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Chapter

Oocyte Activation Failure: Physiological and Clinical Aspects

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Abstract

Despite successful treatment of infertility with assisted reproductive technology (ART), total fertilization failure (TFF) after in vitro fertilization (IVF) and even after intracytoplasmic sperm injection (ICSI) still occurs. In the current chapter, the incidence and etiology of TFF after ICSI are described. The literature on physiology of oocyte activation, electrical properties of gametes' membranes, and ion currents is reviewed. Calcium oscillations play an essential role in fertilization, and calcium ions act as secondary messengers in different metabolic pathways and cellular processes during oocyte activation. The contribution of oocyte- and sperm-related causes of fertilization failure is discussed. Many studies on the physiology of fertilization in mammals have shown that oocyte activation is triggered by the sperm factor. Methods for artificial oocyte activation (AOA) try to bypass fertilization failure by influencing physiological processes that are crucial for successful fertilization. Activation can be induced with the use of electrical, mechanical, or chemical stimuli that elevate intracellular concentrations of calcium ions. Different AOA methods and their success and safety are presented.

Keywords: oocyte activation, total fertilization failure (TFF), calcium oscillations, artificial oocyte activation (AOA), gamete maturation, ion channels, ion currents, calcium signaling, meiosis, intracytoplasmic sperm injection (ICSI), PLCζ, calcium ionophores

1. Introduction

In vitro fertilization (IVF) techniques enabled conception outside the body and led to the birth of the first child conceived in vitro in 1978 [1]. The first laboratory technique used for conception in vitro was "classic" IVF where a suspension of prepared sperm cells is added to oocytes surrounded by cumulus cells and fertilization occurs naturally. The most discouraging result of such assisted reproduction technology (ART) treatment was fertilization failure, occurring often with the male infertility factor or unexplained infertility. The development of a micromanipulation technique named intracytoplasmic sperm injection (ICSI) in 1990 [2] enabled new treatment possibilities for many couples. Bypassing initial steps in the process of natural fertilization, a single spermatozoon is inserted directly into the cytoplasm. Successful fertilization is thus also achieved with low sperm numbers, surgically retrieved sperm, or frozen sperm samples. ICSI was soon globally accepted as a reliable technique leading to successful fertilization, pregnancy, and healthy offspring. Although ICSI can overcome some fertilization problems, total fertilization failure (TFF) still occurs in some ICSI cycles. In some patients, this failure can

repeat in several ART cycles. Some patients have extremely low fertilization rates, which consequently lowers their chances for successful treatment.

Studies of etiology of fertilization failure after ICSI revealed that the predominant cause is oocyte activation failure [3, 4]. In humans, oocyte activation is the transition of the oocyte into a zygote where a series of intracellular calcium (Ca^{2+}) oscillations following the fusion of the gametes play an essential role. Calcium ions are released from intracellular storage in the endoplasmic reticulum; free in the cytosol, they are intracellular messengers and act as modulators of processes in the early steps of fertilization and embryo development. In humans, oocyte activation thus describes a cascade of events that lead to completion of the meiosis, cortical granule exocytosis for prevention of polyspermy, formation of the male and female pronuclei and progression in the first embryonic cell cycle. Both sperm and oocyte defects can cause failed activation.

Artificial oocyte activation (AOA) methods can be used in clinical practice in reproductive medicine in rare cases of TFF or low fertilization. AOA tries to reproduce elevations of calcium ion concentration in cytosol, which are necessary for triggering downstream processes in oocyte activation.

2. Total fertilization failure (TFF) after ICSI

Total fertilization failure after ICSI is complete lack of fertilization at the standard checking time of 17 ± 1 h post ICSI. This means that the obvious sign, female and male pronuclei, is not visible in any of the injected mature oocytes in the metaphase of meiosis II (MII) of the patient.

According to data from the literature, complete fertilization failure occurs in: 2 [5], 1.3 [6], 3 [7], 3 [8], 5.6 [9], and 4.3% [10] of ICSI cycles.

Complete fertilization failure after ICSI is directly correlated with the number of mature oocytes available [9], so the definition is needed. TFF is not surprising in cases of poor ovarian response, nonmotile spermatozoa, or poor sperm morphology. But even if we have a sufficient number of mature oocytes of normal morphology and sperm of good quality, TFF happens and can reoccur in subsequent cycles.

Another problem that also lowers the chances for successful treatment of infertility is an extremely low fertilization rate.

2.1 Results of retrospective analysis of data from our center

We analyzed all consecutive ICSI cycles in the period between years 2011 and 2016. Results are presented in **Table 1**. In this period, we performed 7474 ART cycles (IVF and ICSI) in our center. The majority of these cycles were stimulated with gonadotropins (recombinant FSH or highly purified human menopausal gonadotropin) with pituitary suppression using agonists or antagonists, followed by hCG administration for 36–37 h before ultrasound-guided follicle aspiration. Some of these cycles were natural cycles, as previously described in [11].

In this period, we performed 4533 ICSI cycles with at least one mature oocyte in the metaphase stage of meiosis II (MII) available. Complete fertilization failure (TFF) occurred in 247 (5.5%) of ICSI cycles.

We compared standard characteristics of these cycles regarding the number of oocytes. There were 3550 cycles with 3 or more MII oocytes, TFF occurred in 76 among the (2.14%) cycles. There were 983 cycles with 1 or 2 MII oocytes available, TFF occurred in 171 among them (17.4%). A total of 35 of these TFF cycles were natural cycles.

	Cycles with ≥3MII	**Cycles with <3MII**	**Cycles with >0% and <30% fertilization (≥3MII)**	**Cycles with >70% fertilization (≥3MII)**
N of all cycles	3550	983	175	1980
N of TFF cycles (%)	76 (2.14%)	171 (17.4%)	/	/
Woman age (years)	35.62 ± 4.42	37.26 ± 4.69	34.70 ± 4.96	34.14 ± 4.65
Stimulation protocol				
• Natural cycles	/	35 (20.5%)	/	/
• Long protocol with agonists GnRHa	26 (34.2%)	23 (13.5%)	58 (33.1%)	595 (30.6%)
• Short protocol with antagonists antGnRH	45 (59.2%)	101 (59.1%)	105 (60.0%)	1297 (65.1%)
• Other	5	12	12	88
Duration of stimulation	10.40 ± 1.95	8.77 ± 4.85	9.96 ± 1.76	9.98 ± 1.8
Gonadotropin dose (ampoules)	32.80 ± 11.84	31.53 ± 20.72	28.86 ± 10.75	27.34 ± 9.92
N oocytes	8.04 ± 6.10	2.02 ± 2.46	10.16 ± 5.4	10.16 ± 5.95
MII	5.80 ± 4.13	1.25 ± 0.44	7.91 ± 4.41	8.51 ± 5.07
2PN	0	0	1.35 ± 1.21	7.37 ± 4.33
1PN	0.07 ± 0.25	0.09 ±0.30	0.16 ± 0.40	0.12 ± 0.37
3PN	0.16 ± 0.49	0.26 ± 1.11	0.22 ± 0.64	0.10 ± 0.35
Damaged	0.29 ± 0.82	0.26 ± 1.11	0.57 ± 1.06	0.22 ± 0.55
Male diagnosis				
• Normozoospermia (% of TFF cycles)	15 (19.74%)	61 (35.67%)	31 (17.7%)	371 (18.7%)
• Krypto- and azoospermia (% of TFF cycles)	18 (23.68%)	17 (9.94%)	45 (25.7%)	214 (10.8%)
ET	0	0	1.04 ± 0.76	1.57 ± 0.69
Clinical pregnancy	/	/	39/220 17.7%	702/1822 38.5%
Birth rate	/	/	13.2%	30.5%

Table 1.
Analysis of all consecutive ICSI cycles performed in our clinic in the period between years 2011 and 2016 (N = 4533); cycles with at least one mature oocyte in the metaphase stage of meiosis II (MII) are included. Data are presented in means ± SD or number of cases (percentage of all cases in a group).

We also analyzed the characteristics of cycles with 3 or more MII oocytes and low fertilization; in doing so, we took into account cycles with more than 0% and less than 30% MII oocytes fertilized. There were 175 such cycles (3.9%).

Woman's age is greater in cycles with fewer oocytes, which can be explained with lower ovarian reserve in greater age. Regarding the stimulation protocol of the ART cycles, there were 35 natural cycles in the group with <3 MII oocytes. In our center, natural cycles are mainly performed in patients with extremely low ovarian response, where increasing gonadotropin dosage does not increase the chance to obtain more oocytes.

In TFF cases where 3 or more MII oocytes are available, there is a higher proportion of severe male infertility cases (22.68%) compared to the cycles with less than

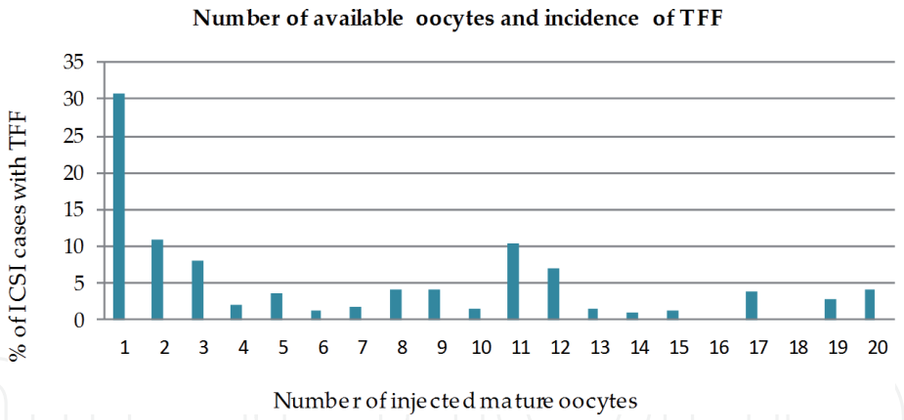

Figure 1.
Number of available oocytes and incidence of TFF. The proportion of ICSI cycles with total fertilization failure regarding the number of mature oocytes available; x = number of injected MII oocytes; y = % of cycles with TFF. 4533 ICSI cycles analysed.

3 MII oocytes (9.94%). When there are more oocytes available, there is statistically less probability for TFF, and more cases of TFF are due to gamete defects. A similar proportion of low fertilization cycles are those with severe male infertility (25.7%). Severe male infertility is described as diagnosis of cryptozoospermia and azoospermia, where individual sperms have to be extracted from semen sediments or testis aspirates/biopsies.

In cases where only one mature oocyte was available, total fertilization failure occurred more often (30.8%) than in those with more oocytes.

In **Figure 1**, the correlation between the number of available mature oocytes and occurrence of TFF is presented. With more oocytes available, there is less probability for TFF.

3. Etiology of failed fertilization after ICSI

Soon after implementation of the ICSI technique, some investigations of possible reasons for unsuccessful fertilization began. It was first speculated that perhaps the proportion of unfertilized oocytes arises from technical limitations of the method itself that cannot deliver the sperm in the cytoplasm, or ejection of the sperm from the cytoplasm occurs after injection. It was established that in only 7 [12], 16.7 [7], 10.6 [13], and 12.6% [14] of unfertilized oocytes after ICSI the sperm DNA was outside the oocytes.

With different staining techniques, visualization of the chromatin, spindle, and other structures was possible, and this enabled a better understanding of at what stage unfertilized oocytes are (**Table 2**). It soon became evident that the majority of unfertilized oocytes are arrested in the metaphase of the meiosis II with different levels of sperm chromatin decondensation, which suggested that oocyte activation and sperm decondensation run independently [15]. In the majority of these oocytes, sperm chromatin is in a decondensed state, which indicates that protamines are usually successfully replaced by histones [14], so unsuccessful decondensation of sperm chromatin can be the underlying cause for only a relatively small proportion of unfertilized oocytes. Premature chromosome condensation (PCC) is a condition when sperm chromosomes are getting condensed in the cytoplasm of oocyte too early and the right synchronization between sperm and oocyte genetic material is compromised. Up to 33% of studied unfertilized oocytes had PCC [15], but it is difficult to conclude whether this indicates sperm- or oocyte-borne defect.

The studies are summarized in **Table 2**.

Study	Method	Number of analyzed specimens	Findings
Flaherty SP, Payne D, and Matthews CD [7]	Hoechst 33342: fluorescent stain for adenine-thymine rich regions in DNA	n = 1005 NF MII	82% of oocytes are arrested in metaphase MII; of these, 74% decondensed SC, 11% intact SC, 15% without sperm in the cytoplasm
			only 17% of oocytes activated; of these 56% decondensed SC, 20% intact SC, 15% without sperm in the cytoplasm
Dozortsev D, Sutter PD, and Dhont M [12]	Giemsa: stain for adenine-thymine rich regions in DNA	n = 82 NF MII	93% oocytes having sperm in cytoplasm and MII chromosomes of the oocyte present; of these 51% SC, 41% intact SC, 8% premature chromosome condensation (PCC)
Yanagida K [13]	Aceto-orcein stain: chromatin staining	n = 76 NF MII	86.8% oocytes having sperm in cytoplasm; of these 68.2% decondensed SC, 4.5% PCC, 27.3% intact SC
Pitsos MA, Nicolopoulou-Stamati P [14]	Chromomycin A3: binds to G-C rich DNA regions, does not bind to DNA coupled with protamines Propidium iodide: fluorescent DNA stain	n = 93 NF MII	74.8% metaphase MII oocytes; of these 63.6% decondensed SC, 23.4% condensed SC, and 13% no sperm in the cytoplasm. In majority of spermatozoa, successful replacement of protamines with histones took place.
Rawe VY, Olmedo SB, Nodar FN, Doncel GD, Acosta AA, and Vitullo AD [16]	Immunofluorescence analysis with immunoglobulins and monoclonal antibodies	n = 150 NF MII	13.3% oocytes with no sperm, 39.9% activation failure, 22.6% defects of pronuclear formation/migration, 13.3% arrest in metaphase of the 1st mitotic division
Kovacic B and Vlaisavljevic V [15]	Hoechst 33258, FITC	n = 180 NF MII	69% oocytes arrested in metaphase MII, 11% completed meiosis, but no PN development

SC = sperm chromatin, MII = mature oocyte in metaphase of meiosis II, NF = non-fertilized oocyte, and PCC = premature chromosome condensation.

Table 2.
Studies of the etiology of fertilization failure.

On the basis of the studies listed in **Table 2**, we can conclude that failed oocyte activation seems to be the predominant reason for fertilization failure. However, it is unclear whether the cause is sperm or oocyte defect, since proteins, organelles, and metabolic paths of both gametes are involved in the activation.

Oocyte activation failure being the main problem was also confirmed by electron microscopy, where unreleased cortical granule at periphery, maternal chromosomes in the metaphase plate, and paternal intact or partially decondensed chromatin were found [3]. These are all signs of failed activation, but it is difficult to conclude on which level in the cascade there is a failure.

Perhaps, in the future, genetic data will give us more information on the etiology of fertilization failure. An interesting case report where researchers investigated possible reasons for fertilization failure on genetic levels analyzed gene expression profiles in unfertilized oocytes of a patient with previous TFF history [17].

4. Oocyte activation

Oocyte activation is a downstream cascade triggered by sperm that causes progression of the oocyte from meiosis arrested in metaphase II toward its completion and beginning of embryonic development. It is a serial of biochemical reactions, organelle redistribution, changes in metabolism, transmembrane potentials, mRNA translation, gene transcription, and cytoskeletal rearrangements.

The role of calcium in fertilization was established very early with a series of experiments on sea urchin eggs where the amount of bound and free calcium was measured in fertilized and nonfertilized eggs [18]. Later, calcium-specific light-emitting protein aequorin injected in fish oocytes enabled visualization of light flash after fusion of oocyte and sperm [19]. It soon became evident that calcium ions play an essential role in activation of the animal oocyte and that the frequencies and amplitudes of these elevations of calcium ions in cytoplasm are species-specific [20].

The term "oocyte activation" probably evolved on the basis of these evident sudden changes that happen during the transition from oocyte to embryo. It describes not only calcium waves that occur but also other processes and morphological changes that happen during fertilization. Intracytoplasmic calcium elevation is essential for fertilization, but it is not always the sperm that triggers it. In some animal species such as fruit flies (*Drosophila*) the calcium wave occurs prior to oocyte-sperm fusion, during ovulation [21]. The focus of our text will be human oocyte activation, but since nonhuman biological material is usually more available or even easier to study, many data on fertilization come from studies on sea organisms such as starfish and sea urchins or different mammalian species. Early studies of the role of calcium in the process of fertilization and even use of ionophores are well documented in the review of Epel [22]. The source of an intracellular rise of calcium ion concentration can be external—calcium enters the cell by influx through calcium channels in the plasma membrane or can be released in cytoplasm from intracellular stores in the endoplasmic reticulum [23].

But it is important to understand that the details vary a lot through the animal kingdom and that these differences can be the reason why the ICSI method can be successful in humans but not in other species. However, the animal studies are the foundation for the development of assisted reproduction techniques in human medicine.

Early studies of fertilization in mammals are well reviewed by Miyazaki [24]; in sum, there is the first hyperpolarization of membrane potential as a result of a change in potassium conductivity across the plasma membrane. This coincides with an increase of free calcium in cytosol; there is no electrical block of polyspermy and a serial of intracytoplasmic rises of calcium concentration follow continuously (oscillations) at intervals of different frequencies and amplitudes, which depends on the species studied. Intracellular calcium first rises near the site of the sperm attachment and spreads like a wave over the entire egg [24]. The model of generating calcium spikes from intracellular stores in the endoplasmic reticulum was described by Igusa and Miyazaki [25]. The techniques used for revealing these processes were measurements with calcium-sensitive microelectrodes, the voltage-clamp technique, aequorin injections, injection of calcium ion chelators, and injection of different compounds that interact with the calcium-releasing system.

The first study of calcium measurements at fertilization in human oocytes showed that the first rise in intracytoplasmic calcium concentration appears 20–35 min after adding sperm suspension in a chamber with oocytes; spikes appear every 10–35 min, with a single spike of amplitude up to 2.25 μM calcium concentration and duration of 100–120 s [26].

Other researchers studied changes of membrane potential across the plasma membrane in human oocytes at fertilization and showed that the increase of potassium ion conductivity of the plasma membrane and outward current of ions, which causes hyperpolarization, is calcium dependent [27]. A study on bovine oocytes gave more information about the relationship between hyperpolarization of the plasma membrane potential and calcium release from intracellular stores and targeted calcium-activated potassium channels as membrane proteins involved in the process [28].

Soon after introducing ICSI, it was of great interest to compare these responses with the classic IVF method, where events such as sperm capacitation and activation, acrosome reaction, and sperm-oocyte membrane fusion happen first. From the work of Tesarik et al. [29], we can see that when performing ICSI in human oocytes, the first intracytoplasmic rise of calcium ion concentration happens immediately; the peak is 10–15 s after penetration with the needle. Sperm then evokes intracellular calcium oscillations. They described that oscillations follow the lag period that lasts 4–12 h. Oscillations are in the form of spikes that last 20 s; the intervals between spikes are 1–5 min. The duration of the oscillatory phase is 30 min–1 h; at the end of the period, the amplitude of calcium spikes gets smaller.

The proposed mechanism through which calcium oscillations are maintained is through the phosphoinositide signaling pathway, where inositol 1,4,5-trisphosphate ($InsP_3$) is generated from phosphatidylinositol 4,5-bisphosphate (PIP_2) [23]. The positive feedback cycle involving calcium-dependent $InsP_3$ generation and $InsP_3$-induced calcium release seem to be responsible for the oscillations [23]. The main protein is $InsP_3$ receptor ($InsP_3R$), a ligand-gated channel found in the membrane of the endoplasmic reticulum that allows calcium release from the ER [30].

That calcium oscillations have a role in long-term embryonic events and provide more than merely a stimulus for meiotic resumption was shown in experiments with different activating agents and subsequent measurements of cell mass of the blastocyst [31].

4.1 The role of free calcium ions in cytoplasm

Calcium is the secondary messenger that regulates different events during fertilization, such as progression of the cell cycle from metaphase II arrest toward chromatid segregation, extrusion of the second polar body and completion of the second meiotic division, and cortical granule exocytosis [32]. The role of calcium in reproduction is preserved through evolution; it is important in plants and animals. Species-specific calcium signatures, like oscillations in mammals, have evolved, which are optimal for activation and development of a specific type of organism [33]. The variations in amplitude, duration, and frequency of oscillations over time are coordinated with the cell cycle, and experimentally changing them also affects development in the later stages when blastocyst forms [31]. Experiments with injecting calcium (Ca^{2+}) chelators in the cytosol of frog eggs demonstrated the blockage of activation [34].

Calcium rises in cytosol are converted in different cellular responses.

4.1.1 Ca^{2+}-dependent process of cortical granule exocytosis

Cortical granule exocytosis enables polyspermy block by altering the zona pellucida with the content of the granule (proteases, peroxidases, and glycosaminoglycans) and prevent more sperms from fertilizing oocytes. The proposed model is that calcium stimulates Ca^{2+}/calmodulin (CaM)-dependent protein kinase

II (CaMKII) and myosin light-chain kinase (MLCK) that phosphorylate a vesicle targeting protein and myosin II to promote exocytosis [33]. It is a quick response; in mouse oocytes, exocytosis starts 15 min after exposure to capacitated sperm, and 30 min after insemination, 78% of cortical granules disappear from cytoplasm [35]. It is proposed that each calcium oscillation cycle moves cortical granule one step closer to the egg plasma membrane toward the fusion with the plasma membrane and exocytosis of the contents [33].

4.1.2 Ca^{2+} is a trigger for cell cycle progression

The completion of meiosis means that the extrusion of the chromatids in the second polar body enables the formation of haploid oocytes that can form a female pronucleus that will be able to combine genetic material with the male pronucleus.

Meiosis is the ground of sexual reproduction where homologous chromosomes recombine—exchange genetic material through chiasmata and generate new genetic combinations that are unique to the offspring. Mammalian oocytes progress through meiosis very slowly; first, the cell cycle is arrested in the dictyate phase of prophase I during the fetal life of a girl and stays in this phase up to 40–50 years. During the menstrual cycle, the recruited oocytes in the ovary progress through the cell cycle under the influence of the hormones. But the meiosis is again arrested at the metaphase of the second meiotic division (metMII). Calcium oscillations at fertilization activate calmodulin-dependent protein kinase II (CaMKII) and switch on the anaphase-promoting complex/cyclosome (APC/C) that leads to securin and cyclin B1 degradation necessary for cell cycle progression and segregation of sister chromatids [33].

Ca^{2+} also plays a role in pronuclear formation by decreasing MAP kinase (MAPK) activity responsible for nuclear envelope assembly [33]. Oscillations terminate with PN formation, and PLCζ localizes into PN [36]. The mechanism by which Ca^{2+} recruits maternal mRNA for translation and genome activation is not well understood at the moment. There are some data indicating that calcium oscillations and mRNA translation are coupled [37]. Not only in cytosol, calcium can also diffuse to the nucleus and control different cell functions by direct nuclear calcium signaling [38].

4.2 Electrophysiology and fertilization

Fertilization potential is a change in the membrane potential across the plasma membrane (PM) of the oocyte that is first observed after oocyte-sperm interaction. In many invertebrates, this is in the form of depolarization of the plasma membrane and is proposed to provide a fast block to polyspermy, described in some invertebrates and only a few vertebrates (such as frogs), but not present in mammals [39]. Lately, there have been some discussions about the nature of electrical block in preventing polyspermy [40]. The role of electrical events in the form of depolarization or hyperpolarization of the plasma membrane at fertilization remains unclear.

In mammals, there is hyperpolarization of membrane potential as a result of change in potassium conductivity across the plasma membrane [24]. In human oocytes, outward current and long-lasting hyperpolarization of the plasma membrane were described [27]. The channels responsible for this hyperpolarization are calcium-activated potassium channels [41]. Species-specific differences in the channels involved in early electrical responses at fertilization are reviewed in [42].

During oocyte maturation, the composition of the channels in the plasma membrane changes, as described in bovine oocytes [28]. The factors regulating

the composition of channels in the plasma membrane, the conductance for different ions, depending on the specificity, gating, and sensitivity of the channels at different stages, are still unclear. The conditions during gamete maturation are very important, and we can imagine that diet and changes in metabolic pathways can affect the performance. It is not just cytoplasmic maturity of the oocyte that is important, but also maturity of the plasma membrane.

The conditions in which gametes are matured are important; the diet and especially taking some medicines in this period can affect the infertility treatment outcome. There are some data from studies of calcium channel blockers used as therapy in various cardiovascular conditions. They affect the movement of free calcium ions across membranes, and dose-dependent reduction in sperm mobility and viability in vitro that can affect fertility treatment was demonstrated [43].

4.3 Sperm factors

Data from studies of fertilization pointed to a sperm component that has to trigger response in the form of calcium oscillations. But the exact component, its nature, and mechanism were long unknown.

There were four main hypotheses, reviewed in [44]. The first one assumed that sperm delivers calcium to the oocyte that further stimulates release of calcium from intracellular stores. The conduit hypothesis assumed that sperm increases the permeability of the plasma membrane for calcium that enters the oocyte with influx from the surroundings. The contact hypothesis predicts that sperm interacts with a receptor on the plasma membrane that causes calcium release from intracellular stores. But success of the ICSI method revealed that there is no need for interaction of sperm and receptors in the plasma membrane for fertilization. The fourth is the sperm factor hypothesis that assumes that there is a component in the sperm cell delivered in cytosol with sperm and that this factor causes calcium release from intracellular stores.

Experiments where soluble sperm extracts are injected into the oocyte coupled with different biochemical approaches enabled the search for unknown sperm factor and were in favor of the sperm factor hypothesis [45, 46].

There were many candidates such as oscillin, a cytosolic sperm factor related to prokaryote glucosamine phosphate deaminase [47]. In nonmammalian species, PLCγ was identified [48] and the role of nitric oxide in fertilization was investigated [49]. In mammals post-acrosomal WW domain-binding protein (PAWP) was described, which is located in the post-acrosomal region of the sperm, from the stage of elongated spermatids onwards, that causes meiosis resumption and PN formation [50].

4.3.1 PLCζ

When it was demonstrated that sperm extracts were related to $InsP_3$ concentrations in cell and PLC activity [51], phospholipases were under investigation. Genetic data from the testis' cDNA library revealed some new isoform of PLC [23] and soon a novel sperm-specific phospholipase C, PLCζ was identified as a trigger of calcium oscillations in mouse eggs [52]. In the work of Saunders et al., it was experimentally demonstrated that PLCζ content in a single sperm evoked oscillations and normal embryonic development [52]. They also prepared PLCζ complementary RNA (cRNA) for injection into MII oocytes that triggered the same effect. When they removed PLCζ from sperm extracts, they no longer induced calcium oscillations.

Important evidence that PLCζ is the necessary trigger for calcium oscillations comes also from the studies of Dpy19l2 knockout mice that have globozoospermic sperm phenotype and absence of or extremely reduced PLC ζ and no ability to trigger calcium oscillations [53]. But proof in the form of the knockout mouse model was missing. By using RNA interference technology that prevents translation of PLCζ mRNA and that reduces the amount of PLCζ protein in transgenic mouse sperm, altered calcium oscillation patterns, lower egg activation, and no transgenic offspring [54] were observed.

A study where whole exome sequencing was performed in patients with previous TFF, homozygous missense mutation in PLCζ was established [55].

PLCζ was identified in many mammalian and nonmammalian species [56, 57] and can act across species. But there are differences in solubility of PLCζ in cytosol that can contribute to differences between species. It is proven in the mouse oocyte activation test (MOAT) that human PLCζ exhibits greater response in mouse oocytes than mouse PLCζ.

The amount of PLCζ measured in a single mouse sperm cell is in the same range as the amount required to cause oscillations experimentally [52]. This can explain why altered frequency of oscillations was established when more than one sperm fertilized an oocyte [58]. PLCζ has to diffuse through cytosol to trigger spatiotemporal response in the form of a calcium wave that spreads from the point of sperm entry to the other pole.

Recently, the knockout mouse model was prepared using CRISPR/Cas9 gene editing [59] and revealed interesting facts: PLCζ-null mouse males have normal spermatogenesis and normal sperm parameters (motility, capacitation, and acrosome reaction) but their sperm cannot elicit calcium oscillations after ICSI. Still, some oocytes can undergo activation in abnormal form or even develop to blastocyst stage. But mating knockout males with normal females shows that they can still produce offspring without PLCζ as a physiological trigger. Males are not infertile, rather subfertile, so there is possibility that apart from PLCζ, there is a second calcium releasing factor delivered to oocyte by sperm, perhaps an alternative that is used only when PLCζ is missing.

Calcium oscillations are normal physiological stimuli for oocyte activation but from parthenogenetic activation at ICSI and from the use of artificial oocyte activation techniques we already know that they are not always necessary to activate oocytes. They can be bypassed on some levels.

There are still many facts about calcium oscillations triggering that are not well understood. The proposed mechanism of activation is reviewed in [23] and represented in a schematic diagram (**Figure 2**): PLCζ diffuses from the sperm head into egg cytosol and hydrolyses the PIP_2 (phosphatidylinositol 4,5-bisphosphate) in the membrane of the vesicle compartment into products $InsP_3$ (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). $InsP_3$ binds to the receptor $InsP_3R$ on the endoplasmic reticulum membrane. The conformation of the channel is changed and it becomes permeable for Ca^{2+} ions that are released from intracellular storage in the endoplasmic reticulum into cytosol. Oscillations are generated through a positive feedback loop.

In a series of experiments on mouse oocytes injected with RNA encoding PLCζ and tagged with a fluorescent protein, it was established that at the time when calcium oscillations terminate and pronucleus is formed, PLCζ protein translocates from the cytosol to the pronucleus [36]. Authors also demonstrated that it is again released in the cytosol in the first mitotic division of an embryo at the time of nuclear envelope breakdown and observed also in the second mitotic division. This suggests the possible role of calcium oscillations not only in oocyte activation but also in early embryonic development.

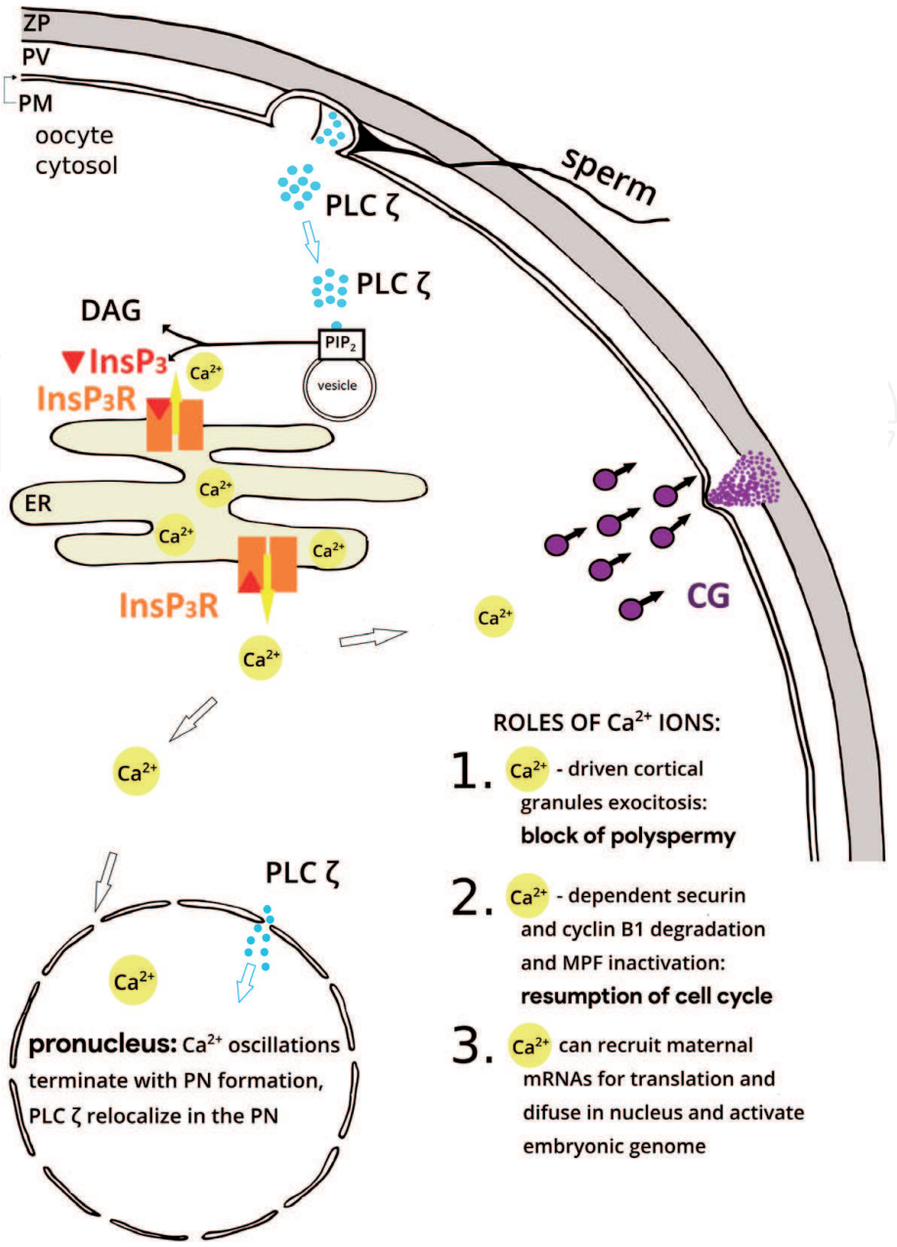

Figure 2.
Schematic diagram of the proposed model of sperm triggered oocyte activation at fertilization in mammals: after the fusion of sperm and oocyte plasma membrane PLCζ diffuses from sperm into oocyte cytosol. Vesicles baring PIP_2 are present in cytosol and PLCζ hydrolyzes PIP_2 into products DAG and $InsP_3$. $InsP_3$ binds to the $InsP_3R$ present in the membrane of ER. $InsP_3R$ is a ligand-gated Ca^{2+} release channel and Ca^{2+} is released from intracellular stores in ER into the cytosol. In mammals, repetitive Ca^{2+} oscillations occur, and several rises of calcium concentration in cytosol take place. Ca^{2+} ions play an essential role in oocyte activation. They enable block of polyspermy by chemically altering zona pellucida with the content of cortical granules that are released in the perivitelline space. Ca^{2+} ions enable cell cycle progression—resumption of meiosis II. Ca^{2+}-dependent inactivation of factors that hold cell cycle in arrested state takes place, by degradation of cyclinB1 and securin and MPF inactivation of cell cycle eventually progresses. Ca^{2+} ions play a role in recruitment of mRNAs and affect their translation. Ca^{2+} can diffuse in the nucleus and play a role in embryonic gene activation. Ca^{2+} oscillations terminate when female pronucleus is formed and PLCζ relocalizes in the pronucleus. PLCζ—phospholipase C zeta, PIP_2—phosphatidylinositol 4,5-bisphosphate, $InsP_3$—inositol 1,4,5-trisphosphate, DAG—diacylglycerol, $InsP_3R$—inositol 1,4,5-trisphosphate receptor, Ca^{2+}—calcium ions, ZP—zona pellucida (the glycoprotein envelope surrounding mammalian oocyte), PV—perivitelline space (space between ZP and plasma membrane of oocyte), PM—plasma membrane, cg—cortical granules, and PN—pronucleus.

All together there is a lot of accumulating evidence that points toward PLCζ as a trigger of oocyte activation cascade in mammals. Soon the idea of using recombinant PLCζ in clinical practice emerged that will be discussed later among other

methods for artificial oocyte activation. But still there are many data missing that are needed to fully understand oocyte activation.

4.3.2 Other sperm-related factors that affect oocyte activation

Spermatozoon has to go through many changes in order to be able to fertilize an oocyte naturally. First, the mechanism of chemotaxis between the gametes plays an important role; capacitation is the process of altering the sperm plasma membrane so that it becomes more permanent for calcium ions, then changes in sperm movements in the form of hyperactivation help to bring the spermatozoon closer to the oocyte. Finally, at acrosome reaction the content of acrosomes (enzymes) facilitates the fusion between the plasma membrane of the oocyte and spermatozoon so that the paternal genetic material can enter the oocyte. Sperm also delivers a centriole into the oocyte that duplicates and forms centrosome, a microtubule-organizing center responsible for mitotic divisions in a growing embryo. ICSI bypasses many of these events and enables fertilization and successful development, but there are still sperm factors other than PLCζ that can contribute to failure of oocyte activation.

Successful sperm chromatin decondensation is a necessary condition for fertilization. The chromatin of the spermatozoon is uniquely packaged in such a way that histones become replaced by protamines during the spermatogenesis. Protamines provide more structural stability but after the entry of the spermatozoon into the oocyte the chromatin of the spermatozoon must be uncoiled and protamines must be replaced by histones. Proteins and other factors in the cytosol of an oocyte play a role in the correct decondensation of male genetic material.

Mitosis-promoting factor (MPF) in the cytosol of an oocyte can cause premature chromosome condensation (PCC), but perhaps it is not only an oocyte-related problem. It was established that protamine-deficient sperm seems to be related to a higher proportion of PCC independent of oocyte cytoplasmic maturity [60, 61]. The cell cycle of spermatozoa is related to chromatin status and protamine-histone remodeling must be synchronized with the oocyte.

4.4 Oocyte factors

If PLCζ depletion in sperm is a good candidate for explaining fertilization failure of male origin, less is known about different oocyte defects that cause unsuccessful activation. It is obvious that oocyte maturation is crucial and competent oocytes of good quality with all the necessary elements in the downstream cascade must be available. In the process of oocyte maturation, not only the elements responsible for generating calcium oscillations must develop but also all other elements such as those responsible for exocytosis of cortical granules, the necessary actin cortical cytoskeleton, and energy resources must be available.

The direct proof of oocyte-borne defects is the results of the mouse oocyte activation test (MOAT) that will be discussed in detail later. It is a heterologous model where mouse oocytes are fertilized with the patient's sperm. Successful fertilization of mouse oocytes points toward oocyte defects that are the underlying cause of previous fertilization failure in ART treatments in a specific couple.

4.4.1 Organelle distribution

Studies of the ultrastructure of unfertilized oocytes with transmission or scanning electron microscopy revealed differences in oocyte ultrastructure that can reflect different stages in oocyte maturation [62].

Cortical granule migration toward the plasma membrane is an important step in cytoplasmic maturation and it is a cytoskeleton-dependent process [63].

Some studies have investigated the relationship between mitochondrial function and fertilization. Unfertilized oocytes exhibit a higher proportion of mtDNA deletions that may contribute to their malfunction and ATP production [64]. As the early embryo requires a lot of energy, it is important that during oocyte maturation a sufficient number and functionality of the mitochondria are prepared.

Reorganization of the endoplasmic reticulum (ER) during maturation seems to play an important role in oocyte competence to generate calcium oscillations. Visualization of the ER in mouse oocytes revealed that in prophase I (in the germinal vesicle stage) the ER is in the form of a fine network with patch-like accumulations in the inner cytoplasm. After the resumption of meiosis, the ER accumulates in the form of a dense ring in the center of the oocyte, around the meiotic apparatus; later the ER rings move together with a meiotic spindle toward the oocyte cortex [65]. In oocytes in metaphase II, the ER ring transforms into clusters in the cortical region; in the central cytoplasm the reticular form is present [65]. These researchers also showed that these relocalizations happen independently from meiotic progression and that microtubules, dyneins, and actins are responsible for the movements.

During oocyte maturation, the Ins_3R receptors responsible for calcium release from the endoplasmic reticulum achieve their functionality. In a mouse model, it was demonstrated that increase in IP_3R1 sensitivity is underpinned, at least in part, by increases in calcium concentrations within the endoplasmic reticulum and receptor phosphorylation but not by changes in IP_3R1 distribution [66].

Distribution of vesicles with PIP_2 is also important. PLCζ diffuses from the sperm head into egg cytosol and acts on PIP_2 that is present in the membrane of small vesicles in the cytoplasm. Defects or deficits of PIP_2 or vesicles could contribute to fertilization failure [30].

4.4.2 Cytoplasmic maturation

Evaluation of oocyte maturity relies on the presence of the first polar body, but it is difficult to evaluate in daily IVF laboratory practice whether the oocyte cytoplasm is mature.

Cytoplasmic maturity is an important factor determining the ability of the oocyte to activate. During oocyte cytoplasmic maturation, the mechanisms responsible for sperm-induced calcium oscillations and oocyte activation develop and are reviewed in [67].

It was experimentally shown in LT/Sv mouse strain (that has abnormal oocyte nuclear maturation arrested at metaphase I) that the ability of these oocytes to be activated by sperm develops gradually during cytoplasmic maturation independent of nuclear maturation [68].

Oocyte cytoplasmic maturity also plays a role in decondensation of the sperm genetic material that is tightly packed with protamines. Oocyte immaturity is correlated to the occurrence of premature chromosome condensation (PCC) of the male pronucleus [69].

Evaluation of cytoplasmic maturity with immunocytochemical methods revealed that metaphase plate rearrangements are more frequent in oocytes showing immaturity [70]. Another study investigated abnormal maturation in patients with a high proportion of immature oocytes [71].

It is obvious that the conditions in which oocytes mature are important and beside patient-related factors there are also cycle-specific factors that have an impact on oocyte maturity, quality, and fertilization. Little is known about the cellular mechanisms of how diet, medicament uptake, or tobacco/alcohol intake affect

oocyte quality. In a review of [72], the environmental impact on oocyte function through mitochondrial level is discussed.

5. Artificial oocyte activation (AOA)

Artificial oocyte activation methods try to induce oocyte activation by using physiological properties of the gametes and in this way interfere in different levels of the cascade of events during fertilization. In general, they try to alleviate intracellular calcium concentration and mimic oscillations. As we are well aware by now that there is big species-specific variability in the mechanisms of oocyte activation, it is not surprising that different AOA methods can be successful in one species, but not in another.

By influencing gamete physiological properties such as electrical excitability and plasma membrane conductivity, the aim is to increase intracytoplasmic calcium concentrations and mimic the frequency and amplitude of the oscillations.

Basically, there are three types of AOA methods: electrical, chemical, and mechanical.

5.1 Electrical methods

By applying direct electrical current within a Petri dish with oocytes, the electrical field stimulates charged proteins to move toward the plasma membrane, and by this, the number of pores in the plasma membrane increases [30]. Calcium conductivity increases, and more calcium enters the oocyte from the surroundings. There is only one large calcium concentration increase.

In the prospective randomized study of [73], an electrical pulse in a special chamber with electrodes 30 minutes after ICSI in 0.3 M glucose drops was used to activate oocytes, and a small increase in the fertilization rate after ICSI was achieved. Successful pregnancy and birth were achieved and reported in the case report [74].

In a study with round spermatid injection coupled with electrostimulation, the electrical pulse triggered not only a single calcium concentration increase, but a series of calcium spikes after spermatid injection [75].

5.2 Chemical methods

Chemical activation is the most commonly used method. Oocytes are exposed to chemical agents that lead to an increase in intracellular calcium concentration in the oocyte. Some agents, such as calcium ionophores cause a single, prolonged calcium transient, while others cause multiple oscillations.

5.2.1 Ionophores

Calcium ionophores, such as ionomycin, A23187 (calcimycin), and gm508 are molecules soluble in lipids, synthesized by microorganisms; today several synthetic compounds are known. They can transport ions across lipid bilayers. They increase membrane permeability for Ca^{2+} ions, thus allowing calcium influx in the oocyte from the surrounding medium and intracytoplasmic rise of calcium concentration. It has been recently established that intracytoplasmic rise of calcium concentration in human and mouse oocytes is not only the consequence of the influx from the surroundings but also from intracellular stores, since this rise appears also in calcium-free medium. However, they are not able to induce calcium oscillations

typical for mammalian species but a single rise. Different protocols are described in the literature, regarding different concentrations used, and intervals of ionophore exposition [76–79].

They are used in cases of repeating TFF or low fertilization, oligoteratoasthenospermia cases, globozoospermia, in vitro maturation of oocytes (IVM), unexplained female infertility, and low ovarian reserve, with patients with Kartagener's syndrome with no response on theophylline, at primary ciliary dyskinesia. They are the most widely used chemical agents for artificial oocyte activation.

5.2.2 Strontium chloride ($SrCl_2$)

It is reported as very efficient in mice and induces not only single calcium concentration elevation, but oscillations [80]. The mechanism by which it induces oscillations is not fully understood.

A study that investigated efficacy of $SrCl_2$ in human oocytes showed significantly increased fertilization rates, when compared with conventional ICSI or calcium ionophore treatment [81].

5.2.3 PLCζ

Soon after the discovery of the role of PLCζ as a trigger of oocyte activation, the ideas of using the protein as an artificial activator emerged. The synthesis of the first recombinant human PLCζ protein was published [82]; when injected into mouse oocytes, calcium oscillations were evoked that closely resembled those initiated by the sperm after fertilization. Later, a study where human recombinant PLCζ was used on human and mouse oocytes was published [83] describing dose-dependent manner of calcium oscillations. These authors also showed that by injecting recombinant human PLCζ the next day in oocytes that failed to fertilize after ICSI, five of eight oocytes were rescued.

Earlier, it was established that PLCζ complementary RNA (cRNA) injection in MII oocytes also triggered oscillations [52] with a time lag that enables protein to synthesize.

The commercial use of recombinant human PLCζ still has to be validated in terms of safety.

5.2.4 Ethanol

In veterinary medicine, ethanol is often used for parthenogenetic oocyte activation. Parthenogenesis is development of an embryo from an unfertilized oocyte, naturally occurring in invertebrates or even some vertebrates. By inhibiting the second polar body extrusion, diploid parthenotes with two maternal genomes can be created and embryos can develop normally for several days, but later die. In several species, artificial parthenogenetic activation was described to be caused by ethanol [84].

5.3 Mechanical methods

Some data from the literature suggest that the modified ICSI technique can give better fertilization in patients with a history of TFF or low fertilization [85, 86]. Vigorous aspiration of cytoplasm and a different position of the pipette tip when ejecting sperm in the oocyte is supposed to increase calcium levels during injection and enable better contact of sperm with intracellular storage of calcium.

6. Diagnostic tools for assessing sperm- or oocyte-dependent activation defects

A proper diagnostic procedure is very important prior to the decision to use artificial oocyte activation, and oocyte or sperm donation is a reasonable treatment option for some couples [87].

There are several diagnostic methods available, but not always accessible to all clinics since legislation can prohibit the use of heterologous human-animal models.

The mouse oocyte activation test (MOAT) is a heterologous ICSI model where mouse oocytes are fertilized with the patient's sperm [78]. As a negative control, mouse oocytes are injected with the medium and as a positive control, they are injected with donor sperm with proven fertilizing ability. It allows discrimination between sperm- and oocyte-borne causes for fertilization failure. According to the ratio of fertilized mouse oocytes, three groups are described: MOAT1 indicating sperm-borne defects, MOAT2 fertilization failure of unknown origin, MOAT3 sperm defects are excluded indicating oocyte defects.

In some patients from groups MOAT2 or MOAT3, capacity to activate mouse oocytes is demonstrated but later ICSI-AOA results in TFF. In these cases, assessment of calcium oscillations can give better answers as to whether the underlying reason is the presence of human sperm activation deficiencies or oocyte-related activation deficiency. Mouse oocyte Ca^{2+} analysis (M-OCA) or even more sensitive human oocyte Ca^{2+} analysis (H-OCA) can be performed before using AOA [88]. In the M-OCA test, the patient's sperm is used and frequency patterns of calcium oscillations are analyzed. H-OCA yields higher sensitivity than M-OCA to detect the presence of human sperm activation deficiencies. It helps detect cases with suspected oocyte-related activation deficiency.

7. Success rates of AOA

The systematic review and meta-analysis of RCTs that compared ICSI-AOA and conventional ICSI first established that there is insufficient evidence available from RCTs to judge the efficacy and safety of ICSI-AOA for couples with previous fertilization failure [89]. A total of 14 articles were assessed and 9 included in meta-analysis. It cannot be concluded that the outcomes are improved using ICSI followed by artificial oocyte activation compared with conventional ICSI. The fertilization rate, cleavage rate, and likelihood of blastocyst formation seem to improve according to some studies, but it is difficult to make a general conclusion.

Recently, important evidence appeared that the conditions in which activation takes place are very important for the success rate and can vary a lot. Varying concentrations of both ionomycin and calcium ions in culture media used during AOA can have significant effects on calcium release and further embryonic developmental potential .

8. Safety of AOA methods

Although AOA methods have been proven efficient to overcome some cases of TFF, the concern around using them in clinical practice is quite big. By artificially increasing intracellular calcium levels we interfere with cellular mechanisms that normally would not occur. Nature has regulatory mechanisms to eliminate errors and when we force events that would not happen spontaneously it is always important to verify all possible negative effects of such procedures.

The number of children born after AOA is relatively small for statistical analysis, but there are accumulating data on the safety of these methods. The study from Ghent analyzed neonatal and neurodevelopmental outcomes of 21 children born after cycles with AOA [90]. For all tests and questionnaires, the mean outcomes lay within the expected ranges, but since the number of studied cases is small, the authors state that AOA should still be performed only in selected couples. In another study, 79 children born following AOA-ICSI and 89 born by ICSI were compared in terms of intrauterine fetal death, preterm delivery, birth weight, growth rate, hospitalization in neonatal intensive care units, abnormal behavior according to age, and the physical and mental health of children born and no significant differences were found [91].

In a study, genetic content of donated oocytes in metaphase II artificially activated with calcium-ionophore was analyzed. By using array comparative genomic hybridization, single-nucleotide polymorphism genotyping and maternal haplotyping chromosome segregation errors in meiosis II were not increased compared to the control group [92].

There are concerns about the effects of AOA on gene expression and later embryonic development coming from animal studies [33, 93]. In the case of the use of $SrCl_2$ for AOA, data in mice show that birth weight of male pups is reduced [80].

9. Conclusions

The problem of failed fertilization is a big burden for patients and clinicians and the pressure to help these patients is enormous. Today, ART methods are generally easily accessible and patients' expectations are very high. In Europe alone, there have been 1,308,289 children born from IVF treatments between the years 1997 and 2013 according to data collected in European IVF monitoring [94]. Global data collection on IVF is a difficult task, but there are reports that in a three-year period, more than a million babies are born worldwide [95]. Despite the great success of ART, there are always some patients facing fertilization failure and the emotional burden of inability to achieve pregnancy is great for these couples. For successful fertilization, sperm must activate a quiescent oocyte to complete meiosis and progress toward embryonic development characterized with repeated mitotic divisions. Oocyte activation is a complex cascade of intracellular processes. Sperm or oocyte abnormalities can contribute to activation failure. In clinical practice, there is a need for safe methods of artificial oocyte activation based on the physiological properties of the gametes that closely imitate calcium oscillations triggered naturally by sperm.

Conflict of interest

The authors confirm that there are no known "conflicts of interest" associated with this publication.

Notes/thanks/other declarations

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References

[1] Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet. 1978;**2**(8085):366

[2] Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;**340**(8810):17-18

[3] Sousa M, Tesarik J. Ultrastructural analysis of fertilization failure after intracytoplasmic sperm injection. Human Reproduction. 1994;**9**(12):2374-2380

[4] Flaherty SP, Payne D, Swann NJ, Matthews CD. Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. Human Reproduction. 1995;**10**(10):2623-2629

[5] Bhattacharya S, Hamilton MPR, Shaaban M, Khalaf Y, Seddler M, Ghobara T, et al. Conventional in-vitro fertilisation versus intracytoplasmic sperm injection for the treatment of non-male-factor infertility: A randomised controlled trial. Lancet. 2001;**357**(9274):2075-2079

[6] Esfandiari N, Javed MH, Gotlieb L, Casper RF. Complete failed fertilization after intracytoplasmic sperm injection--analysis of 10 years' data. International Journal of Fertility and Women's Medicine. 2005;**50**(4):187-192

[7] Flaherty SP, Payne D, Matthews CD. Fertilization failures and abnormal fertilization after intracytoplasmic sperm injection. Human Reproduction. 1998;**13**(suppl. 1):155-164

[8] Liu J, Nagy Z, Joris H, Tournaye H, Smitz J, Camus M, et al. Analysis of 76 total fertilization failure cycles out of 2732 intracytoplasmic sperm injection cycles. Human Reproduction. 1995;**10**(10):2630-2636

[9] Yanagida K, Morozumi K, Katayose H, Hayashi S, Sato A. Successful pregnancy after ICSI with strontium oocyte activation in low rates of fertilization. Reproductive Biomedicine Online. 2006;**13**(6):801-806

[10] Shinar S, Almog B, Levin I, Shwartz T, Amit A, Hasson J. Total fertilization failure in intra-cytoplasmic sperm injection cycles-classification and management. Gynecological Endocrinology. 2014;**30**(8):593-596

[11] Vlaisavljević V, Kovacic B, Reljic M, Lovrec VG, Sajko MC. Results of intracytoplasmic sperm injection of single oocyte in 362 unstimulated cycles. Journal of Assisted Reproduction and Genetics. 2002;**19**(3):127-131

[12] Dozortsev D, Sutter PD, Dhont M. Fertilization and early embryology: Behaviour of spermatozoa in human oocytes displaying no or one pronucleus after intracytoplasmic sperm injection. Human Reproduction. 1994;**9**(11):2139-2144

[13] Yanagida K. Complete fertilization failure in ICSI. Human Cell. 2004;**17**(4):187-193

[14] Pitsos MA, Nicolopoulou-Stamati P. DNA spatial distribution in unfertilized human oocytes by confocal laser scanning microscope. Molecular Reproduction and Development. 2002;**62**(3):368-374

[15] Kovacic B, Vlaisavljevic V. Configuration of maternal and paternal chromatin and pertaining microtubules in human oocytes failing to fertilize after intracytoplasmic sperm injection. Molecular Reproduction and Development. 2000;**55**(2):197-204

[16] Rawe VY, Olmedo SB, Nodar FN, Doncel GD, Acosta AA, Vitullo AD. Cytoskeletal organization defects

and abortive activation in human oocytes after IVF and ICSI failure. Molecular Human Reproduction. 2000;**6**(6):510-516

[17] Gasca S, Reyftmann L, Pellestor F, Rème T, Assou S, Anahory T, et al. Case report: Total fertilization failure and molecular abnormalities in metaphase II oocytes. Reproductive Biomedicine Online. 2008;**17**(6):772-781

[18] Mazia D. The release of calcium in Arbacia eggs on fertilization. Journal of Cellular and Comparative Physiology. 1937;**10**:291-304

[19] Ridgway EB, Gilkey JC, Jaffe LF. Free calcium increases explosively in activating medaka eggs. Proceedings of the National Academy of Sciences of the United States of America. 1977;**74**(2):623-627

[20] Stricker SA. Comparative biology of calcium signaling during fertilization and egg activation in animals. Developmental Biology. 1999;**211**(2):157-176

[21] Kaneuchi T, Sartain CV, Takeo S, Horner VL, Buehner NA, Aigaki T, et al. Calcium waves occur as *Drosophila* oocytes activate. Proceedings of the National Academy of Sciences. 2015;**112**(3):791-796

[22] Epel D. Mechanisms of activation of sperm and egg during fertilization of sea urchin gametes. Current Topics in Developmental Biology. 1978;**12**(C):185-246

[23] Swann K, Lai FA. Egg activation at fertilization by a soluble sperm protein. Physiological Reviews. 2016;**96**(1):127-149

[24] Miyazaki S. Fertilization potential and calcium transients in mammalian eggs. Development, Growth & Differentiation. 1988;**30**(6):603-610

[25] Igusa Y, Miyazaki S. Effects of altered extracellular and intracellular calcium concentration on hyperpolarizing responses of hamster egg. Journal of Physiology. 1983;**340**:611-632

[26] Taylor CT, Lawrence YM, Kingsland CR, Biljan MM, Cuthbertson KSR. Fertilization and early embryology: Oscillations in intracellular free calcium induced by spermatozoa in human oocytes at fertilization. Human Reproduction. 1993;**8**(12):2174-2179

[27] Gianaroli L, Tosti E, Magli C, Iaccarino M, Ferraretti AP, Dale B. Fertilization current in the human oocyte. Molecular Reproduction and Development. 1994;**38**(2):209-214

[28] Tosti E, Boni R, Cuomo A. Fertilization and activation currents in bovine oocytes. Reproduction. 2002;**124**(6):835-846

[29] Tesarik J, Sousa M, Testart J. Human oocyte activation after intracytoplasmic sperm injection. Human Reproduction. 1994;**9**(3):511-518

[30] Yeste M, Jones C, Amdani SN, Patel S, Coward K. Oocyte activation deficiency: A role for an oocyte contribution? Human Reproduction Update. 2016;**22**(1):23-47

[31] Bos-Mikich A, Whittingham DG, Jones KT. Meiotic and mitotic Ca^{2+} oscillations affect cell composition in resulting blastocysts. Developmental Biology. 1997;**182**(1):172-179

[32] Jones KT. Mammalian egg activation: From Ca^{2+} spiking to cell cycle progression. Reproduction. 2005;**130**(6):813-823

[33] Ducibella T, Schultz RM, Ozil JP. Role of calcium signals in early development. Seminars in Cell & Developmental Biology. 2006;**17**(2):324-332

[34] Kline D. Calcium-dependent events at fertilization of the frog egg: Injection of a calcium buffer blocks ion channel opening, exocytosis, and formation of pronuclei. Developmental Biology. 1988;**126**(2):346-361

[35] Fukuda Y. The time of cortical granule breakdown and sperm penetration in mouse and hamster eggs inseminated in vitro. Biology of Reproduction. 1978;**19**:261-266

[36] Sone Y, Ito M, Shirakawa H, Shikano T, Takeuchi H, Kinoshita K, et al. Nuclear translocation of phospholipase C-zeta, an egg-activating factor, during early embryonic development. Biochemical and Biophysical Research Communications. 2005;**330**(3):690-694

[37] Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, Kopf GS, et al. Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. Developmental Biology. 2002;**250**(2):280-291

[38] Oliveira AG, Guimaraes ES, Andrade LM, Menezes GB, Fatima LM. Decoding calcium signaling across the nucleus. Physiology. 2014;**29**(5):361-368

[39] Santella L, Lim D, Moccia F. Calcium and fertilization: The beginning of life. Trends in Biochemical Sciences. 2004;**29**(8):400-408

[40] Dale B. Is the idea of a fast block to polyspermy based on artifact? Biochemical and Biophysical Research Communications. 2014;**450**(3):1159-1165

[41] Dale B, Wilding M, Coppola G, Tosti E. How do spermatozoa activate oocytes? Reproductive Biomedicine Online. 2010;**21**(1):1-3

[42] Tosti E, Boni R. Electrical events during gamete maturation and fertilization in animals and humans. Human Reproduction Update. 2004;**10**(1):53-65

[43] Brezina PR, Yunus FN, Zhao Y. Effects of pharmaceutical medications on male fertility. Journal of Reproduction & Infertility. 2012;**13**(1):3-11

[44] Nomikos M, Swann K, Lai FA. Starting a new life: Sperm PLC-zeta mobilizes the Ca^{2+} signal that induces egg activation and embryo development: An essential phospholipase C with implications for male infertility. BioEssays. 2012;**34**(2):126-134

[45] Swann K. A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. Development. 1990;**110**(4):1295-1302

[46] Stricker SA. Intracellular injections of a soluble sperm factor trigger calcium oscillations and meiotic maturation in unfertilized oocytes of a marine worm. Developmental Biology. 1997;**186**(2):185-201

[47] Parrington J, Swann K, Shevchenko VI, Sesay AK, Lai FA. Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. Nature. 1996;**379**:364

[48] Carroll DJ, Albay DT, Terasaki M, Jaffe LA, Foltz KR. Identification of PLC y-dependent and -independent events during fertilization of eggs. Developmental Biology. 1999;**206**(2):232-247

[49] Kuo RC, Baxter GT, Thompson SH, Stricker SA, Patton C, Bonaventura J, et al. NO is necessary and sufficient for egg activation at fertilization. Nature. 2000;**406**(6796):633-636

[50] Wu ATH, Sutovsky P, Manandhar G, Xu W, Katayama M, Day BN, et al. PAWP, a sperm-specific WW

domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. The Journal of Biological Chemistry. 2007;**282**(16):12164-12175

[51] Rice A, Parrington J, Jones KT, Swann K. Mammalian sperm contain a Ca^{2+}–sensitive phospholipase C activity that can generate InsP3from PIP2associated with intracellular organelles. Developmental Biology. 2000;**228**(1):125-135

[52] Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, et al. PLC zeta: A sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Development. 2002;**129**(15):3533-3544

[53] Escoffier J, Yassine S, Lee HC, Martinez G, Delaroche J, Coutton C, et al. Subcellular localization of phospholipase Cζ in human sperm and its absence in DPY19L2-deficient sperm are consistent with its role in oocyte activation. MHR: Basic Science of Reproductive Medicine. 2015;**21**(2):157-168

[54] Knott JG, Kurokawa M, Fissore RA, Schultz RM, Williams CJ. Transgenic RNA interference reveals role for mouse sperm phospholipase Cζ in triggering Ca^{2+} oscillations during Fertilization1. Biology of Reproduction. 2005;**72**(4):992-996

[55] Escoffier J, Lee HC, Yassine S, Zouari R, Martinez G, Karaouzène T, et al. Homozygous mutation of PLCZ1 leads to defective human oocyte activation and infertility that is not rescued by the WW-binding protein PAWP. Human Molecular Genetics. 2016;**25**(5):878-891

[56] Kashir J, Heindryckx B, Jones C, de Sutter P, Parrington J, Coward K. Oocyte activation, phospholipase C zeta and human infertility. Human Reproduction Update. 2010;**16**(6):690-703

[57] Kashir J, Deguchi R, Jones C, Coward K, Stricker SA. Comparative biology of sperm factors and fertilization-induced calcium signals across the animal kingdom. Molecular Reproduction and Development. 2013;**80**(10):787-815

[58] Faure JE, Myles DG, Primakoff P. The frequency of calcium oscillations in mouse eggs at fertilization is modulated by the number of fused sperm. Developmental Biology. 1999;**213**(2):370-377

[59] Hachem A, Godwin J, Ruas M, Lee HC, Ferrer Buitrago M, Ardestani G, et al. PLCζ is the physiological trigger of the Ca^{2+} oscillations that induce embryogenesis in mammals but conception can occur in its absence. Development. 2017;**144**(16):2914-2924

[60] Nasr-Esfahani MH, Razavi S, Mardani M, Shirazi R, Javanmardi S. Effects of failed oocyte activation and sperm protamine deficiency on fertilization post-ICSI. Reproductive Biomedicine Online. 2007;**14**(4):422-429

[61] Nasr-Esfahani MH, Naghshizadian N, Imani H, Razavi S, Mardani M, Kazemi S, et al. Can sperm protamine deficiency induce sperm premature chromosomal condensation? Andrologia. 2006;**38**(3):92-98

[62] Motta PM, Nottola SA, Micara G, Familiari G. Ultrastructure of human unfertilized oocytes and Polyspermic embryos in an IVF-ET program. Annals of the New York Academy of Sciences. 1988;**541**(1):367-383

[63] Liu M. The biology and dynamics of mammalian cortical granules. Reproductive Biology and Endocrinology. 2011;**9**(1):149

[64] Hsieh RH, Tsai NM, Au HK, Chang SJ, Wei YH, Tzeng CR. Multiple rearrangements of mitochondrial DNA

in unfertilized human oocytes. Fertility and Sterility. 2002;**77**(5):1012-1017

[65] FitzHarris G, Marangos P, Carroll J. Changes in endoplasmic reticulum structure during mouse oocyte maturation are controlled by the cytoskeleton and cytoplasmic dynein. Developmental Biology. 2007;**305**(1):133-144

[66] Wakai T, Vanderheyden V, Yoon S-Y, Cheon B, Zhang A, Parys JB, et al. Regulation of inositol 1,4,5-trisphosphate receptor function during mouse oocyte maturation. Journal of Cellular Physiology. 2012;**227**(2):705-717

[67] Ajduk A, Małagocki A, Maleszewski M. Cytoplasmic maturation of mammalian oocytes: Development of a mechanism responsible for sperm-induced Ca^{2+} oscillations. Reproductive Biology. 2008;**8**(1):3-22

[68] Archacka K, Ajduk A, Pomorsiki P, Szczepanska K, Maleszewski M, Ciemerych MA. Defective calcium release during in vitro fertilization of maturing oocytes of LT/Sv mice. International Journal of Developmental Biology. 2008;**52**(7):903-912

[69] Calafell JM, Badenas J, Egozcue J, Santaló J. Premature chromosome condensation as a sign of oocyte immaturity. Human Reproduction. 1991;**6**(7):1017-1021

[70] Sedó CA, Miguens M, Andreucci S, Ortiz N, Lorenzi D, Papier S, et al. Correlation between cytoplamic oocyte maturation and chromosomal aneuploidies—Impact on fertilization, embryo quality and pregnancy. JBRA Assisted Reproduction. 2015;**19**(2):59-65

[71] Lu Y, Ferrer-Buitrago M, Popovic M, Neupane J, De Vos WH, Lierman S, et al. Patients with a high proportion of immature and meiotically resistant oocytes experience defective nuclear oocyte maturation patterns and impaired pregnancy outcomes. Reproductive Biomedicine Online. 2018;**36**(4):396-407

[72] Grindler NM, Moley KH. Maternal obesity, infertility and mitochondrial dysfunction: Potential mechanisms emerging from mouse model systems. Molecular Human Reproduction. 2013;**19**(8):486-494

[73] Mansour R, Fahmy I, Tawab NA, Kamal A, El-Demery Y, Aboulghar M, et al. Electrical activation of oocytes after intracytoplasmic sperm injection: A controlled randomized study. Fertility and Sterility. 2009;**91**(1):133-139

[74] Egashira A, Murakami M, Haigo K, Horiuchi T, Kuramoto T. A successful pregnancy and live birth after intracytoplasmic sperm injection with globozoospermic sperm and electrical oocyte activation. Fertility and Sterility. 2009;**92**(6):2037.e5-2037.e9

[75] Tanaka A, Nagayoshi M, Takemoto Y, Tanaka I, Kusunoki H, Watanabe S, et al. Fourteen babies born after round spermatid injection into human oocytes. Proceedings of the National Academy of Sciences. 2015;**112**(47):14629-14634

[76] Nikiforaki D, Vanden Meerschaut F, De Roo C, Lu Y, Ferrer-Buitrago M, De Sutter P, et al. Effect of two assisted oocyte activation protocols used to overcome fertilization failure on the activation potential and calcium releasing pattern. Fertility and Sterility. 2016;**105**(3):798-806e2

[77] Tesarik J, Nagy ZP, Mendoza C, Greco E. Chemically and mechanically induced membrane fusion: Non-activating methods for nuclear transfer in mature human oocytes. Human Reproduction. 2000;**15**(5):1149-1154

[78] Heindryckx B, Van der Elst J, De Sutter P, Dhont M. Treatment option for

sperm- or oocyte-related fertilization failure: Assisted oocyte activation following diagnostic heterologous ICSI. Human Reproduction. 2005;**20**(8):2237-2241

[79] Nakagawa K, Yamano S, Moride N, Yamashita M, Yoshizawa M, Aono T. Effect of activation with Ca ionophore A23187 and puromycin on the development of human oocytes that failed to fertilize after intracytoplasmic sperm injection. Fertility and Sterility. 2001;**76**(1):148-152

[80] Vanden Meerschaut F, Nikiforaki D, De Roo C, Lierman S, Qian C, Schmitt-John T, et al. Comparison of pre-and post-implantation development following the application of three artificial activating stimuli in a mouse model with round-headed sperm cells deficient for oocyte activation. Human Reproduction. 2013;**28**(5):1190-1198

[81] Kim J-W, Kim S-D, Yang S-H, Yoon S-H, Jung J-H, Lim J-H. Successful pregnancy after SrCl2 oocyte activation in couples with repeated low fertilization rates following calcium ionophore treatment. Systems Biology in Reproductive Medicine. 2014;**60**(3):177-182

[82] Kashir J, Jones C, Lee HC, Rietdorf K, Nikiforaki D, Durrans C, et al. Loss of activity mutations in phospholipase C zeta (PLCzeta) abolishes calcium oscillatory ability of human recombinant protein in mouse oocytes. Human Reproduction. 2011;**26**(12):3372-3387

[83] Yoon S-Y, Eum JH, Lee JE, Lee HC, Kim YS, Han JE, et al. Recombinant human phospholipase C zeta 1 induces intracellular calcium oscillations and oocyte activation in mouse and human oocytes. Human Reproduction. 2012;**27**(6):1768-1780

[84] Grabiec A, Max A, Tischner M. Parthenogenetic activation of domestic cat oocytes using ethanol, calcium ionophore, cycloheximide and a magnetic field. Theriogenology. 2007;**67**(4):795-800

[85] Ebner T, Moser M, Sommergruber M, Jesacher K, Tews G. Complete oocyte activation failure after ICSI can be overcome by a modified injection technique. Human Reproduction. 2004;**19**(8):1837-1841

[86] Tesarik J, Rienzi L, Ubaldi F, Mendoza C, Greco E. Use of a modified intracytoplasmic sperm injection technique to overcome sperm-borne and oocyte-borne oocyte activation failures. Fertility and Sterility. 2002;**78**(3):619-624

[87] Combelles CMH, Morozumi K, Yanagimachi R, Zhu L, Fox JH, Racowsky C. Diagnosing cellular defects in an unexplained case of total fertilization failure. Human Reproduction. 2010;**25**(7):1666-1671

[88] Ferrer-Buitrago M, Dhaenens L, Lu Y, Bonte D, Vanden Meerschaut F, De Sutter P, et al. Human oocyte calcium analysis predicts the response to assisted oocyte activation in patients experiencing fertilization failure after ICSI. Human Reproduction. 2018;**33**(3):416-425

[89] Sfontouris IA, Nastri CO, Lima MLS, Tahmasbpourmarzouni E, Raine-Fenning N, Martins WP. Artificial oocyte activation to improve reproductive outcomes in women with previous fertilization failure: A systematic review and meta-analysis of RCTs. Human Reproduction. 2015;**30**(8):1831-1841

[90] Vanden Meerschaut F, D'Haeseleer E, Gysels H, Thienpont Y, Dewitte G, Heindryckx B, et al. Neonatal and neurodevelopmental outcome of children aged 3-10 years born following assisted oocyte activation. Reproductive Biomedicine Online. 2014;**28**(1):54-63

[91] Deemeh MR, Tavalaee M, Nasr-Esfahani MH. Health of children born through artificial oocyte activation: A pilot study. Reproductive Sciences. 2015;**22**(3):322-328

[92] Capalbo A, Ottolini CS, Griffin DK, Ubaldi FM, Handyside AH, Rienzi L. Artificial oocyte activation with calcium ionophore does not cause a widespread increase in chromosome segregation errors in the second meiotic division of the oocyte. Fertility and Sterility. 2016;**105**(3):807-814e2

[93] Ozil J-P, Banrezes B, Toth S, Pan H, Schultz RM. Ca^{2+} oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. Developmental Biology. 2006;**300**(2):534-544

[94] Calhaz-Jorge C, De Geyter C, Kupka MS, De Mouzon J, Erb K, Mocanu E, et al. Assisted reproductive technology in Europe, 2013: Results generated from European registers by ESHRE. Human Reproduction. 2017;**32**(10):1957-1973

[95] Dyer S, Chambers GM, De Mouzon J, Nygren KG, Zegers-Hochschild F, Mansour R, et al. International committee for monitoring assisted reproductive technologies world report: Assisted reproductive technology 2008, 2009 and 2010. Human Reproduction. 2016;**31**(7):1588-1609