Oocyte Activation By Calcium Ionophore A23187 in Intra Cytoplasmic Sperm Injection

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ABSTRACT---- ***The present study evaluated the effect of calcium ionophore A23187 on oocytes in intracytoplasmic sperm injection (ICSI) cycles using sperm from different sources. The 80 cycles evaluated were divided into four subgroups according to sperm quality and origin (normospermia- oligoathenoteratospermia – epidermal –testicular), subgroups were further split into experimental groups, depending on whether or not treated with calcium ionophore. For each experimental subgroup, ICSI outcomes were compared between groups. In conclusion we found that treatment the oocyts with calcium ionophoreincrease the fertilization rate in oligoathenoteratospermia, epididymal (PESA) & testicular (TESE) patients without any effect on the embryos quality.***

Keywords--- Calcium ionophore A23187, fertilization rate, embryos quality, oocyte activation

1. INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is a method of micromanipulation used to deposit a sperm directly into oocyte cytoplasm. ICSI is possible with sperm obtained from ejaculate, percutaneous epididymal sperm extraction, or testicular sperm extraction **(Codreanu*et al*., 2013).**

For all ages and with all different sperm types, the fertilization rate after ICSI is reported to be approximately 70-80%. Although the fertilization rates after ICSI are relatively high, some injected oocytes fail to become fertilized despite the presence of a sperm in the cytoplasm and the absence of immaturity or degenerative alterations **(Codreanu*et al*., 2013).**

The terminology "failed fertilization" typically refers to failure of all the available mature MII oocytes to be fertilized. The principal cause of failed fertilization has been attributed to an oocyte activation disruption whether related to female or male gamete dysfunction **(Neri*et al*., 2014).** The Incidence of total fertilization failure (TFF) after ICSI is 1-5 % **(Koyono*et al*., 2012).**

Fertilization failure after ICSI may occur because of the following reasons. First, the injected oocyte may fail to initiate the biochemical processes necessary for oocyte activation **(Codreanu*et al*., 2013).** Second, the biochemical processes are initiated, but they may not occur normally, thus leading to incomplete activation. Third, the sperm may remain poorly accessible to oocyte factors required for chromatin decondensation and formation of the male pronucleus**(Mansour *et al*., 2009).** Both sperm and oocyte factors are believed to be involved in failed oocyte activation after ICSI **(Eftekhar*et al*., 2012).**

Oocyte activation results in the release of the oocyte from its metaphase II (MII) arrested state and leads to further embryo development **(VandenMeerschaut*et al*., 2013).**The association between intracellular ionic increases and release from meiotic arrest was first proposed at the end of the nineteenth century by Loeb and colleagues who observed that initiation of development in sea urchin eggs was possible simply by varying the concentration and composition of the fertilizing medium **(Loeb, 1907).** Subsequently, it was learned that intracellular concentration of calcium ($[Ca^{2+}]_i$) levels dramatically change after fertilization **(Mazia ,1935),** which focused attention on the role of this ion **(Wakai*et al*., 2011).**

Chemical compounds can induce calcium increase and initiate oocyte activation. Chemical oocyte activation has been reported with the use of compounds such as ethanol calcium ionophore A213187, ionomycin, puromycin, strontium

chloride, phorbol ester, and thimerosal. However, the use of these compounds for AOA has been mainly limited to animal models and case reports **(Nasr-Esfahani*et al*., 2010).**

These compounds promote the release of intracellular calcium stores, mobilize intracellular calcium by depletion of calcium stores, and facilitate the influx of extra cellular calcium ions. Some of these compounds may induce a single calcium rise in the oocyte while others may induce multiple calcium rises **(Nasr-Esfahani*et al*., 2010).**

In this study, we evaluated the effect of calcium ionophore A23187 on oocytes, sperms and on both of them together in increasing the fertilization rate in ICSI, with the assessment of the embryos quality. In addition the evaluation of the three routes for achieving higher fertilization rate.

2. MATERIALS AND METHODS

Experimental design

- This study involved 80 ICSI patients, underwent the ICSI program at the International Islamic Center for Population Studies and Research, Al-Azhar University, Cairo, Egypt, between January to September 2014.
- The age of women included in this study is under 38 years at the start of the treatment. Extensive counseling was given beforehand to the couples and the Internal Ethical Committee approved the study.

80 ICSI cycle were divided into four experimental subgroups according to sperm origin and type, 20 ICSI cycles for each subgroup:

1. Normospermia subgroup (ejaculated).
2. Oligoathenoteratospermia (OAT) subgroup (ejaculated).
3. Epididymal subgroup (PESA).
4. Testicular subgroup (TESE).

For each experimental subgroup, calcium ionophore A23187 treated group were compared to control group (non-treated with calcium ionophore A23187).

Ovarian Stimulation

Women received ovarian stimulating drugs according to the ART protocols. Follicular development was monitored by ultra sound scanning and serum estradiol and progesterone levels. Patients received 10,000 IU of Human Chronic Gonodotrophin (HCG) when most of the follicles measured more than 18–20 mm in diameter.

Sperm Preparation

- Sperm obtained from ejaculate, percutaneous epididymal sperm extraction, or testicular sperm extraction.
- The normospermia patient who follow the WHO 2010 parameters.
- Oligoathenoteratospermia patients who have semen parameters lower than the WHO semen count, motility and morphology parameters.
- Sperm preparation done by a discontinuous density gradient centrifugation method using Isolate sperm separation media (Irvine Scientific, Santa Ana, CA, USA) (Makkar et al., 2001).
- Epididymal spermatozoa obtained by PESA, is performed under local anaesthesia using a 27-gauge needle that inserted into the epididymis. Gentle, negative pressure is applied as the epididymal fluid was aspirated.
- Testicular spermatozoa were obtained via TESE,after administration of cord block anaesthesia. Samples extracted from the testis were dissected with small scissors and checked for the presence of spermatozoon.
- PESA and TESE Samples were collected in a Falcon tube and washed by centrifugation at (1800 r.p.m for 10 min) with minimum culture medium. The pellet is suspended in 0.3–0.5 ml Sydney IVF Gamete Buffer (Cook Sydney IVF Limited, National Technology Park, Ireland, UK).

Oocyte Aspiration

Under general anaesthesia, gynaecologist aspirates the oocytes by using a specialized, ultrasound-guided needle (Labotect aspiration catheter, Germany) at 36 h after HCG injection. Warmed HEPESbuffered medium (Irvine Scientific, Irvine, CA, USA) was used for handling and washing of oocytes. Oocyte-cumulus were identified by the embryologist, then washed and incubated in Sydney IVF Fertilization Medium, at 37°C and 6% CO2 for approximately 1 hour.

Oocytes Denudation

Oocytes were placed in a 100 µ1 drop of buffer containing 80 IU hyaluronidase/ml (Irvine Scientific, Irvine, CA, USA) for 30seconds, and then the oocyte was removed and placed in 100 µl drop of Sydney IVF Gamete Buffer. The corona cells were removed by gentle aspiration of the oocyte in and out by a sterile flame polished Pasteur pipette. When stripping was completed, the oocytes washed in Sydney IVF Gamete Buffer. All manipulations are carried on a heated microscope stage.

Oocytes Injection Procedure

The ICSI procedure is carried out using inverted microscope (Olympus 1x71) equipped with Hoffman modulation contrast optics, x4, x10 and x20 objective lenses and 10x eye pieces, with heated stage ,and automatic manipulators (Narashige, Japan)..The sperm cells scanned for the sperm selection. Immobilization of the sperm is done by hitting its tail with the injection micropipette, and then aspirated tail first into the pipette. The oocyte to be injected is attached to holding pipette at 9 o'clock using gentle suction, and then rotated such that the first polar body was located at either the 6 o'clock or 12 o'clock position.

Calcium ionophore A23187 preparation:

Calcium ionophore A23187 (Calcium ionophoreA23187(C7522) Sigma Free Acid; Sigma Chemical Co., St Louis, MO, USA) was dissolved in 1 ml of cell culture-tested dimethylsulphoxide (DMSO (D2650); Sigma–Aldrich Chemie, Belgium), to get the concentration of one mmol/l of Calcium ionophore A23187 stock solution.

The final solution containing 5 µmol/l ionophore was prepared by adding 5 µml from calcium ionophore stock solution to 995 µml of Cook Cleavage medium (Cook Sydney IVF Limited, National Technology Park, Ireland, UK) just before ICSI.

Oocytes Activation:

After oocytes injection, oocytes divided into two parts; the first part is the control; the second part; were incubated in culture Cook Cleavage medium containing 5 µmol/l of the calcium ionophore A23187 (Calcium ionophore A23187, Sigma C7522, EUA) covered with oil at 37°C and 6% CO2 for 5 min,. The oocytes are then washed many times and incubated in Cook Cleavage medium (Cook Sydney IVF Limited, National Technology Park, Ireland, UK) under oil at 37°C and 6% CO2 for further development.

After 18 hours from injection, the oocytes evaluated for fertilization, and evaluated secondary for embryo quality at the day of embryo transfer.

Day 1 Scoring (Assessment of Fertilization)

At about 18–20 h after ICSI, oocytes were examined for the presence of pronuclei and polar bodies by using an X20 objective lens on inverted microscope with Hoffman modulation contrast optics.Fertilization was considered normal when two clearly distinct pronuclei were present.

Assessing Cleavage-Stage Embryos (Days 2 and 3)

Embryo quality was evaluated on inverted microscope with Hoffman modulation contrast optics with X20 objective lens, and the following parameters were be in consideration : (i) the number of blastomeres; (ii) the fragmentation percentage; (iii) variation in blastomere symmetry; (iv) the presence of multinucleation; and (v) defects in the zonapellucida and cytoplasm.

Day 3 embryos classified as described previously from grade 1 to 6, grade 1 and 2 represents the embryos with good quality.

Statistical analysis

The data from all of the experiments described above were tested for statistical significance either by analysis of variance, ANOVA or Duncan's multiple range tests to determine difference in means using Statistical Analyses Systems. Results were considered to be significant at the 5% critical level ($P < 0.05$)**(SAS, 2000).**

3. RESULTS

Sperm Sources (Total Fertilized Oocyte No. /Total Oocyte No.)%			**OocyteTreatedwih Calcium Ionophore A23187**				**Oocyte Non- Treated wih Calcium Ionophore A23187**			
			Total Fertilized Oocyte No. /Total Oocyte No.		No. of Good Embryos Quality		Total Fertilized Oocyte No./Total Oocyte No.		No. of Good Embryos Quality	
Normospermia	132/146	90.4%	59/66	89.3%	31/59	52.5%	66/73	91.4%	36/66	54.5%
Oligoathenoteratospermia	87/142	61.2%	45/62	72.6%	21/45	46.6%	42/80	52.5%	19/42	45.2%
PESA	102/138	73.9%	44/59	74.5%	23/44	52.2%	48/79	60.7%	25/48	52.0%
TESE	76/145	52.4%	37/61	60.6%	12/37	32.4%	39/84	46.4%	12/39	30.7%

- 80 ICSI cycles involved in this group, 20 ICSI cycle in each subgroup.
- Total of 583 oocytes injected in this group, 415 oocytes were fertilized, with fertilization rate is 71.1%.

- Concerning the first subgroup normospermia the fertilization rate of oocytes treated with calcium ionophore A23187 and the fertilization rate of the control group are 89.3%. & 91.4% respectively, as the $P = 0.6213$ ($P < 0.05$); and it is non-significant value. More over the quality of the obtained embryos from the treated group and non-treated group (control group) are 52.5% & 54.5% respectively, which are almost the same, as the P value is 0.5409 ($P < 0.05$) , nonsignificant value.

- For the second subgroup oligoathenoteratospermia (OAT), the fertilization rate of oocytes treated with calcium ionophore A23187 is more than the fertilization rate of oocytes not treated with calcium ionophore A23187 (control group) as they are 72.4%. & 52.5% respectively, as the $P < 0.0001$; and it is highly significant value. About the obtained embryos quality the percentage of embryos with good quality from the treated group is 46.6% and non-treated group (control group) is 45.2%, which are almost the same, as the P value is 0.6440 ($P < 0.05$) , non-significant value.
- For the third epididymal subgroup (PESA), the fertilization rate of oocytes injected with sperm treated with calcium ionophore A23187 is more than the fertilization rate of oocytes not treated with calcium ionophore A23187 (control group) as they are 74.5% & 60.7% respectively , as the P value is 0.0002 ($P < 0.01$); and it is highly-significant value . Concerning the obtained embryos quality, the percentage of embryos with good quality from the treated group and non-treated group (control group) are 52.2% & 52.2% respectively , which are almost the same, as the P value is 0.9506 ($P < 0.05$), non-significant value.
- About the fourth group the testicular subgroup (TESE), the fertilization rate of injected oocytes treated with calcium ionophore A23187 is more than the fertilization rate of injected oocytes which is not treated with calcium ionophore A23187 (control group), as they are 60.6%. & 46.4% respectively, as the $P < 0.0001$; and it is highly significant value. About the obtained embryos, the percentage of embryos with good quality from the treated group is approximately as the percentage of embryos with good quality from non-treated group (control group), which are 32.4% & 30.7% respectively, as the P value is 0.4986 ($P < 0.05$), non-significant value.

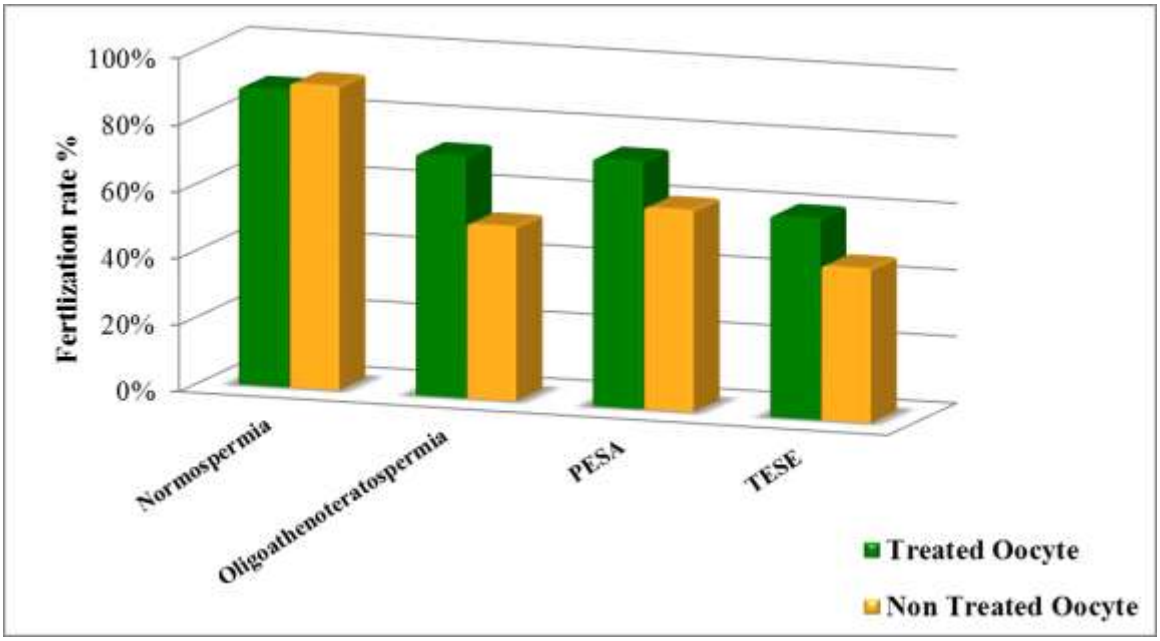

Figure (3): The fertilization rate of oocytes treated by calcium ionophore A23187 and the oocytes not treated by calcium ionophore A23187 (control group), in the four subgroups (Second Group).

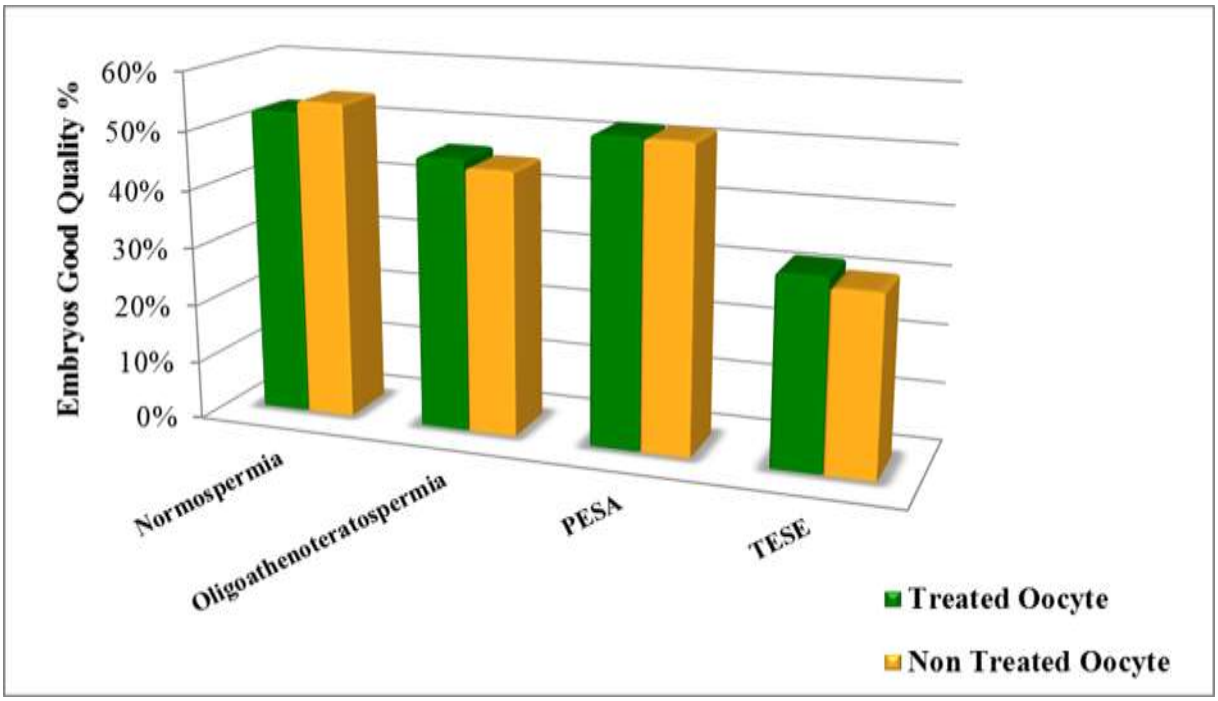

Figure (4): The percentage of embryos with goodquality in oocytes treated by calcium ionophore A23187 and the oocytes not treated by calcium ionophore A23187 (control group), in the four subgroups (Second Group).

4. DISCUSSION

In this study, we tried to assess the effect of calcium ionophore A23187 on the oocyte in enhancing the fertilization rate and embryos quality in ICSI cycles. The 80 cycles evaluated were divided into four subgroups according to sperm quality and origin (normospermia- oligoathenoteratospermia – epidermal –testicular).

Many worked on the effect of calcium ionophore inoocyte activation, although most of them were case studies **(Vanderzwalmen*et al*., 1997, Battaglia*et al*., 1997; Rybouchkin*et al*., 1997, Adams *et al*. 1999).** They resulted in that calcium ionophore A23187 may be useful for enhancing ICSI fertilization rates both for unexpected fertilization failure and for couples with a history of low fertilization.

Concerning the first subgroup with oocytes injected with normal sperm and treated with calcium ionophore A23187 after ICSI, the calcium ionophore A23187 did not enhance the fertilization rate in the normospermia patients; also did not improve the embryos quality. Our results are somewhat different with **Eldar-Geva*et al*. (2003), Chi *et al.* (2004) and Murase*et al.* (2004).**

As **Eldar-Geva*et al*. (2003), Chi *et al.* (2004) and Murase*et al.* (2004),** presented case reported with normospermic patients with low fertilization rate, following calcium ionophore activation, the fertilization rate increased and healthy babies were born. Their results different than this study results, that may be because all of them work on just a case reported patient with low fertilization, but concerning our study we work on non-repeated failure cases, and also may be by more investigation of normospermic patients' sperm as **Chi *et al*. (2004)** did .They found abnormalities in the patient's sperm as many nuclear vacuoles were observed and the expression of some proteins was absent.

Concerning oligoathenoteratospermia patients, we found that treatment of oocytes injected with oligoathenoteratospermia with calcium ionophore increases the fertilization rate. Our results are in agreement with **Tejera*et al.* (2008)** as he worked on globozoospermic patient (teratozoospermic patient), **Sugaya(2010)**who worked on oligozoospermia patient , **Isachenko*et al*. (2010)** who worked on athenoteratozoospermia patients and **Phan*et al*. (2015)** showed a case report of globozoospermia patient. **LU *et al*. (2012)** represented a case with a successful pregnancy and live birth after calcium ionophore A23187 activation on one-day-old unfertilized oocytes in a patient whose husband suffered oligoasthenoteratozoospermia, and who had experienced repeated near-total fertilization failure after ICSI**. Ebner*et al*., 2012** indicate that a ready-to-use calcium ionophore can yield high fertilization and pregnancy rates specially for cryptospermic and azoospermic patients.

Concerning the third subgroup where oocytes are injected with epididymal sperm treated with calcium ionophore we found that the fertilization rate increased with significant value. Our result is in agreement with **Borges *et al*. (2009a).** They results were that the fertilization rate, embryo quality, and pregnancy rate increased in epididymal spermatozoa. Although in our study the embryos quality did not enhanced in the epididymal treated subgroup.

Concerning the fourth subgroup where oocytes are injected with testicular sperm treated with calcium ionophore we found that the fertilization rate increased with significant value. Our result is in agreement with **Ahmady and Michael (2007) and Stecher*et al.*(2011)** As Ahmady and Michael (2007) they presented a case study reported a successful pregnancy and delivery after ICSI with a frozen-thawed nonviable testicular sperm and AOA with a calcium ionophore, also **Stecher*et al*. (2011)** presented a case report of live birth following ICSI with non-vital frozen-thawed testicular sperm and oocyte activation with calcium ionophore.

This in contrast with **Check *et al*. (2007) and Borges *et al*. (2009a&b)** results. As **Check *et al.* (2007)** found that oocytes injected with the testicular sperm of a globozoospermic patient were subsequently exposed to calcium ionophore failed to become fertilized. Concerning **Borges *et al.* (2009a&b)** result about testicular patients as he found no significant result when he treated oocyte injected with testicular sperm with calcium ionophore.

One of the reasons for failed fertilization after ICSI is lack of oocyte activation **(Nasr-Esfahani*et al*., 2008).**

It has been proposed that human oocyte activation during fertilization is mediated by a two-step pattern of increases in intracellular Ca^{2+} concentrations **(Borges *et al.*, 2009a).**

The two-step mechanism, called the trigger and oscillation function, is involved in triggering and maintaining oocyte activation **(Tesarik*et al*., 2002).**

The initial Ca^{2+} rise, which is released from internal stores, is the trigger and is initiated by a receptor-mediated interaction between the sperm and the oocyte plasma membrane **(Heindryckx*et al*., 2005).**

During ICSI this natural trigger is replaced by a so-called 'pseudotrigger' whereby a massive influx of Ca^{2+} into the oocyte is provoked by the injection procedure itself **(Heindryckx*et al*., 2005).**

The second function, the oscillator, is characterized by the development of Ca^{2+} oscillations, resulting from the release of a soluble sperm factor into the oocyte cytoplasm **(Ben-Yosefand Shalgi, 2001).**

In ICSI, once the sperm is injected into the oocyte, the sperm membrane must disintegrate to release the phospholipase Cζ (PLCζ), to generate Ca^{2+} oscillations very similar to those seen during normal fertilization and then initiates the remaining sequence of fertilization events **(Berridge, 2014).**

It is clear that this release of PLCζ from the injected sperm takes some time, because, following ICSI, there is a long latency before Ca^{2+} oscillations begin. In some cases, this latency can extend to 2–10 h before Ca2+ oscillations begin. However, this latency can be greatly reduced if oocytes are treated first with a Ca^{2+}ionophore (A23187), which presumably will enhance the level of intracellular Ca^{2+} , thereby helping to sensitize the IP3 receptors to the point that they will begin to generate Ca^{2+} oscillations **(Berridge, 2014).**

At the cellular level, Ca^{2+}originates from two sources, one external and one internal. Either it is released from internal stores through channels in the endoplasmic reticulum or it enters the cell via sensitive channels that bridge the oolemma. $Ca^{2+}$is only available to a limited extent and once internal stores are deficient or emptied the oocyte/ embryo will be reliant on influx of extracellular Ca^{2+}**(Ebner*et al.*, 2015b).**

It has been demonstrated that Ca^{2+} oscillations in fertilized oocytes regulate not only short-term but also long-term developmental events **(Ducibella*et al.*, 2002).**

The oscillator drives the oocyte's internal calcium stores rendering them capable of supporting the ongoing, largely autonomous series of Ca^{2+} oscillations for several hours **(Ben-Yosef and Shalgi, 2001).**

Thus, it is presumable that the absence of, or aberrant, calcium oscillations at the time of fertilization could lead to failed or low fertilization following ICSI **(VandenMeerschaut*et al.*, 2013).**

Ca^{2+}Ionophores facilitate such mechanisms. When it comes to keeping intracellular calcium level sufficiently high, most embryologists rely on the use of Ca^{2+}ionophores, such as ionomycin or calcimycin (A23187) **(Ebner*et al.*, 2015b).**

Calcium ionophores have proved particularly advantageous because of their ease of implementation. As exposure of human gametes to calcium ionophores has not yet been found to be associated with evidence of toxicity ordetrimental outcome **(Ebner*et al.*, 2012).**

In conclusion we found that using calcium ionophore in oocyte activation may be useful for some patients, as it increased the fertilization rate in oligoathenoteratospermia, epididymal (PESA) & testicular (TESE) patients without any effect on the embryos quality.

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