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Artificial egg activation using calcium ionophore

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Introduction

Artificial oocyte activation, most commonly using calcium ionophore, is a treatment add-on utilised to avoid recurrence of abnormally low or total failed fertilisation following IVF/ICSI. It aims to modify defective physiological processes, specifically calcium-mediated cell signalling that are critical to events required for fertilisation. Routine application of artificial oocyte activation is neither required or recommended, however, it represents a valuable intervention for a subgroup of patients affected by sperm-related oocyte activation deficiency.

Oocyte activation

Human spermatozoa undergo a complex series of biochemical changes and physiological modifications following ejaculation to acquire the capability to fertilise an oocyte. These events were originally described in early 1950s[1,2] and subsequently termed capacitation[3]. Further consideration of the physiology of spermatozoa and function required for fertilisation is beyond the scope of this article, however, it is important to appreciate the wider contribution of sperm over and above paternal DNA, including proteins required for fertilisation and early embryo development[4]. One of the earliest events following gamete fusion is intracellular calcium (Ca^{2+}) signalling within the oocyte[5]. In mammals, this intracellular Ca^{2+} signal is delivered in the form of a series of waves of intracellular Ca^{2+} (oscillations) that begins shortly after fusion of the gametes and persists until completion of meiosis[6]. These oscillations release the oocyte from metaphase 2 meiotic arrest and initiate embryogenesis, cortical granule exocytosis, sperm nucleus decondensation, recruitment of maternal mRNA and pronuclear development; a series of events termed oocyte activation[7].

Phospholipase C zeta

It is now recognised that sperm contribute a soluble factor essential to oocyte activation. A novel, sperm-specific phospholipase C, PLC zeta (PLCζ) was first reported by Saunders[8] to trigger Ca2+ oscillations in mouse oocytes, resulting in oocyte activation and early embryo development. Since then, PLCζ has been detected in many mammalian (and non-mammalian) species. In human sperm, PLCζ appears to be primarily located to the acrosomal, post acrosomal and equatorial region of the sperm head[9], although the pattern of localisation has been reported to change dynamically during capacitation and acrosome reaction[10] and remains a subject of debate, not least due to the heterogeneous nature of human sperm populations but also due to the availability of relatively non-specific antibodies and variable protocols[11]. Nonetheless, PLCζ is almost universally accepted as the sperm-borne activation factor critical to oocyte activation, with both biochemical and clinical evidence to support this[11].

PLCζ is the smallest known mammalian PLC[12] and comprises 2 pairs of EF hand domains (EF1 – EF4), a C2 domain and catalytic X and Y regions[13]. It is highly Ca^{2+} sensitive and induces Ca^{2+} oscillations primarily as a consequence of hydrolysis of lipid phosphotidylinositol 4,5-biphosphate (PIP2), which generates inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 subsequently binds to specific receptors (IP3R) to induce repetitive Ca^{2+} release from the oocyte endoplasmic reticulum Ca^{2+} stores, giving rise to Ca^{2+} oscillations[14-16] (Figure 1). Sperm-induced Ca^{2+}oscillations also stimulate mitochondrial respiration with resultant adenosine triphosphate (ATP) production that maintains sperm-triggered calcium waves. Initial Ca^{2+} oscillations are low frequency, brief calcium spikes that last around 5-50 seconds. These subsequently evolve into extended Ca^{2+} spikes lasting more than a minute. The frequency of Ca^{2+} oscillations depend, in part, on the IP3 concentration[17,18]. As such, it is perhaps not unexpected that oocyte Ca^{2+} oscillation frequency has been reported to be dependent on PLCζ concentration. Ca^{2+} ions also react with cortical granules found around the edges of the cell membrane, initiating the cortical reaction as well as triggering further Ca^{2+} release. The pattern of Ca^{2+} oscillations induced is species specific[19-21] and believed to have implications in terms of embryonic development prior to implantation, as well as pregnancy outcome[22]. Conversely, oocyte activation is triggered by a single Ca^{2+} transient in non-mammalian species such as sea urchins and frogs[23].

Clinical implications

Reduced amounts and abnormal forms of PLCζ have been described in spermatozoa from infertile men in couples affected by low or no fertilisation following IVF/ICSI[24,25], indicating that deficiencies in PLCζ protein expression are likely to underlie unexplained as well as male factor infertility. Similarly, scientific and clinical evidence have now linked globozoospermia and oocyte activation deficiency (OAD) with the abnormal expression, localisation and protein structure of PLCζ[26]. Absent or reduced amounts of PLCζ are common with globozoospermia due to characteristic abnormal morphology (round headed spermatozoa with absent acrosome)[27].

Early signs of oocyte activation include the release of 2nd polar body and pronuclear formation, events routinely observed in the laboratory 17 +/- 1 hour following insemination to confirm fertilisation following IVF/ICSI[28]. IVF fertilisation rates (defined as proportion of COC inseminated with 2 pronuclei (2PN) and 2 polar bodies (2PB) on day 1) are expected to be 60 - 75%. ICSI fertilisation rates (defined as proportion of mature oocytes injected with 2PN and 2PB the day after microinjection) are expected to be 65 - 80%[29], however 5-8% IVF

cycles and 1-3% ICSI cycles are affected by total failed fertilisation (TFF), where no eggs are fertilised[30]. Although TFF is rare, its effect is devastating. Potential causes include technical problems affecting the injection of sperm or laboratory equipment malfunction, although these events are fortunately rare. Oocyte factors, including poor response to controlled ovarian hyperstimulation, low egg numbers and/or poor egg quality may also be implicated in TFF. These factors may be amenable to modification by alteration in stimulation and/or protocol in a subsequent cycle[31]. TFF can also relate to identifiable sperm factors, including a lack of motile spermatozoa, severe forms of teratozoospermia (including globozoospermia) or problems affecting the structure or function of the sperm, including failed head decondensation, premature sperm chromatin condensation or defects of the sperm spindle or aster. However, in a significant proportion of cases it is not possible to easily identify whether fertilisation abnormalities relate to the oocyte or sperm and the main cause of fertilisation failure in this scenario is likely to be failure of oocyte activation[32,33]. By definition, PLCζ deficiency or dysfunction is therefore the predominant underlying cause of unexplained low fertilisation (ULF) and TFF.

It is also worth noting ongoing speculation surrounding post-acrosomal sheath WW domain-binding protein (PAWP) as an alternative oocyte activation candidate[34]. There is currently limited clinical evidence to support this[35,36], although a synergistic interaction between PLCζ and PAWP cannot be entirely dismissed[37,38].

Artificial oocyte activation

Artificial oocyte activation (AOA) can overcome sperm-related activation deficiencies and can be achieved by mechanical, electrical or chemical approaches[22,39]. AOA ultimately aims to mimic the Ca^{2+} oscillations produced at the time of spontaneous fertilisation, resulting in resumption of meiosis, extrusion of secondary polar body and pronuclear formation. Importantly, although various techniques have been developed to simulate human oocyte activation Ca^{2+} events to date, none described achieve the oscillation pulses observed in spontaneous fertilization[40] (Figure 2).

Mechanical activation using osmotic pressure was originally described in Drosophila[41]. Ultraviolet light was subsequently described to stimulate resumption of meiosis in mammalian oocytes[42], although irradiation methods were latterly abandoned due to associated chromosomal DNA mutations. A more precise approach in the form of piezoelectric stimulation was first reported in mouse oocytes[43] and subsequently utilised in a clinical setting with a combination of electrical oocyte activation and ICSI resulting in the

birth of healthy twins[44]. Electrical activation following ICSI with globozoospermic sperm has also been reported, with resultant pregnancy and the livebirth of a healthy female infant[45].

A number of case reports have described the use of strontium (Sr^{2+}), a chemically active alkaline earth metal, to artificially activate human oocytes[46,47]. Notably, incubation of rodent oocytes in media containing Sr^{2+} ions gives rise to Ca^{2+} oscillations. The pattern of oscillations differs from those seen at normal fertilisation, nonetheless, Sr^{2+} represents the parthenogenetic agent of choice for nuclear transfer work[48]. However, the mechanism and safety of oocyte activation via Sr^{2+} remains limited as Sr^{2+} is relatively ineffective at activating oocytes from non-rodent species, although several studies have reported effective oocyte activation, successful pregnancy and livebirth[49-52].

Calcium ionophore

Calcium ionophores such as, ionomycin or calcimycin, are the commonest approach to chemical AOA[53]. The addition of calcium ionophore increases cell membrane permeability to Ca^{2+} and allows extracellular Ca^{2+} to flow into the oocyte[54]. This causes a single Ca^{2+} surge, rather than the oscillations that normally arise following sperm-oocyte fusion. Of note, mammalian eggs activated by a single Ca^{2+} transient have been reported to exhibit poor development following implantation in comparison to eggs activated by Ca^{2+} oscillations[55]. Nonetheless, a sustained increase in Ca^{2+} amplitude is enough to trigger oocyte activation[54,56]. AOA using calcium ionophore A23187 was first reported in 1994[57] with subsequent reports of successful pregnancy and livebirth following ICSI-AOA treatment shortly after[58]. In practice, calcimycin (GM508 Cultactiv; Gynemed) is now the commonest clinical approach to AOA. GM508 Cultactiv is a commercially available media containing Ca^{2+} ionophore A23187 and is ready-to-use following equilibration for 4 hours in 5-7% CO_2. Its use is uncomplicated and easy to perform in most clinics.

Obvious candidates to benefit from ICSI-AOA are patients who have experienced previous ULF or TFF in one or more IVF/ICSI cycles suspected to be as a result of oocyte activation deficiency. As such, ICSI-AOA is an important approach in terms of offering the potential for a biological child, not least for patients whose religious beliefs do not permit sperm donation[59]. In these circumstances, AOA with calcium ionophore treatment after ICSI results in a statistically significant improvement in fertilisation (105 cycles; 65.2% - 68.8% fertilisation following ionophore AOA; p=0.008), as well as clinical pregnancy (23.5 – 25.0%) and live birth rates (LBR)[53]. Nonetheless, patient selection is key, and is probably the biggest challenge to application of AOA as a treatment add-on, particularly given the absence of clinically validated and accessible diagnostic assays for PLCζ. For example, a

recent study where patients with a history of ULF or TFF following ICSI were divided into 3 groups according to mouse oocyte activation test (MOAT) included 19 patients with sperm-related oocyte activation deficiency (OAD) (group 1), 56 patients with poor oocyte activating capacity (group 2) and 47 patients with oocyte-related OAD (group 3)[60]. Fertilisation rates for groups 1, 2 and 3 increased from 9.7%, 14.8%, and 17.7% with conventional ICSI to 70.1%, 63.0%, and 57.3% following ICSI-AOA respectively, demonstrating that couples with sperm-related OAD have the most to gain from AOA treatment add-on but also suggesting possible benefit for a much wider group of patients affected by fertilisation abnormalities, albeit that treatment numbers were small. Preliminary reports also suggest that ICSI-AOA may benefit cycles using frozen sperm[61]. This is perhaps not unsurprising as cryopreservation has been reported to reduce PLCζ concentrations[62]. More controversially, significantly higher implantation, clinical pregnancy and live birth rates (LBR) have been reported following ICSI-AOA compared to ICSI alone in a variety of other patient groups including severe male factor (oligoasthenozoospermia; OAT), unexplained infertility, PCOS and advanced maternal age, but not primary ovarian insufficiency (POI). However, this was despite the fact that ICSI-AOA did not significantly improve fertilisation in most of the patient groups in this study; unexplained infertility (2167/2665 (ICSI) versus 1116/1311 (ICSI-AOA); p=0.3715); PCOS (958/1068 (ICSI) versus 428/477 (ICSI-AOA); p=0.4902); advanced age (968/975 (ICSI) versus 506/558 (ICSI-AOA); p=0.2484). Notably, although a significantly higher fertilisation rate was reported for the severe male factor group following ICSI-AOA (86.4% (2844/3291) versus 75.4% (2471/3277) following conventional ICSI), defective oocyte activation was clearly not an issue affecting the ICSI control group. Despite the apparently unsolicited application of ICSI-AOA, the production of high-quality blastocysts (grades 5AA, 5AB, 5BA, 4AA, 4AB, 4BA) was significantly higher following ICSI-AOA compared to conventional ICSI cycles (31.7% (741/2336) versus 20.4% (469/2288) for unexplained infertility and 38.6% (427/1107) versus 18.1% (335/1850) in OAT), with an increase in implantation rates and a decrease in miscarriage rates demonstrated in all subgroups[63]. Most other reported studies concern patients that have previously experienced ULF or TFF and demonstrate an increase in fertilisation and improved clinical outcomes following ICSI-AOA[51,64], however the reality is that AOA is not a panacea for all fertilisation abnormalities following assisted conception and will not benefit all patients[33].

Current diagnostic challenges

AOA is controversial. It is not routinely practised as part of IVF/ICSI, although treatment of couples affected by previous ULF or TFF have shown encouraging results. One of the biggest challenges is a lack testing for PLCζ expression or activity, and therefore a significant dilemma regarding which patients could benefit and/or should be offered AOA.

Certainly, immunocytochemistry fluorescent approaches to quantify PLCζ expression would be very useful as a diagnostic tool to determine patient eligibility for ICSI-AOA[65] but testing is currently neither robust nor widely accessible. Similarly, the mouse oocyte activation test (MOAT), which involves injection of sperm into mouse oocytes and assessment of fertilisation compared to a fertile sperm control[66] is not routinely available. Samples subjected to MOAT would be expected to achieve >90% fertilisation; those fertilising fewer than 20% oocytes are considered to have an activation deficiency. Moreover, whilst genetic defects of *PLCz1*[31,35,67-73] or associated genes, for example *ACTL9*[74], have been reported in patients affected by fertilisation abnormalities, there is currently no routine genetic testing in clinical practice. Genetic testing may also be fallible as some patients with PLCZ1 deficiency fail to reveal conclusive *PLCZ1* mutations[24].

Risks and benefits of artificial oocyte activation

At the time of writing, no regulations or recommendations have been issued by any reproduction society or policy committee to address the use of AOA. Specifically, the American Society for Reproductive Medicine (ASRM), European Society for Human Reproduction and Embryology (ESHRE), New Zealand's Advisory Committee on Assisted Reproductive Technology (ACART) and Reproductive Technology Accreditation Committee (RTAC) in Australia provide no guidance to support or oppose the use of ICSI-AOA to improve fertilisation rates. Patient information supplied online by the Human Fertilisation and Embryology Authority (HFEA) currently indicates an amber recommendation for calcium ionophore AOA, citing a lack of conclusive scientific evidence for routine patient use and uncertainty regarding safety and improvements to LBR (https://www.hfea.gov.uk/treatments/treatment-add-ons/artificial-egg-activation-calcium-ionophore/). Concerns regarding AOA safety relate to the potential to interfere with normal cell metabolism or embryo development with resultant adverse prenatal, neonatal or childhood outcomes, however, literature published to date does not support this[49,75,76]. It is also notable that similar concerns have been raised more generally regarding the influence of media and in vitro culture on IVF/ICSI, including epigenetic effects and gene expression changes and impact on short and long-term developmental consequences[77-80]. Nonetheless, neonatal and neurodevelopmental outcomes up to aged 10yrs appear to be similar to cognitive, motor and behavioural scores in children conceived following ICSI-AOA compared to other children[81]. Early and primary school age language development also appears to be unaffected[82]. Importantly, there appears to be no increased risk of birth defects, either chromosomal or non-chromosomal, in children born following AOA[75,83]. However, further clinical studies assessing the long-term effects of AOA are still required.

Conclusion

Even with safety concerns addressed, AOA remains controversial. Patient selection is relatively subjective, and AOA does not replicate the normal Ca^{2+} oscillations of normal fertilisation. Nonetheless, it is not unreasonable to offer ICSI-AOA to couples who have undergone one or more cycles of ICSI affected by ULF or TFF where oocyte activation appears to be the primary issue. In the meantime, provided that patient selection criteria are judicious, and assuming appropriate informed consent, ICSI-AOA has immense potential and offers genuine hope to couples challenged by fertilisation failure following IVF/ICSI.

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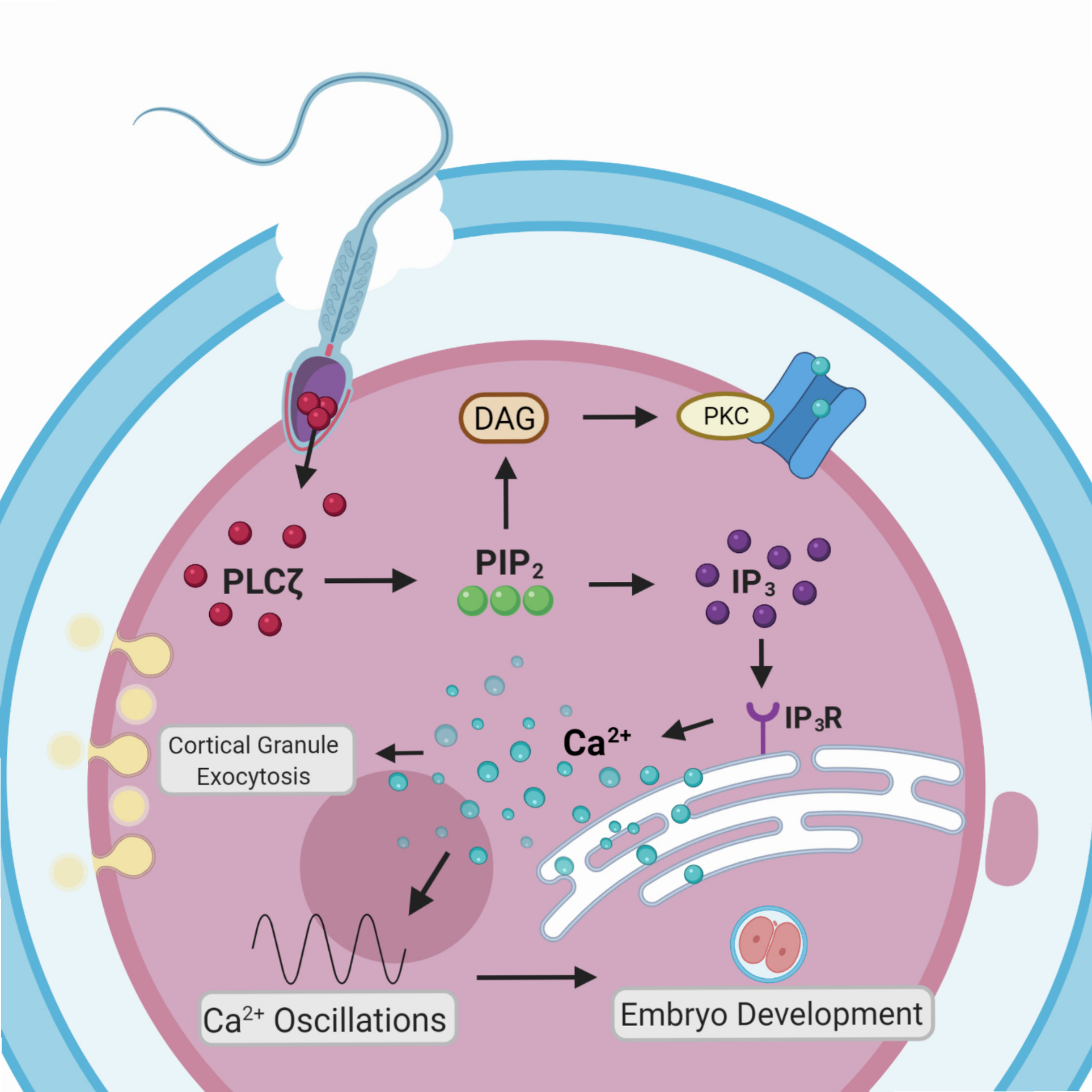
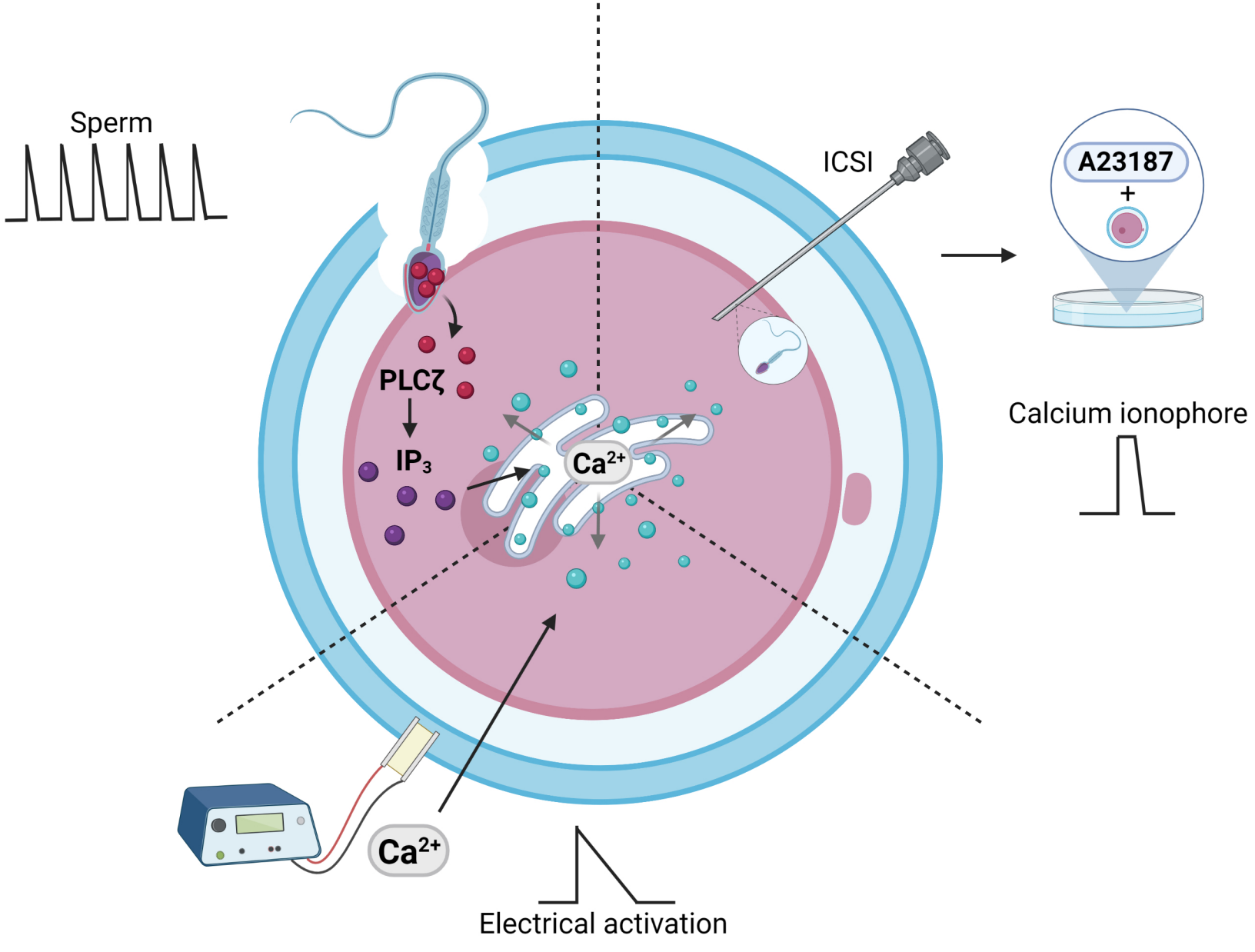
Figure 1

Schematic diagram of mechanism of human oocyte activation. Following sperm-oocyte fusion, sperm-derived PLCζ mediates PIP2 hydrolysis, producing two second messengers, IP3 and DAG. IP3 subsequently binds to IP3 receptors (IP3R) on the endoplasmic reticulum, which triggers repetitive Ca^{2+} release from intracellular stores, giving rise to waves of intracellular Ca^{2+} (oscillations).

Figure 2

Schematic diagram of oocyte activation. Electrical activation or artificial oocyte activation using calcium ionophore (A23187) stimulates a single sustained pulse of intracellular Ca^{2+}, whereas PLCζ gives rise to waves of intracellular Ca^{2+} (oscillations), which continue until meiosis is complete.


DAG
PKC
PLCζ
PIP2
IP3
IP3R
Ca2+
Cortical Granule Exocytosis
Ca2+ Oscillations
Embryo Development


Sperm
PLCζ
IP3
Ca2+
ICSI
A23187
+
Calcium ionophore
Ca2+
Electrical activation