Single Ca$^{2+}$ transients vs oscillatory Ca$^{2+}$ signaling for assisted oocyte activation: limitations and benefits

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Abstract

Oocyte activation is a calcium (Ca$^{2+}$)-dependent process that has been investigated in depth, in particular, regarding its impact on assisted reproduction technology (ART). Following a standard model of signal transduction, Ca$^{2+}$ drives the meiotic progression upon fertilization in all species studied to date. However, Ca$^{2+}$ changes during oocyte activation are species specific, and they can be classified in two modalities based on the pattern defined by the Ca$^{2+}$ signature: a single Ca$^{2+}$ transient (e.g. amphibians) or repetitive Ca$^{2+}$ transients called Ca$^{2+}$ oscillations (e.g. mammals). Interestingly, assisted oocyte activation (AOA) methods have highlighted the ability of mammalian oocytes to respond to single Ca$^{2+}$ transients with normal embryonic development. In this regard, there is evidence supporting that cellular events during the process of oocyte activation are initiated by different number of Ca$^{2+}$ oscillations. Moreover, it was proposed that oocyte activation and subsequent embryonic development are dependent on the total summation of the Ca$^{2+}$ peaks, rather than to a specific frequency pattern of Ca$^{2+}$ oscillations. The present review aims to demonstrate the complexity of mammalian oocyte activation by describing the series of Ca$^{2+}$-linked physiological events involved in mediating the egg-to-embryo transition. Furthermore, mechanisms of AOA and the limitations and benefits associated with the application of different activation agents are discussed.

Introduction: Signal transduction during oocyte activation: role of Ca$^{2+}$

Calcium (Ca$^{2+}$) is an essential component in orchestrating cell signaling. Calcium levels are finely regulated to stimulate diverse cellular programs while avoiding cellular toxicity or death induced by prolonged and sustained Ca$^{2+}$ changes. Calcium also has a particular duality during signal transduction, acting either as the stimulus or the product during the same process (Miyazaki 1993, Bootman et al. 2002). Contributing to this versatility, Ca$^{2+}$ acts as a second messenger to convey diverse stimuli into distinct patterns of Ca$^{2+}$ fluctuations, which can be manifested as transient rises, sustained responses or repetitive oscillatory changes, thereby transducing the specific message into a unique signal (Berridge et al. 2000, Bootman et al. 2001). Oocyte activation is a well-known Ca$^{2+}$-dependent process in which the Ca$^{2+}$ signal can be relayed as a single Ca$^{2+}$ transient (i.e. a Ca$^{2+}$ elevation followed by recovery), further called a ‘single’ response or can attain a more dynamic mode of repetitive Ca$^{2+}$ transients called ‘Ca$^{2+}$ oscillations’. A single Ca$^{2+}$ transient is characterized by an amplitude and duration, whereas Ca$^{2+}$ oscillations are additionally characterized by the frequency of the Ca$^{2+}$ events. Altogether, the occurrence of single Ca$^{2+}$ transients vs dynamic Ca$^{2+}$ oscillations depend on the species, with different species displaying specific Ca$^{2+}$ signatures (Stricker 1999). The molecular basis underlying these differences within the animal kingdom has attracted significant interest.

The hallmarks for oocyte activation among numerous species were compared by (Stricker 1999). The specific Ca$^{2+}$ patterns supporting oocyte activation are not necessarily associated with the lineage origin, since species from the same phyla may manifest different Ca$^{2+}$ patterns, all allowing meiotic resumption. For instance, frogs (sp. Xenopus laevis) display single Ca$^{2+}$ changes while mice (sp. Mus musculus) manifest an oscillatory Ca$^{2+}$ response. Both species have been widely used as models to investigate the cellular mechanistic associated with distinct Ca$^{2+}$ signatures and their impact on oocyte activation. Despite the Ca$^{2+}$ profile differences between both species, the process of oocyte activation is similar (Perry & Verlhac 2008). Briefly, upon fertilization,
the sperm releases the soluble factor phospholipase C zeta (PLCζ) into the ooplasm. PLCζ hydrolyzes phosphatidylinositol bisphosphate (PIP2), located in oocyte vesicle membranes, into inositol trisphosphate (IP3), which diffuses in the cytoplasm, and diacylglycerol (DAG) which remains associated with the membrane and activates the protein kinase C (PKC) (Gonzalez-Garcia et al. 2013). IP3 binds to its cognate receptor (IP3R) embedded on the endoplasmic reticulum (ER), resulting in the opening of Ca2+-permeable channels that facilitate Ca2+ release from intracellular stores, thereby increase concentrations of free cytoplasmic Ca2+ (Miyazaki et al. 1992). Subsequently, a signaling cascade is activated to alleviate meiotic arrest, which is secured by high levels of M-phase promoting factor (MPF) (Tripathi et al. 2010). Interestingly, species that physiologically undergo Ca2+ oscillations during oocyte activation are also capable of responding to a single Ca2+ transient, for instance, as induced by Ca2+ ionophores or ethanol during assisted oocyte activation (AOA). Although the efficiency of these artificial methods is established, it is unsure whether a few Ca2+ transients induced over a period of a few minutes can recapitulate the signaling effects of the long-lasting Ca2+ oscillatory signature. In this regard, the importance of the spatiotemporal aspects of Ca2+ oscillations during oocyte activation and its impact on embryo development is still under discussion. This review starts with a description of the physiology of oocyte activation in species which manifest either a single Ca2+ transient or an oscillatory Ca2+ regime. We then discuss how agents such as Ca2+ ionophores alleviate meiotic arrest by inducing a single or a few Ca2+ transients in mammalian oocytes. Furthermore, we discuss the limitations and benefits of diverse AOA protocols and how they have an impact on both early and late events of embryo development.

**Oocyte activation: Physiology of Ca2+ transients and oscillations**

For clarity, the discussion that follows will be organized in four parts, each linked to a fundamental aspect of the Ca2+ signaling cascade (Fig. 1), as suggested by Berridge and coworkers (Berridge et al. 2000). The model encompasses the stimulus, the ON mechanisms, the effectors and the OFF mechanisms. These four steps allow to structure the numerous findings related to oocyte activation, further outlining which conditions promote single Ca2+ transients or Ca2+ oscillations in different species.

**The stimulus: PLCζ**

It has been well documented that oocyte activation initiates when sperm delivers a specific soluble factor to the ooplasm upon gamete fusion in all mammalian species studied to date (reviewed by Kashir et al. 2013, Swann & Lai 2013). Phospholipase C ζ was identified as the sperm-borne soluble factor that triggers oocyte activation (Cox et al. 2002, Saunders et al. 2002). In the case of non-mammalian vertebrates, the model of the ‘soluble factor’ has been demonstrated only in newt...
Ca\textsuperscript{2+} responses and assisted oocyte activation

(sp. Cynops pyrrhogaster) (Harada et al. 2011). Xenopus fertilization is triggered by a single Ca\textsuperscript{2+} transient (Nuccitelli et al. 1993) and although ‘PLC\textsubscript{ζ} counterpart’ has not yet been identified, a gene identity of 42% to the mouse PLC\textsubscript{ζ} protein has been reported (Runft et al. 1999, 2002). PLC\textsubscript{ζ} triggers Ca\textsuperscript{2+} oscillations in mammals (Cox et al. 2002). It is a soluble and Ca\textsuperscript{2+}-sensitive enzyme that catalyzes the hydrolysis of PIP\textsubscript{2}, at basal concentrations of Ca\textsuperscript{2+} to generate IP\textsubscript{3} and DAG (Miyazaki 1993). The signaling cascade of DAG proceeds, in concert with Ca\textsuperscript{2+}, toward activation of PKC (Ducibella & Fissore 2008). It is a soluble and Ca\textsuperscript{2+}-sensitive enzyme that may potentiate IP\textsubscript{3}-triggered changes in Ca\textsuperscript{2+} concentration, their contribution is not critical in inducing oocyte activation (Ayabe et al. 1995). As a result, IP\textsubscript{3}-induced Ca\textsuperscript{2+} release (IICR) is the predominant mechanism. Experiments in Xenopus suggest that the channel of IP\textsubscript{3}R opens through a conformational transition in response to IP\textsubscript{3} and Ca\textsuperscript{2+} concentrations (Shuai et al. 2009). There are three IP\textsubscript{3}R isoforms described (types 1, 2 and 3) in mice (Fissore et al. 1999), which IP\textsubscript{3}R isoform 1 is particularly expressed (Parrington et al. 1998). The IP\textsubscript{3}R isoform 1 is also predominant in frogs (Kume et al. 1993). Immature oocytes at their germinal vesicle (GV) phase are less sensitive to the IP\textsubscript{3}-induced Ca\textsuperscript{2+} release (IICR) than oocytes at the metaphase II (MII) stage (Mehlmann & Kline 1994). This dramatic increase in sensitivity to IP\textsubscript{3} arises from variations such as an increase of IP\textsubscript{3}R levels, IP\textsubscript{3}R redistribution (Mehlmann et al. 1996, Goud et al. 1999) and post-translational modifications (Vanderheyden et al. 2009a,b), in particular phosphorylation. M-phase kinases, such as polo-like kinase 1 (Plk1) (Ito et al. 2008), mitogen-activated protein kinase (MAPK) (Lee et al. 2006) and cyclin b/cyclin-dependent kinase 1 (Cdk1) (Zhang et al. 2015), play an important role in modulating this increase in the sensitivity of the IP\textsubscript{3}R. Polo-like kinase 1 participates in the early phases of oocyte maturation (Ito et al. 2008). Mouse studies using a Plk1 inhibitor demonstrated that Plk1 phosphorylates an MPM-2 epitope on the IP\textsubscript{3}R1 (Vanderheyden et al. 2009a,b). Also in Xenopus, Plk1 affects the phosphorylation status of the IP\textsubscript{3}R, but in contrast, there is no evidence supporting its role in sensitizing IP\textsubscript{3}R. MAPK mediates the phosphorylation of IP\textsubscript{3}R at MPM-2 during maturation in both Xenopus (Sun et al. 2009) and mouse oocytes (Lee et al. 2006, Vanderheyden et al. 2009b), leading in turn to their sensitization. However, the phosphorylation site appears to differ between the species (Lee et al. 2006, Sun et al. 2009). Moreover, Cdk1, a subunit of MPF, targets the IP\textsubscript{3}R to enhance its sensitivity during mouse oocyte maturation (Wakai et al. 2013). Interestingly, Xenopus data showed that both Cdk1 and MAPK require simultaneous activation to mediate full sensitization of the IP\textsubscript{3}Rs (Sun et al. 2009). Kinases other than Plk1, MAPK and Cdk1 have also been implicated in IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release (Bezprozvannyy 2005, Vanderheyden et al. 2009a,b), including PKA, PKC and in particular, Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII) (Ducibella & Fissore 2008), a key effector during oocyte activation.

**Inositol triphosphate receptors (IP\textsubscript{3}Rs)**

Mouse studies have shown that, although ryanodine receptors are present and may potentiate IP\textsubscript{3}-triggered changes in Ca\textsuperscript{2+} concentration, their contribution is not critical in inducing oocyte activation (Ayabe et al. 1995). As a result, IP\textsubscript{3}-induced Ca\textsuperscript{2+} release (IICR) is the predominant mechanism. Experiments in Xenopus suggest that the channel of IP\textsubscript{3}R opens through a conformational transition in response to IP\textsubscript{3} and Ca\textsuperscript{2+} concentrations (Shuai et al. 2009). There are three IP\textsubscript{3}R isoforms described (types 1, 2 and 3) in mice (Fissore et al. 1999), of which IP\textsubscript{3}R isoform 1 is particularly expressed (Parrington et al. 1998). The IP\textsubscript{3}R isoform 1 is also predominant in frogs (Kume et al. 1993). Immature oocytes at their germinal vesicle (GV) phase are less sensitive to the IP\textsubscript{3}-induced Ca\textsuperscript{2+} release (IICR) than oocytes at the metaphase II (MII) stage (Mehlmann & Kline 1994). This dramatic increase in sensitivity to IP\textsubscript{3} arises from variations such as an increase of IP\textsubscript{3}R levels, IP\textsubscript{3}R redistribution (Mehlmann et al. 1996, Goud et al. 1999) and post-translational modifications (Vanderheyden et al. 2009a,b), in particular phosphorylation. M-phase kinases, such as polo-like kinase 1 (Plk1) (Ito et al. 2008), mitogen-activated protein kinase (MAPK) (Lee et al. 2006) and cyclin b/cyclin-dependent kinase 1 (Cdk1) (Zhang et al. 2015), play an important role in modulating this increase in the sensitivity of the IP\textsubscript{3}R. Polo-like kinase 1 participates in the early phases of oocyte maturation (Ito et al. 2008). Mouse studies using a Plk1 inhibitor demonstrated that Plk1 phosphorylates an MPM-2 epitope on the IP\textsubscript{3}R1 (Vanderheyden et al. 2009a,b). Also in Xenopus, Plk1 affects the phosphorylation status of the IP\textsubscript{3}R, but in contrast, there is no evidence supporting its role in sensitizing IP\textsubscript{3}R. MAPK mediates the phosphorylation of IP\textsubscript{3}R at MPM-2 during maturation in both Xenopus (Sun et al. 2009) and mouse oocytes (Lee et al. 2006, Vanderheyden et al. 2009b), leading in turn to their sensitization. However, the phosphorylation site appears to differ between the species (Lee et al. 2006, Sun et al. 2009). Moreover, Cdk1, a subunit of MPF, targets the IP\textsubscript{3}R to enhance its sensitivity during mouse oocyte maturation (Wakai et al. 2013). Interestingly, Xenopus data showed that both Cdk1 and MAPK require simultaneous activation to mediate full sensitization of the IP\textsubscript{3}Rs (Sun et al. 2009). Kinases other than Plk1, MAPK and Cdk1 have also been implicated in IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release (Bezprozvannyy 2005, Vanderheyden et al. 2009a,b), including PKA, PKC and in particular, Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII) (Ducibella & Fissore 2008), a key effector during oocyte activation.

**ON mechanisms**

The ON mechanisms mediate a cytoplasmic Ca\textsuperscript{2+} increase required for oocyte activation (Kline et al. 1999). Oocyte acquires its ability for Ca\textsuperscript{2+} release during fertilization during final oocyte maturation (Ajduk et al. 2008), with different Ca\textsuperscript{2+} channels controlling the Ca\textsuperscript{2+} release from internal stores, such as type 2 and 3 ryanodine receptors (Ayabe et al. 1995) and type 1 inositol 1,4,5-trisphosphate receptor (IP\textsubscript{3}R1), or the entry of external Ca\textsuperscript{2+}, for instance via store-operated calcium entry (SOCE) (Machaty 2016).

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[Note: For clarity and coherence, the text is broken into meaningful paragraphs, and some sentences are split to ensure natural reading flow. The references are integrated for academic context and are properly cited.]
Moreover, the ability of IP3R to conduct Ca\textsuperscript{2+} has been associated with an increase in the concentration of Ca\textsuperscript{2+} in the ER ([Ca\textsuperscript{2+}]\textsubscript{ER}) (Cheon et al. 2013), which results from the regulation of mechanisms controlling cytoplasmic Ca\textsuperscript{2+} influx and efflux.

**Store-operated calcium entry (SOCE)**

ER Ca\textsuperscript{2+} content increases during oocyte maturation (Cheon et al. 2013) and throughout oocyte activation to maintain a Ca\textsuperscript{2+} oscillatory regime (Wakai et al. 2013). In this regard, store-operated Ca\textsuperscript{2+} entry underlies one of the mechanisms facilitating Ca\textsuperscript{2+} influx in response to [Ca\textsuperscript{2+}]\textsubscript{ER} depletion. In mammals, the two major SOCE players are the stromal interaction molecule (STIM) 1 and 2 (Cahalan 2009), and the Ca\textsuperscript{2+}-release activated Ca\textsuperscript{2+} channel protein 1 (Orai1). STIM 1 and 2 are located in the ER membrane, and Orai1 in the oocyte plasma membrane. STIM2 is activated by limited depletions of Ca\textsuperscript{2+}, while STIM1 is activated when the ER is drastically depleted in Ca\textsuperscript{2+} content. STIM1 functions as a Ca\textsuperscript{2+} sensor that monitors [Ca\textsuperscript{2+}]\textsubscript{ER} by its EF hand domain (Lewis 2007, Carrasco & Meyer 2011). When the [Ca\textsuperscript{2+}]\textsubscript{ER} experiences a drastic decrease, STIM1 responds by clustering into large puncta (Stathopulos et al. 2006, Liou et al. 2007) and migrating toward the ER cortex. STIM1 clusters at the cortex interact with, and open, the Orai1 Ca\textsuperscript{2+} channel, allowing Ca\textsuperscript{2+} entry into the oocyte (Cahalan 2009, Hogan et al. 2010). A recent study demonstrates that Xenopus oocytes, expressing an activated form of SOCE only at metaphase II (MII) stage (Machaca & Haun 2000), can adjust Ca\textsuperscript{2+} signaling to single transients or dynamic oscillations depending also on [Ca\textsuperscript{2+}]\textsubscript{ER} with a drastic Ca\textsuperscript{2+} depletion favoring a single response vs mild Ca\textsuperscript{2+} depletions favoring Ca\textsuperscript{2+} oscillations (Courjaret et al. 2017). SOCE contributes to the increase in intracytoplasmic Ca\textsuperscript{2+} and its function modulates Ca\textsuperscript{2+} oscillations in certain species as swine (Lee et al. 2012). However, several findings suggest that SOCE might not significantly contribute in mediating Ca\textsuperscript{2+} influx to maintain long lasting Ca\textsuperscript{2+} oscillations in for example mouse (Takahashi et al. 2013). Mouse oocytes cultured in Ca\textsuperscript{2+}-free medium failed to get activated after sperm microinjection while inhibition of SOCE in the presence of extracellular Ca\textsuperscript{2+} was associated with normal activation (Miao et al. 2012). These observations indicate that Ca\textsuperscript{2+} influx is essential for oocyte activation and suggest the participation of Ca\textsuperscript{2+} entry pathways other than SOCE during oocyte activation, at least in mouse.

**Effectors: CAMKII**

CaMKII is considered as the major effector during oocyte activation in mammalian vertebrates (Lorca et al. 1993, Backs et al. 2010). This kinase selectively phosphorylates upstream targets like IP3R, resulting in its inhibition, and downstream targets leading to the degradation of the early mitotic inhibitor 2 (Emi2), for instance (Perry & Verlhac 2008). CaMKII is a multifunctional protein sensitive to the Ca\textsuperscript{2+}-calmodulin complex, and it is typically activated in a frequency-dependent manner by Ca\textsuperscript{2+} oscillations as demonstrated in mammals (Tatone et al. 2002). CaMKII can also be activated by high cytoplasmic concentrations of Ca\textsuperscript{2+}, through a cascade involving autophosphorylation and achievement of a sustained active status (Markoulaki et al. 2003, Dupont et al. 2010). In general, CAMKII shows lower activity in oocytes than in other cell types (Markoulaki et al. 2003); its activity depends on the Ca\textsuperscript{2+} oscillations frequency as well as the duty cycle, i.e. the percentage of time that the Ca\textsuperscript{2+} signal is high, relative to the duration of the oscillatory period (Smedler & Uhlén 2014). As a result, CaMKII activity depends on the integration of the preceding Ca\textsuperscript{2+} dynamics, i.e. the time during which the Ca\textsuperscript{2+} signal is high will determine the CaMKII activation status in which the frequency as well as the duty cycle play a role. Thus, low frequencies and short spike durations will trigger little CAMKII activity while continued spiking at higher frequencies will activate and maintain CAMKII activity. The influence of the duration of the Ca\textsuperscript{2+} transients during fertilization has been illustrated in several studies. The first Ca\textsuperscript{2+} transient upon sperm entry always shows a longer duration than subsequent oscillatory spikes (reviewed by Halet 2004, Miao & Williams 2012). In particular, in human oocytes, the duration of the first transient is in the order of 3–4.5 min, whereas subsequent transients have a shorter duration (2.2–2.8 min) (Nikiforaki et al. 2014b). Interestingly, when activation is artificially induced by ionomycin at a low concentration of 2 µM, Ca\textsuperscript{2+} returns nearly to its basal level from its peak value within 5 min, when CAMKII registers 64% of its maximal activity at the occasion of the very first Ca\textsuperscript{2+} transient (Markoulaki et al. 2003). Moreover, long-duration Ca\textsuperscript{2+} transients are not invariably effective; in fact, one study in mouse oocytes has demonstrated that phosphatases can mediate CAMKII inactivation in the presence of sustained Ca\textsuperscript{2+} levels (Ozil et al. 2005). Taken together, these findings suggest that the duration of both isolated Ca\textsuperscript{2+} transients, as well as ongoing oscillatory Ca\textsuperscript{2+} dynamics, contribute to activate and to maintain CAMKII active throughout meiosis completion.

**OFF mechanisms**

In order to sustain an ongoing Ca\textsuperscript{2+} oscillatory activity over several hours, it is necessary to have a set of OFF mechanisms that ensure restoration of the Ca\textsuperscript{2+} levels to baseline after each spike (Brandman et al. 2007, Cahalan 2009). In general, OFF mechanisms include refilling of the ER Ca\textsuperscript{2+} stores by sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+} ATPases (SERCA pumps), extrusion of Ca\textsuperscript{2+} by low-affinity, high-capacity Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange
proteins (NCX) and high-affinity, low-capacity Ca\textsuperscript{2+} ATPases in the plasma membrane, as well as and Ca\textsuperscript{2+} uptake into mitochondria.

**PMCAs and SERCA pumps**

Presence of NCX has been reported in mouse oocytes (Carroll 2000). However, Ca\textsuperscript{2+} efflux mediated by PMCA\textsubscript{s} is suggested to be more relevant. Studies in Xenopus show that PMCA\textsubscript{s} experience an internalization during oocyte maturation leading to a sustained single Ca\textsuperscript{2+} rise mediate fertilization (El-Jouni et al. 2008). The impact of PMCA internalization on modulating Ca\textsuperscript{2+} changes is observed as follows. While an immature oocyte manifests a sharp and fast Ca\textsuperscript{2+} spike upon sperm microinjection, a mature oocyte displays a Ca\textsuperscript{2+} rise that lasts for at least 5 min (El-Jouni et al. 2008). These observations suggest the contribution of PMCA to Ca\textsuperscript{2+} efflux. Additionally, intracytoplasmic Ca\textsuperscript{2+} is taken up into ER lumen by SERCA\textsubscript{pumps} (Wakai et al. 2012), and it occurs after Ca\textsuperscript{2+} influx. Both events, thus Ca\textsuperscript{2+} influx and ER replenishment, play an important role in guaranteeing the persistence of Ca\textsuperscript{2+} oscillations during mammalian oocyte activation.

**Mitochondria**

Mitochondria are also actively involved in Ca\textsuperscript{2+} homeostasis during fertilization (Rizzuto et al. 1998). Mitochondrial ATP production is necessary for SERCA activity (Fissore et al. 2002), a mechanism involved in ER replenishment (Wakai et al. 2013). Mitochondria are associated with the maintenance of low basal cytoplasmic Ca\textsuperscript{2+} levels contributing to the occurrence of the Ca\textsuperscript{2+} oscillations (Dumollard et al. 2004). In addition, mitochondrial Ca\textsuperscript{2+} uptake has also been shown to be essential in the regulation of IP3-induced Ca\textsuperscript{2+} release (IICR) during Ca\textsuperscript{2+} signaling in both, Xenopus and mice oocytes (Jouaville et al. 1995, Wakai et al. 2013). In this regard, the protein MICU1 (mitochondrial Ca\textsuperscript{2+} uniporter) is involved in the mitochondrial matrix (Perocchi et al. 2010). Interestingly, when mitochondrial function was uncoupled by either the protonophore FCCP (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone) or AMA (antimycin A), both sperm and Sr\textsuperscript{2+}-induced Ca\textsuperscript{2+} transients were disrupted in mouse oocytes, resulting in a sustained and long Ca\textsuperscript{2+} increase, which resulted in apoptosis (Liu et al. 2001).

To this point, we have discussed the physiology associated with single transients and oscillatory Ca\textsuperscript{2+} signaling using Xenopus and mouse oocytes as reference models. The mechanisms mediating oocyte activation in both species show particular similarities with several findings demonstrating the role of sperm in triggering Ca\textsuperscript{2+} changes and the imperative role of the oocyte’s machinery in modulating and shaping the final Ca\textsuperscript{2+} signature. The capability of oocytes in integrating distinct Ca\textsuperscript{2+} signals during fertilization is particularly observed using strategies to artificially induce oocyte activation. In this regard, disparate Ca\textsuperscript{2+} responses have demonstrated their efficiency in allowing egg-to-embryo transition, particularly in mammals. The following section aims to discuss the mechanisms of action of diverse activating agents and their impact in the physiology of mammalian oocyte activation subsequently affecting embryonic development.

**Ca\textsuperscript{2+} signaling for assisted oocyte activation (AOA)**

Protocols used for AOA can be classified based on whether the mechanism evoking the Ca\textsuperscript{2+} trigger that promotes fertilization is mechanical, electrical or chemical. Apart from the various mechanisms of action of these protocols, the response can also differ between different species (Vanden Meerschaut et al. 2014b). The mechanical approach involves the introduction of the sperm into the oocyte followed by vigorous ooplasm aspiration and re-injection during ICSI. In this way, the Ca\textsuperscript{2+} introduced from the extracellular medium is sufficient to restore the fertilization rates to normal, generally assumed as >70% oocytes showing two pronuclei (Tesarik & Sousa 1995, Tesarik et al. 2002). Electrostimulation involves the application of a pulsed high-voltage electrical field, which modifies the plasma membrane lipid bilayer leading to the formation of pores, and therefore, allowing Ca\textsuperscript{2+} entry from the extracellular medium (Versieren et al. 2010, Vanden Meerschaut et al. 2014b). However, chemical artificial activation is the most used strategy among practitioners. Chemical activation agents are classified based on the Ca\textsuperscript{2+} response they elicit in mammalian oocytes: single Ca\textsuperscript{2+} transients, dynamic Ca\textsuperscript{2+} oscillations and oocyte activation independent of the initial Ca\textsuperscript{2+} trigger. Table 1 summarizes AOA protocols commonly applied in mice and human. The mechanisms of action of the different activation agents as well as their limitations and benefits are further discussed.

**Agents inducing single Ca\textsuperscript{2+} transients**

**Ca\textsuperscript{2+} ionophores**

Calcium ionophores such as ionomycin and calcimycin are two carboxylic antibiotics, synthesized by the Streptomyces bacterial species Streptomyces conglobatus and Streptomyces chartreusensis, respectively. Ca\textsuperscript{2+} ionophores confer high permeability to cell membranes allowing Ca\textsuperscript{2+} ions to penetrate through. Oocytes exposed to Ca\textsuperscript{2+} ionophores experience an increase of free intracytoplasmic Ca\textsuperscript{2+}, resulting from Ca\textsuperscript{2+} influx as well as from Ca\textsuperscript{2+} release from the intracellular stores, particularly the ER, as described in starfish (Vasilev et al. 2012); no data specific to mouse and human oocytes are currently available.

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Table 1  Chemical-based AOA protocols used in mouse and human studies.

<table>
<thead>
<tr>
<th>Activation agent</th>
<th>Species</th>
<th>Ca²⁺ response</th>
<th>Activation rate</th>
<th>Blastocyst formation rate</th>
<th>Protocol</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionomycin</td>
<td>Mouse</td>
<td>Single transient</td>
<td>73.0%</td>
<td>10 µM for 10 min (2 exposures, 30 min interval)</td>
<td>2 µg/mL cytoD for 3 h</td>
<td>Heytens et al. (2008) a,1</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>66.3%</td>
<td>10 µM for 10 min (2 exposures, 30 min interval)</td>
<td>2 µg/mL cytoD for 4 h</td>
<td>Nikiforaki et al. (2016) a,1</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td>85.0%</td>
<td>10 µM for 10 min (2 exposures, 30 min interval)</td>
<td>2 mM 6-DMAP for 3 h</td>
<td>de Fried (2008) 1</td>
</tr>
<tr>
<td>Calcimycin</td>
<td>Mouse</td>
<td></td>
<td>86.1%*</td>
<td>10 µM for 6 min (1 exposure)</td>
<td>1 µg/mL cytoD for 5–7 h</td>
<td>Uranga et al. (1996) a,1</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td>74.2%</td>
<td>10 µM for 10 min (2 exposures, 30 min interval)</td>
<td>Human globozoospermic sperm</td>
<td>Nikiforaki et al. (2016) a,1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Mouse</td>
<td></td>
<td>76.2%</td>
<td>5 µM for 5 min (1 exposure)</td>
<td>2 µg/mL cytoD for 2 h</td>
<td>Winston et al. (1991) 3</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td>11.8%</td>
<td>5 µM for 5 min (1 exposure)</td>
<td>Human globozoospermic sperm</td>
<td>Balakier and Casper (1993) 3</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>Mouse</td>
<td>Oscillatory signaling</td>
<td>82.7%</td>
<td>7% for 6 min (1 exposure)</td>
<td>Human donor sperm</td>
<td>Liu (2014) 2</td>
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<tr>
<td>Human</td>
<td></td>
<td></td>
<td>61.7%</td>
<td>10 mM for 1 h (1 exposure)</td>
<td>Human sperm with low or complete failed fertilization</td>
<td>Rogers et al. (2006) c,1</td>
</tr>
<tr>
<td>PLCζ</td>
<td>Mouse</td>
<td></td>
<td>54.5%</td>
<td>10 mM for 20 min (1 exposure)</td>
<td>2.5 mM 6-DMAP for 1.5 h</td>
<td>Kim et al. (2014) 3</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td>78.0%</td>
<td>cRNA injection: 0.02 mg/mL</td>
<td>2 µg/mL cytoD for 4 h</td>
<td>Saunders et al. (2002) c,1</td>
</tr>
<tr>
<td>Puromycin</td>
<td>Human</td>
<td>In absence of the initial Ca²⁺ trigger</td>
<td>75%*</td>
<td>cRNA injection: 0.1 µg/mL</td>
<td>5 µg/mL cytoB for 6 h</td>
<td>Nomikos et al. (2013) c,1</td>
</tr>
<tr>
<td>Roscovitine</td>
<td>Mouse</td>
<td></td>
<td>81.0%</td>
<td>10 µg/mL for 5–24 h (1 exposure)</td>
<td>2 µg/mL cytoD for 2 h</td>
<td>Phillips et al. (2002) a,1</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Mouse</td>
<td></td>
<td>80.0%</td>
<td>100 µg/mL for 7–8 h (1 exposure)</td>
<td>/</td>
<td>Balakier and Casper (1993) 3</td>
</tr>
<tr>
<td>TPEN</td>
<td>Mouse</td>
<td></td>
<td>&gt;20%</td>
<td>50 µM for 8 h (1 exposure)</td>
<td>Mouse sperm</td>
<td>Rogers et al. (2006) c,1</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td>&gt;20%</td>
<td>20 µg/mL for 4 h (1 exposure)</td>
<td>2 µg/mL cytoD for 2 h</td>
<td>Suzuki et al. (2010) a,1</td>
</tr>
</tbody>
</table>

Activation agents are classified based on the induced Ca²⁺ response. Activation rate is evaluated at day 1 after oocyte activation. For mouse studies: % 2-cell embryos. For human studies: % 2 pronuclei (pn) (*) Activation rate as % 1 pronuclei. Activation rate as % 2-cell embryos. Protocols describe (#1) concentration of the activation agent and time of exposure and repetitions, (#2) strategy used to maintain diploidy. Mouse strains: aB6D2F1, bOF1, cMF1, dCF1. Source of oocytes: 1 in vivo matured MII oocytes 2 IVF/ICSI failed to fertilize 3 in vitro matured MII oocytes. (nd) non available data.
**Ethanol**

Similarly, ethanol evokes a single Ca\(^{2+}\) transient, presumably caused by altered membrane fluidity followed by Ca\(^{2+}\) leakage through the membrane (Rybouchkin et al. 1996). As with Ca\(^{2+}\) ionophores, the ethanol-induced Ca\(^{2+}\) flux occurs during the time that ethanol is applied and recovers after ethanol washout.

The Ca\(^{2+}\) changes induced by Ca\(^{2+}\) ionophores or ethanol have not been reported to result in spontaneous dynamic Ca\(^{2+}\) oscillations. Interestingly, both stimuli have been demonstrated to activate mice and human oocytes leading to high rates of oocyte activation and blastocyst formation (Table 1). However, their competence in inducing oocyte activation and supporting blastocyst formation varies, particularly in humans, as described in several studies (Vanden Meerschaut et al. 2014b).

**Agents inducing oscillatory Ca\(^{2+}\) signaling**

**Thimerosal**

The sulfhydryl reagent thimerosal has been proposed as an activating agent given its capacity to induce Ca\(^{2+}\)-induced Ca\(^{2+}\) oscillations, as shown in mouse oocytes (Mehlmann & Kline 1994). Thimerosal induces fertilization-like oscillations by oxidizing protein thiol groups at the IP3R. As a consequence, a thimerosal-induced activation in human oocytes is observed (Swann 1991). Despite evoking Ca\(^{2+}\) sensitization of the receptor to cytosolic concentration protein thiol groups at the IP3R. As a consequence, a thimerosal-induced activation in human oocytes is observed (Swann 1991). Despite evoking Ca\(^{2+}\) sensitization of the receptor to cytosolic concentration.

The Ca\(^{2+}\) changes induced by Ca\(^{2+}\) ionophores or thimerosal have not been reported to result in spontaneous dynamic Ca\(^{2+}\) oscillations. Interestingly, both stimuli have been demonstrated to activate mice and human oocytes leading to high rates of oocyte activation and blastocyst formation (Table 1). However, their competence in inducing oocyte activation and supporting blastocyst formation varies, particularly in humans, as described in several studies (Vanden Meerschaut et al. 2014b).

**Recombinant phospholipase C \(\zeta\) (PLC\(\zeta\))**

The capacity of both, mouse and human PLC\(\zeta\) proteins to induce Ca\(^{2+}\) oscillations and subsequently oocyte activation have been demonstrated (Kouchi et al. 2004, Rogers et al. 2004). It is worth considering that, PLC\(\zeta\) being the physiological stimuli to induce oocyte activation, the Ca\(^{2+}\) response would be expected to result in viable embryos. In this regard, the use of PLC\(\zeta\) as an oocyte activation agent seems promising, particularly in cases where sperm is devoid of PLC\(\zeta\), such as in globozoospermia (Escoffier et al. 2015) or in patients carrying punctual mutations in PLC\(\zeta\) gene (Nomikos et al. 2013, Sanusi et al. 2015).

**Oocyte activation in absence of the initial Ca\(^{2+}\)-trigger**

Oocyte activation occurs as an immediate consequence of the complete degradation of the MPF, cdk1-cyclin b protein complex responsible for keeping the oocyte in meiotic arrest (Shoji et al. 2006). Therefore, designing an AOA approach requires targeting MPF degradation. As described earlier, numerous strategies are based on promoting intracellular Ca\(^{2+}\) changes to prime the initiation of the oocyte activation. However, several approaches have demonstrated their capacity to disrupt MPF function in the absence of the initial Ca\(^{2+}\) trigger. CAMKII cRNA allowed oocyte activation in mouse (Knott et al. 2006), supporting further embryonic development. Further downstream of CaMKII activation, a prominent zinc ion (Zn\(^{2+}\)) efflux, known as the Zn\(^{2+}\) spark, occurs during the course of oocyte activation, particularly after Emi2 inactivation (Bernhardt et al. 2011). This
Zn$^{2+}$ spark is required for cyclin B degradation and the subsequent meiotic release (Suzuki et al. 2010). Artificial Zn$^{2+}$ sequestration, bypassing the Ca$^{2+}$ mobilization and mediated by the specific Zn$^{2+}$ chelator N,N,N',N'-tetrakis (2-pyridylmethyl)ethane-1,2-diamine (TPEN), allows oocyte activation in several mammalian species, including human (Suzuki et al. 2010, Lee et al. 2015, Duncan et al. 2016). In addition, full-term embryonic development was achieved in mice and pigs by using TPEN as the only agent for oocyte activation. Moreover, different protein synthesis inhibitors such as puromycin (De Sutter et al. 1992, Lu et al. 2006) and cycloheximide (Moses & Kline 1995, Rogers et al. 2006) have been shown to alleviate meiotic arrest by interrupting the continuous synthesis of cyclin B. Roscovitine, in turn, was shown to induce the meiotic release by direct inhibition of the cdk1 function (Phillips et al. 2002).

**AOA: clinical applications**

AOA is a prerequisite to perform certain methodologies such as somatic cell nuclear transfer (SCNT) (Campbell et al. 2007) or parthenogenesis. However, AOA is mainly used as a complementary ART procedure to overcome fertilization failure after ICSI (Heindryckx et al. 2008, Vanden Meerschaut et al. 2012), and the application of Ca$^{2+}$ ionophores is the most widely used method. Moreover, cases experiencing recurrent molar pregnancies have been identified as a population that could benefit from AOA application, as suggested in a recent study describing aberrant Ca$^{2+}$ patterns upon in vitro fertilization by sperm involved in partial hydatidiform moles (Nikiforaki et al. 2014a). In these cases, Ca$^{2+}$ ionophores might restore the lack of a normal sperm-induced Ca$^{2+}$ response and provide the oocyte with sufficient Ca$^{2+}$ for the accomplishment of cellular events associated with the process of oocyte activation, in particular granule exocytosis to block polyspermy (Ducibella et al. 2002). AOA has been recently proposed as a strategy to improve embryonic development in cases experiencing pronuclear and embryonic arrest (Darwish & Magdi 2015, Ebner et al. 2015). This application is supported by several findings demonstrating that oocyte activation and subsequent embryonic development might be mediated by the total summation of Ca$^{2+}$ spikes during the oscillation period (Ozil et al. 2005, Tóth et al. 2006). The benefit of AOA in restoring fertilization rates is more evident in cases with sperm activation deficiencies. However, it is worth noting that few of the cases studied thus far did not benefit from the treatment (Vanden Meerschaut et al. 2012), possibly due to an underlying oocyte quality issue. In this respect, methods intended to overcome the oocyte-related fertilization failure after ICSI remain to be further explored (Yeste et al. 2016). Moreover, AOA strategies remarkably differ between IVF centers. As previously suggested by our group, the variability observed among protocols confounds the comparison of the efficiency of these methods (reviewed by Vanden Meerschaut et al. 2013). Therefore, randomized comparative studies would be of benefit to select the most appropriate AOA strategy, contributing in turn to a robust standardization of the method. In this respect, a recent study compared Ca$^{2+}$ release after exposing mouse and human oocytes to the Ca$^{2+}$ ionophores ionomycin and calcimycin (Nikiforaki et al. 2016). Ionomycin induced higher amplitudes of the Ca$^{2+}$ transient than calcimycin. As a result, ionomycin also led to significantly higher activation rates. Moreover, ionomycin resulted in higher blastocyst formation rates as evaluated in mouse. It is worth noting that calcimycin failed to activate oocytes pre-injected with mouse wobbler sperm, an animal model used for the study of globozoospermia (Heytens et al. 2010).

Mice are a popular translation model for human oocyte activation and AOA protocols and are commonly used to study the mechanisms involved in oocyte activation in mammals. However, other species such as the horse could closer reflect the fertilization events observed in humans, given the similarity of equine and human Ca$^{2+}$ signatures observed during oocyte activation (Leemans et al. 2015).

**Limitations and benefits of AOA strategies**

The oocyte, unlike other cell types in which the initiation of diverse cellular pathways occurs in response to a certain frequency of Ca$^{2+}$ oscillations, is capable of integrating different types of Ca$^{2+}$ signatures to successfully resume the cell cycle (Dupont et al. 2010). Methodologies that evade dynamic Ca$^{2+}$ signatures (e.g. Ca$^{2+}$ ionophores) have been shown to activate mammalian oocytes and consequently, to stimulate the embryonic development, merely by inducing single Ca$^{2+}$ transients (Ozil 1998). However, Ca$^{2+}$ oscillations are essential to completely inactivate MPF as well as to impede its rebound (Ducibella et al. 2002, Tóth et al. 2006). Early and late embryo developmental events occurring during the process of oocyte activation, such as cortical granules exocytosis or the maternal mRNAs recruitment, are stimulated by a different number of Ca$^{2+}$ transients (Ducibella et al. 2002). As a result, these cellular events require a lower number of Ca$^{2+}$ oscillations for their initiation (e.g. 4 or 8 transients) than for their completion (e.g. 24 transients). Moreover, the impact of Ca$^{2+}$ signature patterns on the subsequent embryonic development, ranging from the first embryonic interphase at the pronuclear stage to the blastulation, has been repeatedly described (Ozil et al. 2005, 2006, Tóth et al. 2006). Manipulating the Ca$^{2+}$ oscillatory pattern during oocyte activation did not impair the activation or the blastocyst formation rates in mouse. However, when an early cessation of Ca$^{2+}$ oscillations was provoked, gene expression profiles showed that preimplantation potential was
Ca\textsuperscript{2+} responses and assisted oocyte activation

Ca\textsuperscript{2+} responses and assisted oocyte activation (Ozil et al. 2006). Furthermore, blastocysts resulting from a Ca\textsuperscript{2+} hyperstimulation revealed gene expression profiles associated with post-implantation failure (Ozil et al. 2006). The impact of the absence of an initial Ca\textsuperscript{2+} trigger to induce oocyte activation has also been evaluated. Oocytes stimulated by agents such as cycloheximide (Rogers et al. 2006) or TPEN (Suzuki et al. 2010) showed normal activation rates, in the absence of Ca\textsuperscript{2+} rises. However, their efficiency in supporting subsequent embryonic development to blastocyst stage was remarkably low (Fig. 2). Interestingly, the additional application of a treatment that promotes cytoplasmic Ca\textsuperscript{2+} increase such as ethanol that causes single Ca\textsuperscript{2+} transients, or SrCl\textsubscript{2}, that causes Ca\textsuperscript{2+} oscillations, restored embryonic development rates to normal (Rogers et al. 2006). These findings highlight the prominent role of Ca\textsuperscript{2+} in fertilization and particularly in supporting embryonic development (Fig. 2). Following the hypothesis in which the total amount of Ca\textsuperscript{2+} released would determine for a minimum threshold required to achieve oocyte activation (Ozil et al. 2005, Tóth et al. 2006), it is not difficult to understand that the single Ca\textsuperscript{2+} transients induced by Ca\textsuperscript{2+} ionophores are sufficient to trigger oocyte activation. In support, a predictive minimal mathematical model reflected the capacity of single Ca\textsuperscript{2+} transients to induce meiotic alleviation in mammals (Dupont et al. 2010).

As described earlier, Ca\textsuperscript{2+} ionophores use is the most common AOA strategy used in ART. Besides the substantial cellular stress that Ca\textsuperscript{2+} ionophores might exert by altering membrane permeability, safety concerns remain regarding the induction of a single Ca\textsuperscript{2+} transient which differs remarkably from the physiological oscillatory Ca\textsuperscript{2+} response (Santella & Dale 2015, van Blerkom et al. 2015). Accumulating evidence supports the biosafety of ionomycin as an activating agent. First, high oocyte survival rates are observed following AOA in mouse (Heytens et al. 2008) and in human (Heindryckx et al. 2008) oocytes. Moreover, ionomycin did not increase the incidence of meiotic errors of maternal origin in human oocytes (Capalbo et al. 2016). Most importantly, the follow-up studies of children born after AOA support the safety of this methodology (D’haeseleer et al. 2014, Vanden Meerschaut et al. 2014a, Miller et al. 2016). Together, these data endorse the readiness of AOA for clinical applications (Ebner & Montag 2016). However, defining a proper indication requires further investigation (van Blerkom et al. 2015). Diagnostic tools to identify cases that could benefit from AOA are needed to help clinicians give appropriate medical advice. To this end, heterologous ICSI was introduced as a valuable approach to determine whether an oocyte-related or sperm-related factor is causing the fertilization failure (Rybouchkin et al. 1995, Heindryckx et al. 2005). Accordingly, the mouse oocyte activation test (MOAT) is an efficient assay to reveal the presence of sperm-related activation deficiencies. The MOAT is used for patients experiencing failed fertilization after ICSI and involves injection of human sperm into mouse oocytes, to classify the sperm activation capacity.

![Figure 2](https://www.reproduction-online.org)  
**Figure 2** Schematic diagram of impact of Ca\textsuperscript{2+} signature during AOA on blastocyst formation rate. Data represent the efficiency of different AOA strategies to support blastocyst formation compared to in vivo-fertilized oocytes (further cultured in vitro after zygote collection). AOA strategies that supply the oocyte with Ca\textsuperscript{2+} during activation reflect similar capacity to support blastocyst formation than oocytes that were fertilized in vivo. This capacity is affected by the absence of Ca\textsuperscript{2+} during activation, as observed in studies that used TPEN and cycloheximide as activation factors. The figure has been designed based on available data and includes only mouse studies. Normalization has been performed based on the blastocyst formation rate (%) observed after in vivo fertilization. Reference data were obtained from studies that used the same mouse strain and, where possible, were performed in the same lab. Experiments with MF1 mice (light gray), are normalized to blastocyst formation rate obtained by Saunders et al. (2002), which was 87%. Experiments with B6D2F1 mice (dark gray) are normalized to blastocyst formation rate obtained by Neupane et al. (2014), which was 91%. Only blastocyst formation rate reported after the use of TPEN was obtained in same mouse strain (B6D2F1) but in a different lab.
into one of three categories (Heindryckx et al. 2005). The activation capacity is defined by observing the percentage of 2-cell embryos 24 h after ICSI and results are compared to a fertile control. MOAT groups range from low-to-high activation potential. MOAT group 1 (0–20% of 2-cell embryos 24 h after ICSI) includes patients with a sperm-related activation deficiency, such as cases with globozoospermia (Heytens et al. 2010) or carriers of mutations in the PLCζ gene (Heytens et al. 2009, Kashir et al. 2012). MOAT 2 (21–84%) includes patients showing diminished activation capacity and MOAT 3 (>85%) includes patients indicating a normal activation capacity, comparable to a positive control and consequently pointing more to an oocyte-related activation deficiency (Heindryckx et al. 2005). Additionally, further investigation revealed an interesting correlation between the Ca2+ oscillatory patterns and the MOAT groups. The outcome obtained by the mouse oocyte calcium analysis (MOCA) defined a finer classification on the human sperm activation potential and highlighted the importance of studying Ca2+ patterns in cases experiencing ICSI failures. Both heterologous tests, MOAT and MOCA, are valuable approaches to use for in medical counseling (Vanden Meerschaut et al. 2013). Considering that activation potential is directly related to PLCζ properties (Nomikos et al. 2014a), the MOAT and MOCA tests can be considered as useful tools for evaluating PLCζ functionality. However, it is worth noting that there are interspecies differences, since human PLCζ shows greater Ca2+ oscillation-inducing potency than the mouse PLCζ in conspecific oocytes (Cox et al. 2002, Nomikos et al. 2014b). Performing Ca2+ analysis using human oocytes to measure human sperm activation capacity has been proposed as an add-on methodology to the heterologous tests (Ferrer-Buitrago 2016). However, the scarcity of human oocytes donated for research purposes is a major limitation.

**Conclusion**

The process of oocyte activation is firmly associated with a specialized Ca2+ signal, which occurs as single Ca2+ transients or Ca2+ oscillations depending on the species (Stricker 1999). The prominent role of the oocyte’s Ca2+ machinery in modulating the Ca2+ signature is clear from *Xenopus* sperm extract studies, which provoke a single Ca2+ transient in *Xenopus* oocytes but induced Ca2+ oscillations in mouse oocytes (Dong et al. 2000). In this regard, the regulation of the oocyte’s machinery plays a crucial role in modulating Ca2+ profile in preparation to the egg-to-embryo transition. Moreover, mammalian oocytes can respond to artificially induced single Ca2+ transients or strategies, which mediate meiotic alleviation in the absence of the initial Ca2+ trigger, both resulting in oocyte activation (Table 1). This is probably because molecular events, in particular, the activation of effectors such as CAMKII, occur in response to a minimum threshold of Ca2+ levels (Ozil et al. 2005, Tóth et al. 2006). Moreover, MPF degradation and subsequent meiotic alleviation can occur bypassing the increase of Ca2+ or presence of oscillations, in response to the destabilization of proteins responsible of the meiotic arrest. However, the use of strategies that induce Ca2+ oscillatory responses probably would show further benefits as they would more closely mimic the physiological situation. The importance of Ca2+ oscillations in achieving high blastocyst formation rates have been demonstrated in certain mammalian species (Ozil 1998). Moreover, specific Ca2+ oscillatory patterns have an impact on events required for full-term mammalian embryonic development, both early (e.g. oocyte activation) (Ducibella et al. 2002, Tóth et al. 2006, Ducibella & Fissore 2008) and late (e.g. post-implantation viability) (Ozil et al. 2006). Methods to intervene more accurately the frequency of Ca2+ oscillations would allow discerning whether the presence of Ca2+ oscillations has additional impact in cellular processes occurring during the egg-to-embryo transition, such as the late events of parental chromatin remodeling: chromatin decondensation within the male pronucleus and the import of nuclear proteins (McLay & Clarke 2003). Despite the benefits reported on the presence of Ca2+ oscillations for subsequent embryonic development, Ca2+ oscillatory responses are not regularly used for clinical applications in the human. In this regard, Ca2+ ionophores, inducing single Ca2+ transients, are nowadays mostly used for AOA in ART, in particular to overcome fertilization failure after ICSI. To our knowledge and experience, the use of Ca2+ ionophores is the most efficient AOA strategy currently known for application in the human. Accumulating evidence supports their efficiency in achieving embryonic development and pregnancies to term. However, developing strategies to artificially induce Ca2+ oscillatory activity in human oocytes are urgently needed. Given the inconsistency of the data reported after the use of Sr2+ in human, the intracytoplasmic injection of human PLCζ represents a promising AOA approach to achieve a more physiological Ca2+ response, which may have substantial clinical impact (Swann & Lai 2016). Mouse studies have reported on the efficiency of PLCζ cRNA to induce oocyte activation (Cox et al. 2002, Yu et al. 2008). The minimum concentration of human PLCζ associated with mouse oocyte activation was calculated as 1 fg after the injection of a solution containing human PLCζ cRNA concentrations of 0.05–0.05 µg/µL (Yu et al. 2008). Moreover, the direct injection of 80 fg purified recombinant human PLCζ protein showed its potential to rescue oocyte activation failure in mouse oocytes pre-injected with cRNA encoding for mutant forms of PLCζ (Nomikos et al. 2013). Although oocyte activation occurs over a wide range of PLCζ levels, embryonic development is supported within a narrow window of protein concentrations (Yu et al. 2008), in correlation...
with the total number of Ca\(^{2+}\) oscillations observed during oocyte activation. The study performed by Yu and coworkers (Yu et al. 2008) evaluated mouse blastocyst formation potential in association with PLC\(_{ζ}\) levels expressed as counts of luminescence per second (cps). With 1 cps corresponding to \(-250\) fg, higher blastocyst formation rates were achieved within the range of 0.12–2.5 cps, thus from 30 to 625 fg of human PLC\(_{ζ}\). Of note, low and high levels of PLC\(_{ζ}\) were associated with low oocyte activation potential and embryo development arrest, respectively (Yu et al. 2008). Moreover, the direct injection of recombinant human PLC\(_{ζ}\) at a concentration of 80 fg allowed mouse blastocyst formation in a proposed prototype for male infertility based on failed fertilization observed after the injection of PLC\(_{ζ}\) cRNA encoding known point mutations (Kashir et al. 2012, Nomikos et al. 2013). A definite protocol describing whether exogenous PLC\(_{ζ}\) would induce a real benefit for human oocyte activation and embryo development has not been yet established. However, the activation capacity of human PLC\(_{ζ}\) cRNA (Rogers et al. 2004, Nomikos et al. 2013) has also been demonstrated in human oocytes. As observed in mouse studies, although oocyte activation is associated with a broad range of PLC\(_{ζ}\) cRNA concentrations (10–0.1 \(\mu\)g/ml), higher blastocyst formation rates were obtained after the injection of PLC\(_{ζ}\) cRNA at the lowest concentration (0.1 \(\mu\)g/ml) (Rogers et al. 2004). Further research is required to standardize this methodology and evaluate long-term safety in offspring.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Authors’ contribution statement

M F B and D B were responsible for the literature search and making the table. M F B was responsible for making the figures. M F B, D B, L L and B H were responsible for writing of the manuscript. P D S, L L and B H were responsible for the supervision and the approval of this manuscript.

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