* values for **Chi square test** for comparing between the two groups; U, *p*: U and *p* values for **Mann Whitney test** for comparing between the two groups; *: Statistically significant at $p \leq 0.05$.

Table 3. Comparison between the two studied groups according to pregnancy.

	Treated (n = 30)		Control (n = 30)		χ^2	*p*
	No.	%	No.	%		
Pregnancy						
Negative	13	43.3	14	46.7	0.067	0.792
Positive	17	56.7	16	53.3		

χ^2, *p*: χ^2 and *p* values for **Chi square test** for comparing between the two groups.

may affect further development [44]. Although possible risks associated with exposing human oocytes to chemical agents may still be uncovered, calcium ionophore treatment has been widely applied in human oocytes with no evidence of toxicity.

In the present study, fertilization and post fertilzation reproductive outcomes were not significantly improved by its use of ca ionophore. Although the ca ionophore treated group and the control group showed insignificant difference in the number of fertilized oocytes, the treated group was slightly higher than control group in the number of fertilized oocytes (6.93 ± 3.85 vs. 6.20 ± 3.95).

Regarding the fertilization rate and implantation rate, the present study showed insignificant difference between the treated and the control groups, although the fertilization rate was slightly better in the control group, but the implantation rate was better in the treated group. This is in accordance with some difference with the findings of Yoon *et al.*, [45] who demonstrated that Ca io-

nophore was efficient only in couples with low fertilization (<50%) in previous cycles. In their study, a total of 185 ICSI cycles with a history of no or low fertilization was included in retrospective study. They compared outcomes of AOA after ICSI with ejaculated-normal, ejaculated-oligo-astheno-terato or extracted-testicular spermatozoa. They found that, fertilization, cleavage, pregnancy and implantation significantly improved; whereas in couples with fertilization more than 50%, no effect was noted.

The difference that, Yoon *et al.*, [45] compared outcomes of AOA after ICSI with ejaculated-normal, ejaculated-oligo-astheno-terato or extracted-testicular spermatozoa, and their divided into two groups according to fertilization in the previous ICSI cycles (complete fertilization failure and low fertilization groups), while in the present study the patient in both groups was NOA, divided randomly into control and treated groups.

Another largest similar study, [23] including children born on AOA with calcium ionophores, which involved 89 patients, concluded that AOA with calcium ionophores could be efficacious for patients with a fertilization rate that was below 30% in their previous standard ICSI cycles. However, cases of testis-retrieved spermatozoa were excluded. The present study, by contrast, mainly included patients for whom the spermatozoa was testicular (surgically retrieved sperms), and compared the clinical outcomes.

Hee Jung Kang *et al.*, [46] studied 29 intracytoplasmic sperm injection (ICSI)-AOA cycles involving male factor infertility, Patients were divided into two groups (control, n = 480; AOA, n = 29). They suggested that, oocyte activation is a useful method to ensure fertilization in TESE-ICSI cycles regardless of restoration of sperm motility after pentoxifylline (PF) treatment and concluded that, ca ionophore did not improve the outcome in TESA ICSI, except in cases with previous fertilization failure and this in agreements with our results.

The difference in their study, they studied male factor infertility, used large number in the control group (n = 480), and they split the treated group into two subgroups according to sperm motility after (PF) treatment, while in the present study, we strict the study on NOA patients, divided randomly into two equal groups (n = 30) with no PF treatment.

In the present study, calcium ionophore treatment slightly improved ICSI outcomes only in some embryo parametrs, which suggests that not only the spermatozoa but also the oocyte plays a role in oocyte activation. It has been postulated that PLC-z is inactive inside spermatozoa, and that it is activated upon introduction into the oocyte [47]. In addition, it has been postulated that the action of the sperm factor is not a passive process, but instead depends on oocyte agents, such as sperm nucleus decondensation factor (SNDF) [48].

Even though it has been demonstrated that AOA can promote a rise in intracellular calcium concentration, which can result in higher fertilization rates, in the present study, no improvement in fertilization was found when a calcium ionophore was applied. Nevertheless, further embryonic development was found

to be positively affected by AOA.

The hypothesis that Ca^{2++} oscillations may provide more than merely a stimulus for meiotic resumption and that they may play a role in long-term embryonic events was previously discussed [49] [50]. It has been shown that differences in Ca^{2++} signaling patterns can have effects not only on implantation and post-implantation development but also on long-term fetal morphology [50] and weight variation in offspring [25].

The exact mechanism by which intracellular Ca^{2++} influences embryonic development is not completely understood; however, it has been postulated that the calcium oscillation pattern may partly act through gene expression regulation [50].

In the study by Ebner *et al.*, [51] Ca ionophore treatment during ICSI failed to improve fertilization rates, but instead, it improved the number of embryos going to the blastocyst formation in patients with previous embryo development problems and this is in agreements with the finding of the present study.

Finally, although the findings of this study are interesting, the present trial has some limitations, especially when the groups were done only on non-obstructive azoospermia with surgically retrieved sperms. Therefore, a well-conducted, prospective randomized study with wider types of sever male factors infertility with larger number of patients is needed to give the definitive answers.

5. Conclusion

AOA by Ca^{2++} ionophore didn't improve the outcome of ICSI cycle in cases of non obstructive azoospermia in terms of fertilization, implantation and pregnancy rate.

Declaration

This study was approved from the **Ethics committee**, faculty of medicine, Alexandria University. **The number is**: 0105188.

Written informed consent for publication of their clinical details and/or clinical images was obtained from the patient. A copy of the consent form is available for review by the Editor of this journal.

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Funds

Personal.

Authors' Contributions

ME analyzed and interpreted the patient data regarding the ICSI cycle parameters. EK, YO, SM and ME performed the ovarian stimulation, patients follow-up, ovum pick up and embryo transfer. ME and YO were contributors in writing the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare that they have no competing interests.

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