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COMMENTARY

# Assisted yes, but where do we draw the line?

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**Abstract** In a recent report in *Reproductive Biomedicine Online* by Ebner et al., a comprehensive multi-centre study was presented on the use of a calcium ionophore, A23187, to artificially activate oocytes from patients who had poor fertilization rates in previous cycles. Under physiological conditions, the calcium increase in oocytes at activation is caused by influx and release from specific stores and ion channels, and has precise temporal, quantitative and spatial patterns. Calcium ionophores may release $Ca^{2+}$ in an uncontrolled fashion from intracellular stores that would not normally be involved in the activation process. Ionophores, including A23187, have a multitude of effects on cell homeostasis, not yet defined in oocytes, that may have long-term effects, for example on gene expression. We suspect that the successful births reported by Ebner et al. are a result of the overriding influence of the injected spermatozoa, rather than the effect of the ionophore; nevertheless, such an invasive non-physiological approach to assisted reproduction techniques is worrying, especially as epigenetic effects may result in future generations.


**KEYWORDS:** calcium increases, caution, ionophore, oocyte activation

## Dynamics of the calcium increase at oocyte activation

Calcium release is one of the first indications of oocyte activation in all animals (Stricker, 1999), with many studies showing aesthetically pleasing images of oocytes loaded with calcium-sensitive dyes 'lighting up' at activation. It is not so simple. The calcium increase is caused by both influx and release (Santella et al., 2004) from specific stores and ion channels, with temporal, quantitative and spatial patterns that need to be respected. The signal transduction pathway generating the intracellular $Ca^{2+}$ liberation is still unknown. Several different calcium release mechanisms exist in oocytes, depending on the type of receptor located on the intracellular calcium store. Furthermore, the calcium increase does not occur alone. Simultaneously, and obviously in conjunction, there is a massive cortical re-organization of this large cell involving cytoskeletal elements, the plasma membrane and a cascade of cell–cycle kinases. All activation events are propagative and also have temporal, quantitative and spatial patterns (Dale et al., 2010). Fortunately, much has been published on animals across the animal kingdom that is allowing us to build up a larger picture.

In echinoderms, ascidians and mammals, intracellular $Ca^{2+}$ increases occur in the form of a single wave or oscillations starting at the sperm attachment site and then spreading to the antipode. It is thought that the spermatozoon triggers activation locally by releasing a soluble sperm factor into the cortex. Candidates are a sperm-specific phospholipase, PLC zeta, a complex of several non-specific factors, or a post-acrosomal WW domain-binding protein (Dale et al., 2010). Whatever the mechanism, the signal originates at the point of sperm–oocyte fusion and traverses the oocyte in a wave. Several studies in mammals have shown that alterations in the precise pattern of calcium release or influx at oocyte activation influences later developmental traits (Miao et al., 2012). When sperm–oocyte membrane interaction is bypassed, as in intracytoplasmic sperm injection (ICSI), the $Ca^{2+}$ oscillations show a delayed and truncated pattern, with the $Ca^{2+}$ increases starting from an arbitrary cortical region, not far from the vicinity of the injected sperm head, indicating that the oocyte cortex where sperm oocyte interaction normally

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occurs, is more sensitive than the cytoplasm. In hamster oocytes, the increase in calcium is caused by the release of $Ca^{2+}$ from the IP3 receptors localized on the endoplasmic reticulum, which, however, also contains ryanodine receptors, whose activation is regulated by $Ca^{2+}$ influx and by cyclic ADP ribose. Although ryanodine-sensitive stores are found in mammalian oocytes, they have not yet been shown to be directly involved in the mechanism of sperm-induced $Ca^{2+}$ oscillations.

In mouse oocytes, $Ca^{2+}$ oscillations activate CaMKIIγ causing decreases in maturation-promoting factor and mitogen-activated protein kinase, which, in turn, promote the metaphase to anaphase transition and exocytosis of cortical granules (Miao et al., 2012). We also have to take into consideration that the modulation of $Ca^{2+}$ spikes in mammalian oocytes is likely to be more complex, as the maintenance of $Ca^{2+}$ oscillations requires external $Ca^{2+}$ to replenish the intracellular $Ca^{2+}$. In ICSI-induced mouse oocyte activation, this $Ca^{2+}$ influx across the plasma membrane, which is activated by the emptying of intracellular $Ca^{2+}$ stores following the first $Ca^{2+}$ transients, replenishes the $Ca^{2+}$ stores and also activates a cortical signalling pathway that is required for the actin-based functions necessary for meiotic spindle rotation and polar body extrusion (Miao et al., 2012). Mitochondria also participate in intracellular $Ca^{2+}$ homeostasis. It has been shown that sperm-triggered $Ca^{2+}$ oscillations stimulate repetitive cycles of reduction and oxidation in the mitochondria, both of which lead to generation of adenosine-5′-triphosphate, which is essential to maintain the $Ca^{2+}$ oscillations (Dumollard et al., 2009). Pharmacological inactivation of mitochondria alters the pattern of $Ca^{2+}$oscillations, resulting in sustained increases in cytosolic $Ca^{2+}$ levels. This suggests that, under normal conditions, mitochondrial $Ca^{2+}$ sequestration contributes to returning $Ca^{2+}$ levels to the baseline level between the $Ca^{2+}$ spikes.

In echinoderms, fine regulation of the actin cytoskeleton in the oocyte cortex is essential for normal maturation and fertilization. Perturbation of the actin cytoskeleton in the oocyte cortex by several agents can alter all the events associated with the fertilization process, i.e. sperm–oocyte interaction, the $Ca^{2+}$ response, cortical granules exocytosis and cleavage of the zygote (Puppo et al., 2008; Vasilev et al., 2012).

## Ionophore-induced calcium increases in oocytes

Calcium ionophore, A23187, is a carboxylic antibiotic that binds divalent cations, such as $Ca^{2+}$ and $Mg^{2+}$. It was developed in the 1960s, and was shown to affect mitochondrial function in rat liver cells by perturbing calcium and magnesium content leading to inhibition of ATPase and glutamate oxidation (see Reed and Lardy, 1972 for a review). It is thought to freely transport divalent cations across all biological membranes in all organelles. A second ionophore used in oocyte activation, ionomycin, is more specific for $Ca^{2+}$, and can activate $Ca^{2+}$ or calmodulin-dependent kinase and phosphatase to stimulate gene expression. In human T cells, ionomycin induces hydrolysis of phosphoinositides and activates protein kinase C to mediate T-cell activation, whereas, in human B cells, it induces the activation of calcium-dependent endonuclease and results in apoptosis.

In starfish, the ionomycin-induced $Ca^{2+}$ rise is a centripetal cortical $Ca^{2+}$ wave lasting for several minutes, whereas the sperm-induced cortical $Ca^{2+}$ release is very rapid, lasting several seconds and is followed by a $Ca^{2+}$ wave propogating from the sperm–oocyte interaction site to the opposite pole (Puppo et al., 2008). Ionophore activation was followed by a rapid alteration of the actin cytoskeleton, including a retraction of microvilli at the oocyte surface. In addition, cortical granules were disrupted or fused with other vesicles a few minutes after the addition of ionomycin. These structural changes prevented cortical maturation of the oocytes, despite the normal progression of nuclear envelope breakdown, inhibition of the fertilization envelope elevation and upon sperm incorporation, the monospermic zygotes were blocked at the first cell division or displayed a problem in cell cleavage at later stages (Vasilev et al., 2012).

Ascidian oocytes may be partially activated by calcium ionophore A23187, leading to a surface contraction and a wave of $Ca^{2+}$ increase only by applying a localized high concentration of the ionophore, in a micropipette.

$Ca^{2+}$ ionophore treatment has been widely applied in human oocytes. Determination of intracellular $Ca^{2+}$ concentration changes in human oocytes using two different $Ca^{2+}$ dyes showed that activation of the oocytes with A23187, or ionomycin, induced a $Ca^{2+}$ elevation in the form of a single transient, which was not followed by further $Ca^{2+}$ oscillations. A combination of calcium injection and $Ca^{2+}$ ionophore treatment can also activate human oocytes injected with round-headed sperm cells and acrosome-less human sperm that are incapable of penetrating oocytes *in vivo* and *in vitro*.

## Clinical concerns

Ebner et al. (2015) selected patients that had undergone previous ICSI cycles with poor fertilization rates, where the reason for failure was not defined. Their concept was to aid activation by exposing the oocytes to 10 μM solutions of calcium ionophore. Although the dose is quite low, the non-physiological action of this chemical, which is capable of releasing calcium from all intracellular stores, including those that would not normally be involved in the activation process, in an uncontrolled fashion, both spatially and temporally, is, in our opinion, and that of others (van Blerkom et al., 2015), the object of concern. Non-physiological activation of oocytes could affect the activity of specific proteins downstream of the signal transduction pathway, which is strictly dependent on the spatial and temporal regulation of the $Ca^{2+}$ increases (Ducibella and Fissore, 2008). In addition, ionophores, including A23187, have a multitude of effects on cell homeostasis, not yet defined in oocytes that may have long-term effects such as on gene expression.

In most species, gametes are produced in large numbers, and most fail to produce a viable offspring. Human reproduction is no exception: mature spermatozoa are plentiful, the female fetus is endowed with hundreds of thousands of immature oocytes, and several hundred oocytes may be ovulated; however, it has been estimated that only 5% of mature oocytes produced in in-vitro programmes lead to a viable birth. Of the gametes that do interact, failure at fertilization may be due to the spermatozoon being unable to trigger activation events or to the oocyte being unable to respond to the activating trigger. Although the former may be due to a deficiency in the soluble activating factor thought to be present

in spermatozoa, the latter may be attributed to an innumerable number of factors, from deficiency in the calcium release mechanisms, cytoskeletal defects, any of many cell cycle kinases, and intracellular pH, glutathione. Oocytes from women with polycystic ovary syndrome, while often appearing morphologically normal, are notoriously disorganized at the organelle level. Elsewhere, we have pointed out the heterogeneity of ovulated oocytes which, despite apparent synchrony in nuclear maturity, express asynchrony in cytoplasmic maturity (Dale et al., 2015).

Finally, we disagree with the concept that the end-point for success in IVF is a live birth. The successful activation and embryogenesis reported by Ebner et al. (2015) in our opinion is not due to the ionophore, but rather to the fortuitous selection of better gametes in the re-trial. In any case, the non-physiological approach to assisted reproduction techniques, is worrying, especially as epigenetic effects may appear in alternate generations. We believe that recovering defective gametes is not the way forward in assisted reproduction techniques; rather, we should be dedicating resources to ways of improving gametogenesis, *in vivo* and *in vitro*.

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