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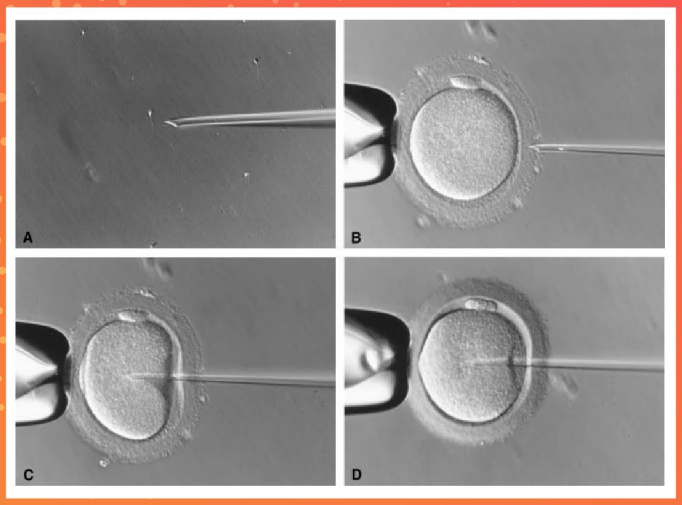
NEXUS

Indian Fertility Society & ORIGIO India Initiative

Artificial Oocyte Activation



Chief Editor: Dr. Pankaj Talwar

Dr M Gouri Devi
President - IFS

It is a great privilege and pleasure to write this message for this E-bulletin of IFS – Nexus dedicated to Artificial Oocyte Activation. The idea of Nexus has been initiated to bridge the gap between ART Clinicians and Embryologists. It aims to enhance the awareness about quality control, basic IVF techniques and lab protocols within the IVF community. Artificial Oocyte Activation using Ca2+ ionophores or similar compounds is a widely applied technique in IVF laboratories. Ca2+ peak caused by ionophores is appropriate to rescue cycles showing severe male factor infertility, deficient oocyte maturation. This remains a controversial topic and widely debated. In this bulletin the authors would cover at length the nuances of AOA.

On behalf of the Indian Fertility Society I sincerely thank "ORIGIO India Private Ltd" to partner with us in this great academic endeavor. My heartiest congratulations to the editorial team at IFS, ORIGIO and the entire team and very best wish fort the future.

Prof (Dr) Pankaj Talwar
Secretary General - IFS
Editor NEXUS

Dear all,

Indian fertility Society feels proud and congratulates the editors on the launch of this edition of Nexus E-Bulletin. We take it as our duty and responsibility to train and educate our budding embryologist and infertility specialists right from the basics and this bulletin is a step forward in this direction. It has been known for decades that periodic changes in internal Ca2+ mediate a large range of cell functions involved in oocyte activation and subsequent cell function. Different procedures for AOA have been established and are commonly divided into three subtypes -Mechanical, electrical, and chemical stimuli that elicit one or several calcium transients and help in successful fertilization. The effectiveness of these calcium oscillations depends on their number, frequency, temporal modulation and amplitude. These all would be discussed in this E bulletin and I am sure that you would all benefit by reading this material. I thank the guest editors for this excellent compendium.

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In this modern era , an interested reader can easily find information from book, electronic media and journals. when it comes to Assisted Reproductive Techniques quality of information is vital. I am extremely pleased and gratified that IFS bulletin helps fertility practising specialists and embryologist not only to enhance their knowledge but to improve the success rate.

We all are aware that with tremendous improvement in ART and use of sophisticated facilities for IVF lab, fertilization rate approaches 80-90%. **However, fertilization failure still exists as a frustrating experience- which is really a challenge for clinician and embryologist. Total Fertilization Failure** [TFF]occurs in 5- 10% of conventional IVF cases and 2-3% in ICSI cases. TFF is related to sperm and/or,egg abnormalities and oocyte activation defects.

Identifying and addressing right cause might help to improve the outcome. Artificial Oocyte Activation is a choice of method to address oocyte activation defects.

In this manual three different methods of Oocyte Activation have been explained, among which chemical oocyte activation is most popular and easy to incorporate in our practice. Ionophore will be a handy tool for Artificial Oocyte Activation and practical tips will help embryologist to enhance fertilization rate.

I am thankful to IFS for giving me this opportunity to compose and compile this manual.

Warm regards

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Special Acknowledgement

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Editorial team

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Part - 1

Basics of Oocyte Activation

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Part 1: Basics of Oocyte Activation

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1 Basics of Oocyte Activation

In assisted reproduction programs, decisions concerning the treatment techniques, conventional IVF or ICSI, are usually taken after examination of the male factors and the result of previous IVF attempts into account. There are no fixed criteria for treatment of IVF and/or ICSI. ICSI is a choice of treatment with male factor of infertility or sub-fertility.[1] In spite of all the preliminary testing and recommendations, fertilization failure or low fertilization can still occur. Low or failed fertilization can be due to sperm factors, egg factors or both or environmental factors. Fertilization is a complex biological process and while some steps are known, many other steps involved are still poorly understood. Poor or failed fertilization may be due to a failure at any step in the process of fertilization, hence a better understanding of gamete physiology and fertilization process is needed to improve the fertilization rate in ART treatment.

Fertilization can be described through the following steps, viz., Sperm capacitation, sperm-zona pellucida binding, the acrosome reaction, penetration of the zona pellucida, sperm-oocyte binding, egg activation and the cortical reaction, zona reaction and post-fertilization events.

The changes in sperm behavior and morphology, known as sperm capacitation, are necessary to ensure oocyte fertilization was reported in 1952[2]. Capacitation is a multistage process involving changes in the form and function of the sperm that are induced by oocyte extracellular structures. The changes include motility, chemotaxis, binding, **Acrosome Reaction (AR)** and fusion of the two plasma membranes [3]. Acrosome reaction renders spermatozoa to be able to penetrate the **Zona Pellucida (ZP)**. Once the sperm cells find their way past the ZP, cortical reaction takes place. Cortical granules inside the secondary oocyte fuse with the plasma membrane of the cell, causing enzymes inside these granules to be expelled by exocytosis to the ZP that makes the whole matrix hard and impermeable to the sperm, preventing fertilization of an egg by more than one sperm. The cortical reaction and acrosome reaction are both essential to ensure that only one sperm fertilizes an egg. When a signaling cascade leads to the cortical granule reaction, oocyte activation takes place. The oocyte completes its second meiotic division and releases a second polar body. The nucleus of the oocyte is called a pronucleus. The sperm's tail and mitochondria degenerate with the formation of the male pronucleus which migrates toward the center of the oocyte. Rapid replication of its DNA prepares the zygote for the first mitotic division [4].

Although ART plays a critical role in reducing infertility, there are still several infertile couples for whom ART has not yet been successful. Particular outstanding concern are infertile males whose sperms are unable to activate oocytes, even following ICSI, or idiopathic male factor infertility. [5]

Oocytes that are arrested at metaphase of second meiotic division biologically progress only after oocyte activation. Activation initiates release from meiotic arrest, cortical granule exocytosis, progression of the cell cycle, pronuclear formation, maternal mRNA recruitment, and involves repeated oscillations of free cytosolic calcium.[6] This calcium is a universal secondary messenger in cells controlling diverse biological processes.

There exist two hypotheses pertaining to oocyte activation. They are the **PLCz (Protein Lipase C zeta)** and the **PAWP (Post-Acrosomal Sheath WW Domain-Binding Protein)** hypothesis. According to the former hypothesis, sperm triggers rise in intracellular calcium concentration and consequently awakens the oocyte which is blocked at the metaphase of the second meiotic division. In mammalian oocytes, Ca^{2+} oscillations are triggered upon sperm entry. The Ca^{2+} oscillations are necessary to relieve M II arrest and to provoke all the other events of oocyte activation like cortical reaction, maternal mRNA recruitment, pronuclear development and mitotic cleavage. M II arrest is characterized by a high level of the cyclin **B/cdk1** complex, also known as **Maturation Promoting Factor (MPF)**. MPF prompts M II and is involved in several central features of cell division like dis-assembly of the nucleus, chromosome condensation, cytoskeletal rearrangements and arrest in transcriptional activity. In the surface-mediated model of oocyte activation according to this hypothesis, the sperm may trigger egg activation via the interaction between a sperm protein and an egg surface receptor. This receptor is activated by the sperm binding and a possible signaling pathway could be the activation of a **Tyrosine Kinase** which then activates **Phospholipase C (PLC)**. In the ooplasm hydrolysis of **Phosphatidylinositol 4,5-Biphosphate (PIP2)** is induced which generates two second messengers: **Inositol Triphosphate (IP3)** and **Diacylglycerol (DAG)**. Release of Ca^{2+} is induced by binding of IP3 to its receptor **(IP3R)** and Ca^{2+} osillations start. [7] **(Fig: 1)**

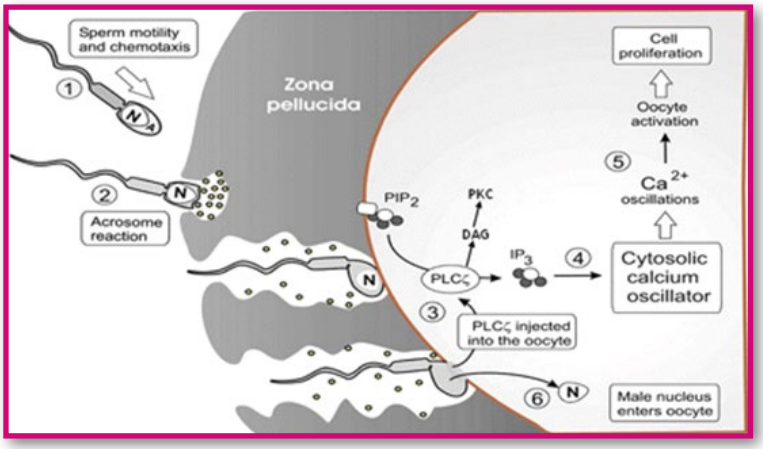

Fig: 1

According to the PAWP hypothesis, another possible candidate for the sperm factor is the **Post-Acrosomal Sheath WW Domain-Binding Protein (PAWP)**, which resides in the post-acrosomal sheath region of the perinuclear theca. It plays role in meiotic resumption during fertilization. Although the molecular mechanisms underlying the precise function of PAWP are still unknown, it was suggested that both PAWP and PLCz pose a double role in the oocyte activation mechanism or alternatively, that the PAWP-mediated signalling pathway may act upstream or downstream of Ca^{2+} signalling. [8,9]

Part - 2

Frequently Asked Questions (FAQs)

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1 What are the steps of fertilization?

Step 1:
Sperm penetration in expanded cumulus cells.

Step 2:
Requires sperm recognition of the zona pellucida which is dependent on the four ZP proteins (ZP1, 2, 3, 4), as well as linked oligosaccharides. The sperm bind to ZP3, undergos the acrosome reaction and bind to ZP2 and becomes competent to penetrate the zona pellucida.

Step 3:
Involves sperm/oocyte fusion events where the sperm bind to the oolema through interactions with microvilli and associated membrane proteins subsequently forming a fusion pore.

Step 4:
Encompasses the process of oocyte activation, wherein a signaling cascade leads to the cortical granule reaction and block polyspermy through modification of ZP2 and ZP3, leading to completion of the second meiotic division and extrusion of the 2nd polar body.

Step 5:
Includes processes required for processing of the sperm to release its nuclear contents.

Step 6:
Fertilization process is completed through formation of the pronuclei and their migration as they prepare for syngamy [10]. **(Fig: 2)**

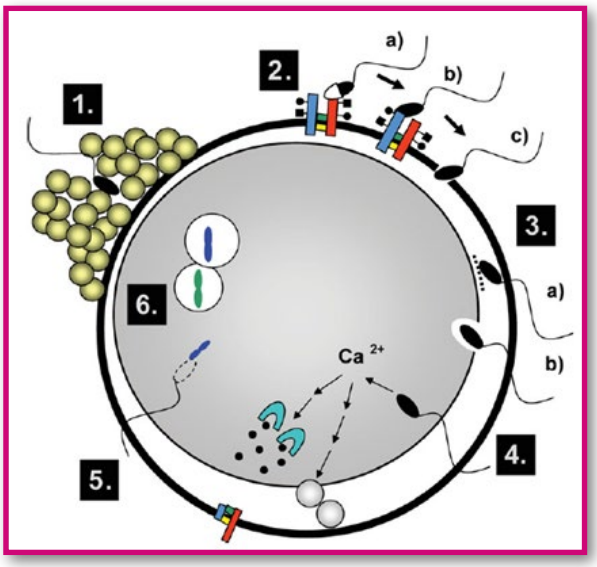

Fig: 2

2 What is the difference in procedure between conventional IVF and ICSI to achieve fertilization?

In ICSI the egg is held in place while a single sperm is injected into the cytoplasm of the egg using a very fine needle. On the contrary, in conventional IVF, the eggs and sperm are mixed together in a dish for fertilization of the egg naturally. **(Fig 3)**

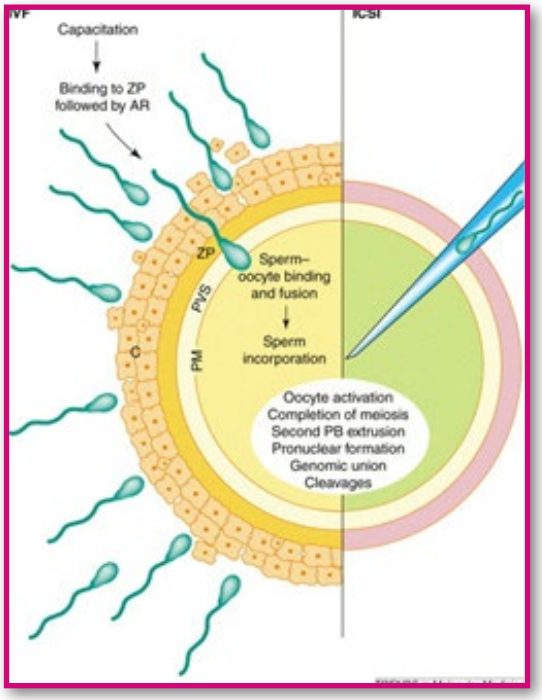

Fig: 3

3 What are the reasons for fertilization failure in Conventional IVF and ICSI?

Total Fertilization Failure (TFF) occurs in 5–10 % of IVF cycles while it is in 2–3 % of ICSI cycles [11,12]. If any event of fertilization process like cumulus cell penetration, sperm/oocyte binding and penetration, sperm/oocyte fusion, oocyte activation, sperm processing or pronuclei formation goes wrong there occurs failure in fertilization. In case of ICSI the first three reasons are bypassed.

Summary of failed fertilization etiologies and their prevalence are as under

I. Pre-sperm penetration

a. No sperm incorporation - 15–56 % [13]
   Aberrant protein secretion, increased apoptosis and attached carbohydrate moieties affects sperm penetration or acrosome reaction.

b. Maternal chromosomal defects - 10 - 30% [14]

II. Post-sperm penetration

a. Failed oocyte activation - 15 - 66% [15]
   (Inadequate or abnormal Ca^{2+} oscillations caused by malfunctioning endoplasmic reticulum, mitochondria or IP3 signaling pathways, resulting in aberrant kinase/phosphatase signaling cascades, lead to failed cortical granule exocytosis or failure to correctly complete the second meiotic division).

b. Failed sperm head de-condensation - 4 - 45%)[16]
   (Failed sperm head de-condensation occurs due to failed fusion or activation events or abnormal reversible phosphorylation. Failure to remodel chromatin is due to insufficient amount of oocyte-derived proteins **(Glutathione, Histones).**

c. Premature sperm chromatin condensation - 2- 23% [17]

d. Spindle defects/sperm aster defects - 6- 18% [13]

e. Sperm ejection -6- 23% [18]

III. Miscellaneous

Number of oocytes retrieved, stimulation protocol and ICSI technique might also affect fertilization.

4 What is Artificial Oocyte Activation (AOA)?

A human oocyte enters the first meiotic division during embryonic life and gets arrested in this phase for an extended period of time. Upon resumption of the first meiotic division, the oocyte is subsequently arrested at the second metaphase (MII) where it waits for fertilization. Upon fertilization, spermatozoa overcome the second meiotic arrest by inducing a series of cellular events within the oocyte that are essential for normal development, collectively called oocyte activation. These events include an early intercellular rise in calcium concentration from endoplasmic reticulum stores. This increase occurs 1 to 3 minutes after fusion of the sperm with oolemma and originates at the point of sperm entry.

The first transient rise in Ca^{2+} is followed by a series of shorter calcium transient rises of high amplitude, known as calcium oscillation. As fertilization progresses, the amplitude and frequency of calcium transient decreases while their duration increases until absolute cessation. Induction of calcium oscillations from intracellular stores in the human oocyte is believed to be triggered by inositol triphosphate, which is catalyzed by **Sperm-Specific Phospholipase C** named **PLCζ**, present in the perinuclear theca of sperm. The induced calcium oscillation leads to resumption of meiosis, decondensation of sperm nucleus, maternal RNA recruitment, formation of male and female pronuclei, initiation of DNA synthesis, and cleavage.

PLCζ are responsible for induced calcium oscillation and subsequent oocyte activation. It is of interest to note that, unlike in IVF, calcium oscillation in ICSI begins after a delay of approximately 30 minutes to several hours. Sometimes oocyte activation does not occur and it may result in failed fertilization as calcium transient is the key trigger of oocyte activation.

Different procedures for AOA [19] have been established and are commonly divided into three subtypes, viz., mechanical, electrical, and chemical stimuli that elicit one or several calcium transients and help in successful fertilization.

5 What are the indications for Artificial Oocyte Activation?

The indications includes

1	**Oocyte related activation deficiency**
2	**In Vitro Maturation- IVM oocytes**
3	**Low numbers of oocytes at retrieval**
4	**Severe teratozoospermia**
5	**Severe oligoasthenoteratozoospermia- OAT**

6	**Globozoospermia**
7	**Surgical sperm collection either for azoospermia or high sperm DNA fragmentation index**
8	**Previous fertilization failure or poor fertilization**
9	**Unexplained infertility**
10	**Frozen Thawed oocytes**

6 What is Mechanical Oocyte Activation?

In ICSI, two steps are considered essential for successful fertilization. These are immobilization of the spermatozoon and rupture of the oolemma. A sperm-associated factor is likely to be responsible for initiation of egg activation in ICSI. Oocyte–sperm interaction like ooplasmic factors triggering sperm chromatin decondensation is essential.

Due to impaired semen characteristics or very low oocyte numbers some patients may face repeated fertilization failures. In such patients modified ICSI or mechanical stimulation might be useful. Modified ICSI techniques involving repeted dislocation of central ooplasm to the periphery, thereby increasing the intracellular free calcium concentration either by influx or release from cell organelles.

Modified ICSI is based on the hypothetical accumulation of highly polarized mitochondria, with a high inner mitochondrial membrane potential from pericortical regions (9 O'Clock position) to the centre of the oocyte, thus, theoretically, supplying more ATP (energy) directly to the place where the spermatozoon was injected. In this regard, it appears that aggregation patterns of mitochondria correspond well to the light microscopic appearance of the oocyte.

Mitochondria are maternally inherited organelles that are functionally heterogeneous and highly polarized, and thus of high metabolic ATP activity. These mitochondria maintain adequate ATP level while other mitochondria move to the center of the ooplasm during fertilization and play a key role in oocyte activation.

Modified ICSI causes an influx of calcium and calcium stored in endoplasmic reticulum will be set free due to mechanical process of this oraganelle and trigger oocyte activation. This method is more beneficial for couples with failed fertilization in their previous IVF cycles. [19]

Procedure:

1	Keep sperm at the tip of micro injection pipette. Oolemma breakage should achieved by vigorous aspiration of the ooplasm into the needle.
2	Aspiration will be done with the needle tip located in the oocyte central area to reduce the risk of disturbing the area occupied by oocyte chromosomes. **(Fig. 4)**
3	The ooplasm will be aspirated until the distance between the spermatozoon in the needle and the outer surface of the zona pellucida is equal to the distance between the outer surface of the zona pellucida and the center of the oocyte.

4	The needle tip will be subsequently pushed as close as possible to the oocyte periphery opposite to the puncture site, and the aspirated ooplasms is re-injected into the oocyte until the spermatozoon reached the needle tip
5	The needle then pulled back until the needle tip reached the central area of the oocyte, and the same aspiration and re injection procedure will be repeated once more

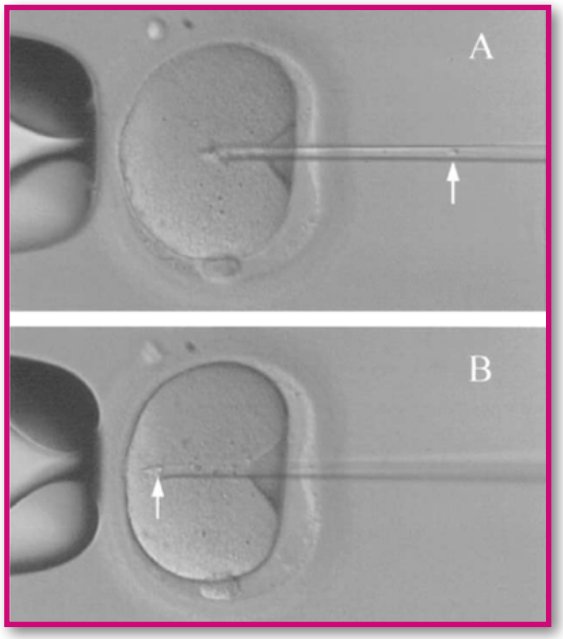

Fig: 4

7 What is Electrical Oocyte Activation?

In ICSI, because the sperm is injected inside the ooplasm, failure of pronuclear formation and division most probably is the result of the failure of oocyte activation. The key event of oocyte activation involves a temporal rise in the intracellular Ca^{2+} concentration. The typical pattern in a Ca^{2+} rise is repetitive until the pronuclear stage. A single long-lasting rise in Ca^{2+} concentration can be induced by certain agents like ethanol, calcium ionophore, or a single electric pulse. It has been reported that about 70–80% of unfertilized oocytes after ICSI responded to electro-activation and form two pronuclei. [19]

1	Oocyte Electrical Activation is performed 30 minutes after ICSI.
2	The oocytes will be suspended in 0.3 M glucose drops, with pH at 7.3, and placed between two parallel electrodes (2 mm apart) in an electric slide chamber (BTX micro slide P/N 450, 0.5-mm gap; BTX, San Diego, CA).
3	A double-square direct-current pulse (130V, 50 µc apart) is generated by using an electro cell manipulator (BTX) to achieve the desired field strength of 2.6–2.8 kv/cm

4	The activation setting is adjusted as follows: Mode, HV (99 per sec per 3 KV); Volage, 130–140 V; Pulse length, 50 µs and Pulse number of two
5	The electrically stimulated oocytes immediately will be transferred back to the tissue culture media, to be rinsed and then incubated under oil in 5% CO_2 in air, at 37^0C.
6	The oocytes will be checked 16–18 hours after injection to determine the presence of pronuclei.

8 What is Chemical Oocyte Activation?

Most cases of fertilization failure following ICSI can be traced back to a lack of oocyte activation. From the morphokinetic point of view, fertilization failure typically results from an insufficient number of mature oocytes with normal morphology, irregular spermatozoon morphology, or poor motility characteristics.

At the intracellular level, the reason for the lack of oocyte activation may be due to a deficient cytosolic S**perm-Associated Oocyte Activating Factor (SAOAF)** resulting in a partial or complete inability of the sperm to activate the oocyte, or to the inability of the oocyte to decondense the sperm. These events include an early intracellular rise in calcium concentration from endoplasmic reticulum stores. This increase occurs 1 to 3 minutes after fusion of the sperm with oolemma, and it originates at the point of sperm entry. This first calcium transient rise is followed by a series of shorter calcium transient rises of high amplitude, known as calcium oscillations. As fertilization progresses, the amplitude and frequency of calcium oscillations decrease while their duration increases until absolute cessation after 2-3 hours.
Assisted Oocyte-Activation (AOA) techniques, aiming at artificially increasing the intracellular calcium, whether mechanical, electrical or chemical have been suggested as potential tools to overcome total fertilization failure but with variable success. Chemical stimulation, the most commonly used method for AOA includes brief exposure of the injected oocytes to one of the agents like ethanol, strontium chloride, calcium ionophore or ionomycin. An ionophore is a lipid soluble molecule usually synthesized by microorganisams to transport ions across the lipid layer of the cell membrane.

Calcimycin (Ionophore A23187), also a calcium ionophore that was first isolated from the fermentation reactions of *Streptomyces Chartreusis*. It is a mobile ion carrier that forms stable complexes with divalent cations and can cause cell activation. After ICSI procedure injected oocytes are exposed to 10 mol/lit ionophore solution for 5 minutes and cultured in IVF media after three washes. **Ionomycin** is also an ionophore produced by the bacterium *Streptomyces conglobatus.* In mammals cells ionomycin acts as a potent and selective Ca^{2+} ionophore. It induces hydrolysis of phosphoinositides. Sigma catalogue number 13909 is a ready to use solution. After ICSI procedure injected oocytes are incubated in ionomycin solution for 5 minutes under oil in CO_2 incubator and then washed twice before culture in IVF media.

Strontium chloride action mimics the effects of sperm on the oocyte, and seemed to be mediated through the inositol trisphosphate receptors for Ca^{2+} release from endoplasmic reticulum.

9 What are the follow up studies and is it safe to use?

Chemical activation of oocytes does not appear to be cytotoxic when used in an optimum concentration and about 80% of unfertilized oocytes revealed normal chromosomes. Some studies revealed no physical or mental developmental disorders in babies born through these procedures, even 12 months after birth, [22] Although use of these agents must be limited to indicated cases as their potential cytotoxic, teratogenic, epigenetic and mutagenic effects on oocytes and embryos is not yet very clear. It should be noted that issues of genetic safety and abnormal imprinting have not been addressed for the combined use of these oocyte activation methods.

10 How does injection procedure effects an oocyte?

- Since 1992, when the first pregnancy reported, ICSI has rapidly become choice of treatment for severe male factor sub fertility.The procedure seems to be safe,but still concern has been raised about the potential danger of the ICSI procedure. Theoretically , both the injection technique it self, as well as the possible injection of abnormal spermatozoa can affect oocyte.

Possible factors that might affect an oocyte during ICSI are as under:

a. Small quantity of medium is injected into an oocyte along with sperm can either cause oocyte degeneration or failure to activate. Embryologist need to maintain sperm at the tip of injection pipette and careful about loading of sperm into

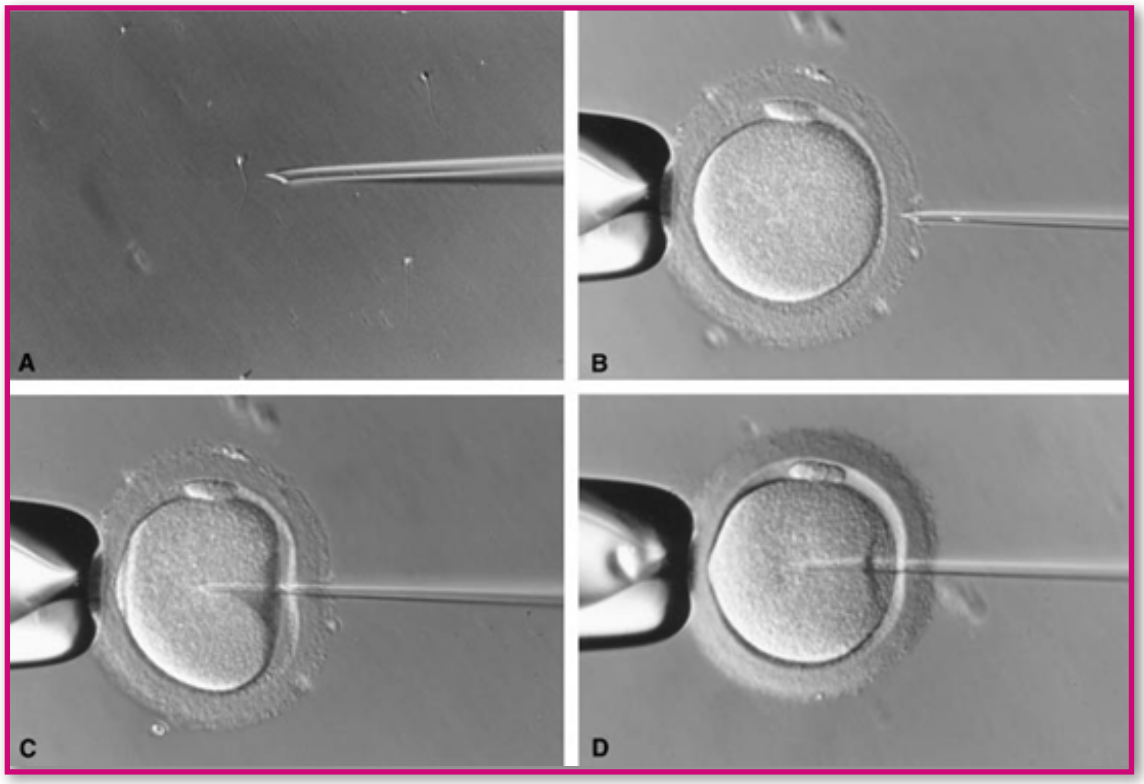

Fig: 5

b. Cellular injury may happen during cytoplasm aspiration- which might result in failure of oocyte activation. Do not aspirate too much of the cytoplasm. **(Fig: 5)**
c. In the group of oocytes where oolemma is broken immediately after an injection or it did not break can also be the possible reason for activation failure.
d. Positioning of polar body during ICSI procedure also affect fertilization rate. It is recommended to keep polar body either at 12 or 6 O'Clock position to prevent spindle injury.
e. Picking up the morphological normal sperm will help to improve activation process. Motility enhancer like theophyline can be used to improve motility.

11 What are the precautions an embryologist should take to improve fertilization rate?

- Ensure ICSI timing , it should be at-least 40 hours post trigger injection.
- While denuding an oocytes be fast and do not stress an oocytes. Use 140 or 150 µm diameter denuding pipette and pre warm Hyaluronidase enzyme.
- Set micromaipulator and align injecting and holding pipette before hand.
- Check the timing of ICSI procedure, load fewer numbers of oocytes per ICSI dish to minimize timing of keeping oocyte outside the incubator.
- If Surgically retrieved sperms or severe oligoasthenoteratozoo sperms are used for an ICSI, first find the normal sperms and then load the oocytes in ICSI dish.
- Hold the oocyte gently and avoid aspirating large volume of cytoplasm.
- Keep polar body either at 12 or 6 O'Clock position.
- Observe oocyte and sperm morphology and save pictures in system to study later.
- Check channel formation , breakage of oolemma and cytoplasm characteristics.
- Write ICSI start and finish time.

12 What are the different protocol for Artificial Oocyte Activation with calcium ionophore as per available literature studies?

Study	Study Type	AOA Criteria	Procedure
Tesarik et al 1995[23] **2000**[22]	Case study	Unfertilized oocytes after ICSI Globozoospermia	10 µM Ca^{2+} ionophore - Sigma A 23187 in DMSO for 10 mins after 30 mins of ICSI 10 µM ionophore A 23187 for 10 mins
Battaglia et al 1997[25]	Case report	Globozoospermia	10 µM ionophore A 23187 for 10 mins
Eldar-Geva et al 2003[26]	Case report	Previous failed fertilization	10 µM/L ionophore A 23187 after 1 hr of ICSI
Chi et al 2004 [27]	Case Report	-	8 µM/L ionophore A 23187 for 8 mins after 30 mins of ICSI
Ahmady et al 2006 [28]	Case report	Testicular sperm extractions	10 µM/L ionophore A 23187 for 10 mins after ICSI
Borges et al 2008[29]	Prospective Study	Surgical sperm extractions	5 µM ionophore A 23187 for 30 mins

Study	Study Type	AOA Criteria	Procedure
Tejera et al 2008 [30]	Case report	Globozoospermia	5 picoliters of 0.1 µM/L ionophore aspirated and injected with sperm into oocyte
Isachenko et al 2010 [31]	Case report	Asthenoteratozoopermia	10 mM calcium ionophore (end concentration in culture medium) just after ICSI for 20 min
Montag et al 2012 [32]	Retrospective cohort study	Fertilization failure in previous cycle	10 mM/L calcium ionophore A 23187 for 15 mind immediately after ICSI
Maryam et al 2013[33]	Prospective Study	Teratozoospermia	5 µM of ionophore A23187 for 5 min immediately after ICSI
Kim et al 2015 [34]	Case report	IVM oocytes	10 mM of calcium ionophore A23187 for 30 min after half an hr of ICSI
Seda Karabulut et al 2018 [33]	Case report	Frozen thawed sperms	10 µM ionophore A23187 for 5 min, 30 min post ICSI

13 What are the different protocol for Artificial Oocyte Activation with ionomycin as per available literature studies?

Study	Study Type	AOA Criteria	Procedure
Moaz et al 2006 [36]	Prospective Study	Previous failed fertilization	10 µg/ml ionomycin - ICN USA for 10 mins after an hr of ICSI
Nasr-Esfahani et al 2008 [37]	Prospective Study	Asthenoteratozoospermia	10 µM ionomycin for 10 mins

14 What are the different protocol for Artificial Oocyte Activation with commercially available ready to use ionophore as per available literature studies?

Study	Study Type	AOA Criteria	Procedure
Ebner et al 2012[38]	Prospective, Multicenter study	Male factor infertility	Oocytes were incubated for 15 min in 200 mL of a readyto-use A23187solution called GM508 CultActive (Gynemed) immediately after ICSI
Caglar Aytac et al 2015[39]	Prosptive, Randomized controlled study	Previous failed fertilization	Injected oocytes were incubated in 50 mL GM 508 CultActive calcium ionophore solution for 15 min (Gynemed)

15 What are the different protocol for Artificial Oocyte Activation with Strontium chloride ($Sr Cl_2$)as per available literature studies?

Study	Study Type	AOA Criteria	Procedure
Yanagida et al 2006 [40]	Case Report	Previous failed fertilization	10mM $SrCl_2$ (Sigma) for 60 min after 30 min of ICSI
Kyono et al 2008 [41]	Case Report	Previous failed fertilization	10mM $SrCl_2$ (Sigma) for 60 min after 30 min of ICSI
Jun Woo Kim et al 2012[42]	Case Report	Frozen thawed testicular sperm	10mM $SrCl_2$ (Sigma) for 60 min after 30 min of ICSI

Part - 3

Artificial Oocyte Activation step by step

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Part 3: Artificial Oocyte Activation step by step

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1 Procedure -1 Artificial Oocyte Activation with Ionomycin solution from Sigma

Media Requirements	Disposable Requirements
	• Tissue culture grade Center well or Four well dish • Transfer pipette • Denuding pipette - 170 µm • Micro pipette tips

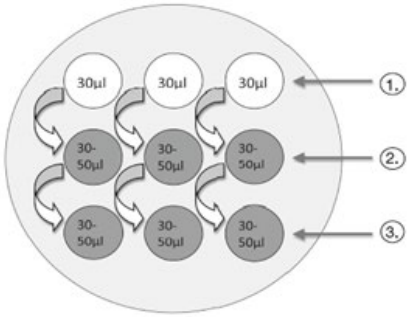
Procedure	Steps
	• In four well dish add 500 µl of IVF media and cover with paraffin oil. Keep the dishes for equlibration in incubator for minimum 8 hours • Add 5 µl ionomycin solution in first well to get final solution of 10 µM /litre. Keep the dishes in incubator for 15 - 20 minutes. • 30 minutes after ICSI, expose oocytes to pre warmed ionomycin solution for 10 minutes. • Wash the oocytes twice in remaining two wells and culture it in fourth well till pronuclear check. • 16-18 hours post ICSI check for presence of pronuclei.

2 Procedure -2 Artificial Oocyte Activation with Calcium Ionophore from Gynemed

Media Requirements	Disposable Requirements
Calcium ionophore solution - Gynemed product - GM508 CultActive 	• Tissue culture grade Center well or Four well dish • Transfer pipette • Denuding pipette - 170 µm • Micro pipette tips
IVF culture media Paraffin oil	• Pipette handle

Instructions for use:

- GM508 CultActive must be shaken directly before use for approximately 30 sec.
- GM508 CultActive must be equilibrated 4 hours in a vial not firmly closed at 5-7 % CO_2 and 37°C prior to use.
- Equilibrate culture medium for washing for 4 hours in a vial not firmly closed at 5-7 % CO_2 and 37°C prior to use.

Procedure	Steps
1. Activation - GM 508 CultActive - 30 µl 2. Washing Step 1 - IVF media - 30-50 µl 3. Washing Step 2 - IVF media - 30-50 µl 	• Prepare for each oocyte 1 drop (30 µl) GM 508 CultActive and 2 drops (30-50 µl) MOPS and HEPES-free culture medium (oil overlay is not necessary due to the short exposure time). • Immediately after the ICSI procedure incubate the oocytes for 15 minutes in the pre-equilibrated Ca^{2+}Ionophore GM 508 CultActive drops. (Step 1 in Fig) • Remove the oocytes from the GM 508 CultActive drop and wash them twice in culture media. This has to be done in a HEPES-or MOPS-free medium, e.g. GM 501 Cult. (Step 2 and 3 in Fig) • Keep the oocytes in IVF culture media for further culture. • Assess fertilization on specific time points

Part - 4

Indian Vendors

S. No.	Nomenclature	Cat No.	Price	Manufacturer / Distributor's Address and Contact No.
1	Sigma	13909	24000 ≈	www.sigmaaldrich.com +91 80 66219600
2	Gynemed	GM-508 CultActive	6955+ 18%GST	• Mr. Karan Kalra omikenterprises@gmail.com WZ-97/204, 2nd floor, sunder Palace jwala Heri market, Paschim Vihar New Delhi - 110063 Mobile: +91 9891166997 Tel: 011 25273118 • Mr. Prashant Kadam info@sscientifics.com F-96, 1st Floor, Dreams the Hall CBS Road, Bhendup (West) Mumbai - 400078 Mobile: +91 9220710738 Tel: +91 22 400566677

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Notes

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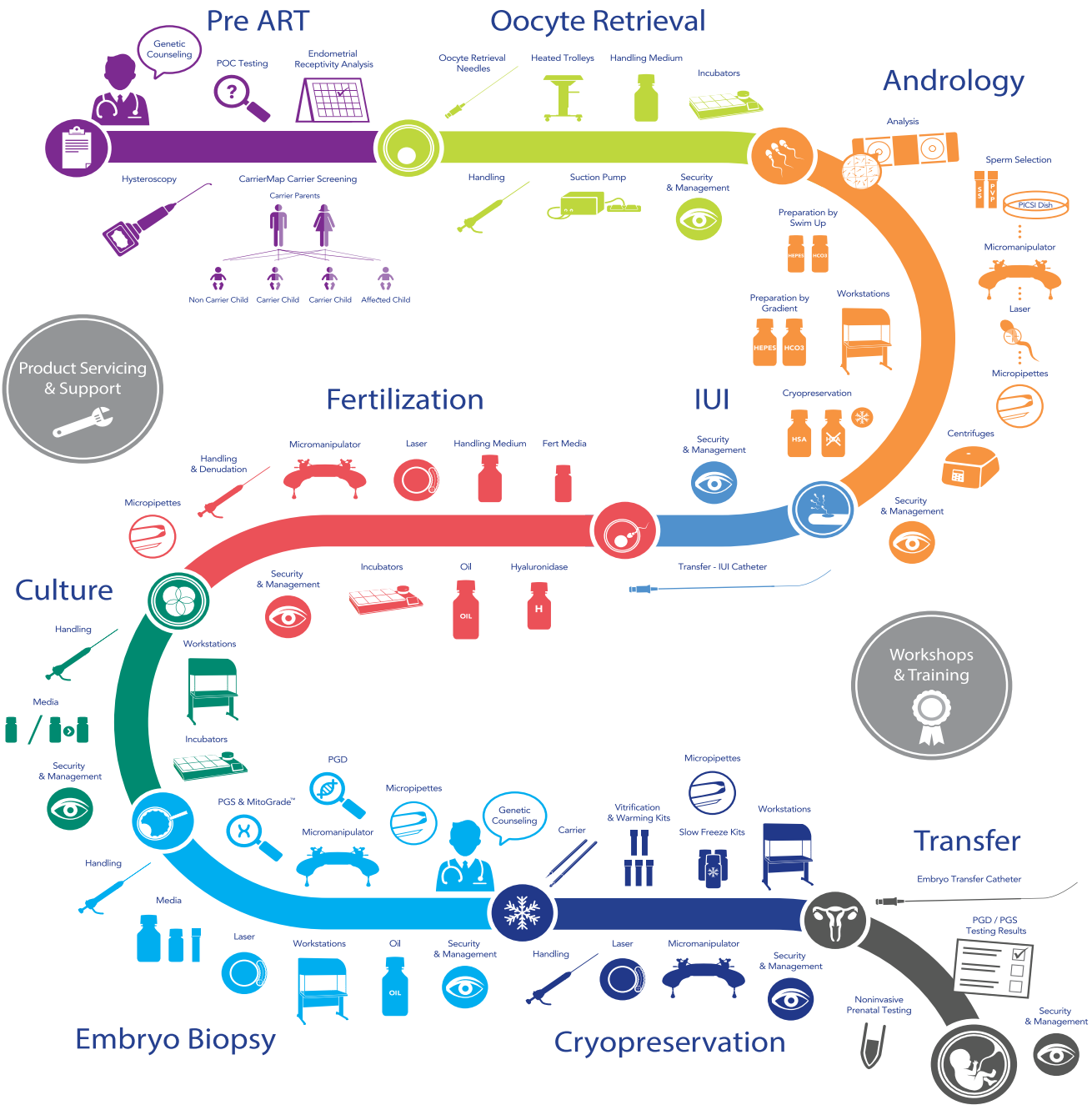

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