The role of Ca^{2+} in oocyte activation during *In Vitro* Fertilization: insights into potential therapies for rescuing failed fertilization.

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Abbreviations: ICSI, intracytoplasmic sperm injection; PLCζ, phospholipase Czeta; CDK1, cyclin dependent kinase 1; MPF, maturation promoting factor.

Abstract

At fertilization the mature mammalian oocyte is activated to begin development by a sperm-induced series of increases in the cytosolic free Ca^{2+} concentration. These so called Ca^{2+} oscillations, or repetitive Ca^{2+}, are also seen after intracytoplasmic sperm injection (ICSI) and are primarily triggered by a sperm protein called phospholipase Czeta (PLCζ). Whilst ICSI is generally an effective way to fertilizing human oocytes, there are cases where oocyte activation fails to occur after sperm injection. Many such cases appear to be associated with a PLCζ deficiency. Some IVF clinics are now attempting to rescue such cases of failed fertilization by using artificial means of oocyte activation such as the application of Ca^{2+} ionophores. This review presents the scientific background for these therapies and also considers ways to improve artificial oocyte activation after failed fertilization.

1. Introduction

At fertilization in mammals, the sperm initiates a series of events that activates the development of the mature metaphase II-arrested oocyte into a zygote and early embryo. In metaphase II mammalian oocytes (often just called 'eggs') there are a distinctive series of transient rises in free cytosolic Ca^{2+} that persist for several hours after sperm oocyte interaction [1]. These rises are referred to as Ca^{2+} oscillations, or repetitive Ca^{2+} spikes (see Fig.1). The Ca^{2+} oscillations activate the oocyte and hold the key to understanding how the sperm starts embryo development. We know a considerable amount about how the sperm generates the Ca^{2+} transients and the role of Ca^{2+} in oocyte activation [1-3]. This knowledge has the potential to have a direct clinical benefit, but this has yet to be fully realised. In this review I outline our knowledge of Ca^{2+} in oocyte activation at fertilization in mammals, with a particular note to studies on human oocytes. I will then consider the scientific basis for the use of treatments that attempt to promote oocyte activation as part of human IVF treatment.

In mammals the ovulated oocyte is arrested before fertilization at the second metaphase of meiosis (MII). The activation of the oocyte at fertilization involves the resumption of meiosis as indicated by a metaphase to anaphase transition, and the emission of a second polar body [3]. This occurs about an hour after sperm oocyte fusion in the mouse. The formation of two pronuclei (one from the sperm and one

from the retained set of maternal chromosomes) represents the completion of meiosis and entry into the first interphase of the zygotic cell cycle [2,3]. This occurs several hours after second polar body emission. As well as meiotic resumption, mammalian oocyte activation involves the exocytosis of cortical granules which lead to modification of the zona pellucida to prevent sperm penetration [2]. Both of these key events are physiologically triggered by increases in cytosolic Ca^{2+} which stimulate calmodulin-dependent kinases, or myosin light chain kinases [1-3]. The step initiating meiotic resumption is the Ca^{2+}-dependent inactivation of cyclin B and its cyclin dependent kinase (CDK1) activity in the oocyte, which then leads to a release from meiotic metaphase arrest [3]. If CDK1 activity is kept low, the oocyte will complete meiosis and enter interphase as indicated by the formation of pronuclei. For the purposes of this review, oocyte activation will imply the completion of meiosis, hence second polar body emission and pronuclear formation. If pronuclear formation is not the end point of 'oocyte activation' it will be specifically stated.

2. The pattern of Ca^{2+} increases in mammalian oocytes during fertilization.

Studies in the 1970s first showed that a single transient cytosolic Ca^{2+} increase occurred during activation at fertilization in sea urchin and fish eggs and oocytes. Studies on mammalian oocytes were not far behind. A series of oscillatory increases in cytosolic free Ca^{2+} were first demonstrated in mouse oocytes during fertilization using the photoprotein aequorin [4]. In hamster oocytes it was also shown that fertilization induces a series of Ca^{2+} oscillations that can be readily monitored by Ca^{2+} activated membrane potential hyperpolarizations which occur in synchrony with Ca^{2+} increases [1,5]. Thanks to use of Ca^{2+}-sensitive fluorescent dyes, we now know that Ca^{2+} oscillations also occur at fertilization in cow, pig, and rat oocytes [1,2,6]. In addition, it has been shown that cytosolic Ca^{2+} oscillations also occur in human oocytes during *in vitro* fertilization, IVF [7]. The Ca^{2+} oscillations in fertilizing mammalian oocytes are distinctive in being of low frequency with one Ca^{2+} 'spike' occurring about every 10 minutes, as in the mouse oocytes, or about one spike once every 30 to 60 mins, as in human, pig or cow oocytes. The initial Ca^{2+} increase at fertilization in the mouse is seen a few minutes after sperm-oocyte fusion and travels as a wave crossing the oocyte in about 10 seconds [1,8]. However, the subsequent Ca^{2+} spikes occur as very rapid waves of Ca^{2+} release that cross the oocyte in about

1 second [8]. Hence, Ca^{2+} oscillations in mammalian oocytes are distinctive in that they have very prolonged intervals between spikes, and yet each Ca^{2+} increase is initiated by a fast wave of >50 μm/sec.

The mechanism of Ca^{2+} oscillations in mammalian oocytes involves the production of inositol (1,4,5) trisphosphate production ($InsP_3$). This leads to the stimulation of Ca^{2+} release via $InsP_3$ receptors (IP_3Rs). The primary stimulus for $InsP_3$ appears to be the sperm specific phospholipase Czeta (PLCζ) which can enter the oocyte after gamete fusion [1,2,6]. Microinjecting PLCζ RNA into oocytes causes Ca^{2+} oscillations very similar to fertilization in mouse and humans [9,10]. The existence of PLCζ inside the sperm head can also explain the success of intracytoplasmic sperm injection (ICSI) in mice [11]. With ICSI, a whole sperm is first 'damaged' slightly with the injection pipette and then injected into the oocyte which then activates and can develop to term. ICSI also causes Ca^{2+} oscillations similar to those seen at fertilization in both mouse and human oocytes and the 'damaging' of the sperm promotes the occurrence of oscillations presumably by facilitating the release of PLCζ [12-14].

There is substantial evidence that PLCζ is physiologically significant in causing the Ca^{2+} oscillations at fertilization. In mouse it has been shown to be present in sperm in the post-acrosomal region, which is the first part of the sperm to contact the oocyte cytosol [1,2,6]. PLCζ is also present in an amount that is able to cause a normal pattern of Ca^{2+} oscillations at fertilization [9]. Recently the phenotype of PLCζ knock-out mice was reported by two independent groups. In both studies it was found that sperm lacking a functional PLCζ protein were unable to cause Ca^{2+} oscillations after ICSI [15,16]. Hence PLCζ for ICSI is synonymous with so-called SOAF (sperm-borne oocyte activating factor) that was characterized using ICSI [11]. However, both studies using different types of PLCζ knock out mice, also found that males can still produce offspring, albeit of much reduced litter size [15,16]. In addition, it was found that in IVF sperm lacking PLCζ can still give rise to about 70% oocyte activation with very much delayed pronuclear formation and a high degree of polyspermy. Interestingly, a limited number of Ca^{2+} oscillations, which were delayed by an hour after gamete fusion, can be seen in oocytes fertilized by PLCζ KO sperm [16]. This suggests that the sperm may have an alternative factor that can cause Ca^{2+} release in oocytes. For reasons that are unclear this factor is not active in ICSI,

but is active when freshly prepared sperm fertilize oocytes in IVF. The nature of the alternative factor remains to be determined. It does not appear to be as potent as PLCζ in causing Ca^{2+} release. Since its effects are delayed it is still true to say that PLCζ is the primary stimulus for oocyte activation. Nevertheless, this secondary factor may represent a novel agent for triggering Ca^{2+} release in oocytes. Its existence in humans remains unknown.

It is worth noting that PLCζ is the only sperm specific protein that has been reproducibly shown to cause Ca^{2+} oscillations in mammalian oocytes. It was reported in 2015 that a protein called PAWP (post acrosomal WW-domain binding protein) was able to cause Ca^{2+} oscillations in mouse and human oocytes [17]. The authors of this study suggest that PAWP might be sperm factor active in IVF and ICSI [17]. However, the key findings in this paper are not reproducible [18]. Sperm from PAWP knock-out male mice also cause completely normal Ca^{2+} oscillations after ICSI and *in vitro* fertilization, so a role of PAWP in oocyte activation seems highly unlikely [19]. It is clearly not the secondary sperm factor.

3. The importance of Ca^{2+} oscillations in activation of mammalian oocytes.

It was first shown in the 1970s that oocytes from hamsters can be stimulated to activate by applying the Ca^{2+} ionophore A23187 [20]. These initial studies showed that A23187 works in a variety of eggs and oocytes by releasing Ca^{2+} from intracellular stores [20,21]. Subsequently it was shown that direct injection of Ca^{2+} ions also activates mouse oocytes, and in this case the parthenogenetic embryos were shown to develop to the blastocyst stage [22]. It was later shown that Ca^{2+} ionophore A23187 can also activate human oocytes [23]. Hence Ca^{2+} influx by injection, or Ca^{2+} release by A23187 act as parthenogenetic stimuli. However, studies on human oocytes were inconsistent because other groups found that the Ca^{2+} ionophore, ionomycin, which acts like A23187 and causes a single large Ca^{2+} rise, did not lead to oocyte activation [24]. Another report also found that A23187 was ineffective in activating human oocytes [25]. Instead it was found that the protein synthesis inhibitor puromycin could activate human oocytes [25]. So, from the earliest studies the effectiveness of ionophore in human oocyte activation appeared variable.

The conflicting data on human oocytes is not surprising since many studies have found that Ca^{2+} ionophores are not very effective when used on their own as a parthenogenetic stimulus in mammals. To activate oocytes, ionophores are combined with a protein synthesis inhibitor, or a protein kinase inhibitor such as 6-dimethyl aminopurine [26]. Such double treatments is standard practice for parthenogenetic oocyte activation in domestic animals and is commonly used as an oocyte activation method after nuclear transfer. The reason there is a need for two stimuli appears to be the dynamic nature of cyclin B synthesis. The activity of CDK1 holds the oocyte in meiotic arrest and CDK1 activity depends on continuous cyclin B synthesis [3]. A single Ca^{2+} increase leads to proteolysis of cyclin B and a reduction in the CDK1 activity that promotes meiotic resumption [27]. However, if there is only a single Ca^{2+} rise cyclin B is resynthesized and CDK1 activity returns, leading to oocyte re-arrest. The return of CDK1 activity is prevented by inhibiting protein kinases, or protein synthesis, which explains why activation is most effective with ionophore plus a protein kinase or protein synthesis inhibitor [3,27]. It's worth noting here that the very first study on the effect of ionophores in hamster oocytes found that A23187 was more effective in activating oocytes with a longer incubation in Ca^{2+} free media [20]. This is probably because long incubations in Ca^{2+} free media leads to inhibition of protein synthesis and hence this protocol effectively provides a double stimulus [28].

An issue that became clear in these early studies is that most parthenogenetic activation agents do not mimic the response at fertilization. For example, ethanol (7% in culture media) that was once commonly used to activate mouse oocytes causes just one very large increase in Ca^{2+} in the oocyte [29]. As noted above, Ca^{2+} ionophores also only cause a single Ca^{2+} increase [29]. Exposing oocytes to high intensity electric fields (electroporation) in the presence of Ca^{2+} can also lead to a large rise in Ca^{2+}, due to Ca^{2+} influx across the plasma membrane. However, again this only causes a single large Ca^{2+} increase [29,30] (see Fig.2). Hence causing artificial Ca^{2+} influx, or artificial Ca^{2+} release, in mammalian oocytes does not generally trigger the Ca^{2+} oscillations as seen at fertilization [29].

The first parthenogenetic stimulus shown to cause Ca^{2+} oscillations in mouse oocytes is incubation in medium where Sr^{2+} replaces the Ca^{2+} [31]. Ca^{2+} oscillations occur for as long as Sr^{2+} is present, which is typically for 2 hours in order to activate

mouse oocytes [31]. Significantly, Sr^{2+} media alone is an effective activator of mouse oocytes, even with 'freshly ovulated' oocytes that usually show a poor response to parthenogenetic stimuli [28]. Sr^{2+} is also effective in stimulating development to the blastocyst stage after failed fertilization with ICSI. For example, if sperm are heat treated, or taken from a mutant mice, and used in ICSI, the activation and development rates are very low [32,33]. These oocytes that have failed to fertilize after ICSI can be 'rescued' by treatment with Ca^{2+} ionophore, but a 'rescue' with Sr^{2+} medium is better at inducing activation and development to blastocyst. These data imply that the Ca^{2+} oscillations induced by Sr^{2+} are a more effective way of stimulating early development than the single large Ca^{2+} increase induced by ionophore. Unfortunately, these studies with Sr^{2+} have been restricted to mice because Sr^{2+} only appears to cause Ca^{2+} oscillations in rodent oocytes (see later).

Whilst a single electroporation pulse causes a singular large Ca^{2+} rise in mammalian oocytes, multiple electroporation pulses can be used to cause more than one Ca^{2+} transient [30]. In order to do this the oocytes are placed between two electrodes in a medium of low ionic strength, such as 300mM glucose, to which is added a small amount (50μM) of $CaCl_2$ [30]. A brief voltage pulse is then applied across the electrodes. It is worth noting that if this is done in a standard electroporation device with a static chamber, multiple electroporation pulse leads to oocyte lysis. However, if oocytes are instead placed in a rapid perfusion microfluidic device, and then after each electroporation the oocyte is rapidly perfused (~200ms) with culture media, oocyte integrity is preserved. If the procedure of electroporation and rapid perfusion is repeated then a series of Ca^{2+} rises of predefined amplitude and duration can be induced [30]. With regards to understanding the Ca^{2+} oscillations, this type of electrical stimulation method has been used to show that multiple Ca^{2+} rises are required to bring down both the CDK1 and MAP kinase activity for long enough to allow the zygote to enter interphase and form pronuclei [34]. It has also been shown that the pattern of electroporation-induced Ca^{2+} increases also influences the timing of activation events such as pronuclear formation in mice [30].

This rapid perfusion electroporation device has also been used on fertilized animal oocytes (i.e. zygotes). In rabbit zygotes it was also found that providing extra Ca^{2+} pulses increased implantation rates [35]. In mice it was further shown that varying the number of Ca^{2+} spikes from 2 or 3 spikes (spontaneous) to over 20 Ca^{2+} spikes

(imposed by electroporation), leads to different rates of implantation compared to control embryos [35]. The main conclusion from these variations is that either too few or too many Ca^{2+} spikes, compared to IVF, resulted in fewer pregnancies [36]. These data clearly suggest that Ca^{2+} oscillations can affect later development of embryos as well as oocyte activation. It also implies that there is an optimum stimulus range for later development and implantation.

Microinjecting PLCζ RNA or PLCζ recombinant protein can also cause robust Ca^{2+} oscillations in mouse, pig, cow and human oocytes [1,6, 9,10,37-39]. It leads to high rates of oocyte activation and development to blastocysts. When it is used toa 'rescue' oocyte activation after ICSI with heat-treated sperm, it is as effective as Sr^{2+} in triggering development to blastocyst stages [32]. This reinforces the idea that it is the Ca^{2+} oscillations that are key to developmental success. With PLCζ injection into mouse or cow oocytes it has also been found that, whilst low frequency Ca^{2+} spikes supported development to the blastocyst stage, injecting higher amounts of PLCζ RNA causes a high frequency of Ca^{2+} oscillations that are associated with embryo arrest during cleavage stages [38,40]. It clearly suggests that high frequency Ca^{2+} oscillations inhibit pre-implantation embryo development.

Taken together these various studies have shown that stimulating oocytes with a very large Ca^{2+} increase can be effective in activating mammalian oocytes. However, the more general finding is that a single large Ca^{2+} increase that lasts typically less than 10 minutes only activates a fraction of a cohort of mammalian oocytes. Parthenogenetic stimuli such as Ca^{2+} ionophores are also found to be more effective when used on oocytes that are aged *in vitro* after ovulation, probably because cyclin B levels decline with age *in vitro* [3,28,29]. The most consistent way to activate oocytes and obtain good later development is to induce multiple, low frequency Ca^{2+} oscillations that, perhaps not surprisingly, mimic the Ca^{2+} response seen at fertilization. In human oocytes it is likely that about 10 to 20 Ca^{2+} transients at intervals in the range 30-60 minutes is the type of physiological pattern associated with successful development [7,14].

4. **Human IVF and failed oocyte activation.**

In human IVF or ICSI, oocytes or zygotes are examined about 16 to 18 hours later for signs of pronuclear formation. The lack of pronuclei is a sign of failed fertilization

and still occurs to some extent in all IVF clinics [41]. ICSI is now widely used as a means to overcome many cases of fertilization failure after conventional IVF, since the sperm is directly placed in the oocyte cytoplasm [42]. ICSI is used in a wide variety of cases such as those where there is a low sperm count, or where oocytes are cryopreserved. I shall concentrate on failed fertilization after ICSI since it is clear that the sperm is within the oocyte, and that any lack of fertilization is due to factors associated with the sperm processing or oocyte activation. The major cause of failed fertilization after ICSI appears to be failed oocyte activation [41-44]. There are two classes of failed fertilization in ICSI that may both be related to a lack of a Ca^{2+} signal. One is widely recognized, the other more insidious.

The widely recognized fertilization failure after ICSI is referred to as total fertilization failure which means that all the oocytes collected in a treatment cycle fail to form pronuclei after injection of sperm. It is reported to occur in 1-4% of ICSI treatment cycles and these cases are generally associated with failed, or abortive oocyte activation [41-44]. There are numerous reports showing that failed fertilization after ICSI is associated with either a relative lack of PLCζ in the sperm, or otherwise with mutations in PLCζ that affects its activity in oocytes [45,46]. In some cases the human sperm has been shown to be deficient in its ability to cause Ca^{2+} oscillations after ICSI in mouse oocytes, which can be used as an effective bioassay of the sperm Ca^{2+} releasing ability [43,45]. There are also a number of cases reported where a mutation in PLCζ has been identified in men with repeated failed ICSI. In these cases the PLCζ mutation has been shown to inhibit PLCζ's enzyme activity [46]. In one particularly notable case, two brothers were shown to have a homozygous mutation in the C2 domain of PLCζ [47]. This mutation disrupts PLCζ activity and reduces its expression in sperm. Whole genome sequencing showed that this was the only mutation of a known gene predicted to be disruptive of a protein's structure. This suggests that a mutation in PLCζ alone in humans can cause male factor infertility. Other pronounced cases where PLCζ is lacking is in globozoospermia where the sperm lacks an acrosome and many other structures around the sperm head [43,46]. Many cases of globozoospermia are associated with a mutation in the Dpy1912 gene. In a mouse model where the Dpy1912 gene is deleted, the sperm are shown to lack PLCζ in the sperm head, and sperm from males fail to activate oocytes [48]. Again this suggests that a lack of PLCζ can play a

causal role in fertilization failure after ICSI. PLCζ deficiency in human sperm could account for many cases of total fertilization failure in human ICSI.

The second and more insidious type of failed fertilization occurs during routine clinical ICSI. With ICSI the typical fertilization rates for a group of oocytes are around 70% [41]. This is true even with morphologically normal sperm and oocytes [49]. Hence on average about 30% of human oocytes do not fertilize after ICSI. This represents a recurrent failure rate per oocyte, which is distinct from the total fertilization failure issue [41]. This may not be critical for generating embryos if there are 10 or more oocytes collected in a cycle, but it becomes significant if fewer oocytes are ovulated. It is also obvious that IVF clinics would prefer it if more oocytes would fertilize after ICSI since this would give more embryos to select for embryo transfer.

There are indications that one of the problems with this recurrent fertilization failure is a lack of an adequate activation stimulus. For example, human sperm have been assessed for their ability to cause Ca^{2+} oscillations in oocytes after ICSI [14,50]. In cases of male factor infertility with total fertilization failure, the sperm generally fail to cause any Ca^{2+} oscillations. What is intriguing is that even sperm from control 'fertile' males are still variable. In a recent study, about 70% of control sperm were shown to cause more than 3 Ca^{2+} spikes in human oocytes [14]. With about 20% of sperm there were only 1 or 2 spikes, which is unlikely to activate oocytes, and 10% of control sperm did not cause any Ca^{2+} spikes at all. Hence, 30% of control sperm are not very effective at stimulating effective Ca^{2+} signals in human oocytes. Even with the sperm that did cause multiple Ca^{2+} spikes only 50% (overall) could be induced more than 10 Ca^{2+} spikes [14]. Hence, overall these data show that the PLCζ activity levels, or at least the ability of normal human sperm to cause Ca^{2+} signals are likely to be highly variable. This is consistent with antibody staining of human sperm which showed variable localization and often the absence of PLCζ, even in sperm from fertile males [51]. These data suggest that variable PLCζ activity from fertile sperm samples could well account for the recurrent ICSI failure.

5. The use of Ca^{2+} ionophores in human oocyte activation.

The above discussion suggests that a deficient activation stimulus, most notably a lack of PLCζ activity and a lack of Ca^{2+} signal, could underlie many cases of failed

fertilization after ICSI. As a result some clinics have tried to rescue cases of failed fertilization after ICSI by providing a separate stimulus in the form of Ca^{2+} ionophores such as A23187 or ionomycin. This can involve using ionophores on oocytes just after they have failed to activate (~16 hours), or else using ionophores for all oocytes in the subsequent treatment cycle. There are now a number of reports showing that an incubation of 'failed to fertilize' oocytes in Ca^{2+} ionophores for ~ 10 mins can improve activation rates with ICSI [52-55]. A recent meta-analysis on the use of Ca^{2+} ionphore in ICSI concluded that ionophore treatment could significantly improve clinical pregnancy rates as well as oocyte activation [52-55]. Many such studies with Ca^{2+} ionophore have been retrospective studies where all oocytes in a treatment cycle were treated with ionophore. This is usually because the fertilization rate was extremely low in the previous cycle. Nevertheless, whilst improvements in activation and development rates are promising, in many cases the only 'control oocytes' have been those that failed to fertilize in the previous cycle. One randomized control trial where sibling oocytes were used as controls found that the use of a protocol involving Ca^{2+} injection and then double ionomycin treatment was effective in improving fertilization and pregnancy rates in patients where there was evidence for a lack of Ca^{2+} releasing activity in the sperm [57]. Another study, again using sibling oocytes as controls for A23187 activation after sperm selection for ICSI found no obvious improvement in fertilization rates [58]. So, it is possible that only certain patients will benefit from oocyte activation with ionophore, or that benefits are more likely seen when more than one Ca^{2+} increase is induced [57]. In addition the composition of the medium affects the size of the Ca^{2+} response and its ability to activate oocytes so comparisons between clinics need to take into account any differences in protocols and preparations used [59]. It is also notable that clinics sometimes consider using multiple treatments with Ca^{2+} ionophore [57]. One assumes that this begins to mimic in some way the multiple Ca^{2+} increases at fertilization. However, one problem that arises is that the ionophore-induced Ca^{2+} increases are smaller with each application, and prolonged incubation in ionophores will probably destroy an oocyte [20].

Since they can be toxic with prolonged exposure [20], concerns exist about the use of Ca^{2+} ionophores in clinical IVF. However, when Ca^{2+} ionophores are applied to mouse zygotes after fertilization, with a brief exposure designed to mimic the effect

of the extra Ca^{2+} rise, then development to blastocysts stage, and to term was normal [60]. Ca^{2+} ionophores have also been applied after ICSI with wobbler mouse sperm and development to term appeared to be normal [33]. Consequently, there are no direct indications that the extra Ca^{2+} rise caused by ionophore has an overt detrimental effect upon mouse development. Early indications are that ionophore activation in human oocytes also does not have obvious effect on the health of babies [61,62]. This should not be a surprise since during the ICSI procedure there is a large and long lasting Ca^{2+} increase [12], and oocyte cryopreservation is almost certainly associated with a significant Ca^{2+} increase [63]. If a single extra Ca^{2+} rise had a dramatic detrimental effect on embryo development then it is hard to see how ICSI or oocyte cryopreservation could be successful as clinical procedures. Nevertheless, the question remains as to whether ionophore treatment as some more subtle effect on embryos, or upon the long-term health of offspring. This will require longer-term studies with larger numbers of patients.

As noted above, when Ca^{2+} ionophores are applied on their own they have limited capacity to activate mammalian oocytes, but when used in combination with ICSI the situation may be a little different. In failed ICSI, a sperm has been injected and this might introduce a small amount of PLCζ, which could sensitize the oocyte to Ca^{2+} elevating agents such as ionophores. It has been found that applying ionophore after ICSI leads to a large Ca^{2+} increase followed by smaller Ca^{2+} oscillations [64]. It is not clear how common this type of response may be, nor whether these smaller oscillations are effective in promoting oocyte activation.

6. Electrical activation of human oocytes.

Another way to achieve human oocyte activation is to use an electroporation pulse in the presence of Ca^{2+}. This type of electrical activation can rescue oocyte activation failure after ICSI. In one study that used sibling oocytes as a control group it was found that there was a small increase in the fertilization rate after ICSI [65]. It has also been shown that electrical activation can stimulate human oocyte activation and live births after injection of round spermatids [66]. In this latter study it was shown that the electrical pulse triggers a series of Ca^{2+} spikes after spermatid injection. The electrical pulse only causes a single Ca^{2+} increase in control oocytes, so it is likely

that a small amount of PLCζ introduced by the round spermatid may be sensitizing the oocyte to the electroporation induced Ca^{2+} influx [30]. Human round spermatids probably possess a small amount of PLCζ because they can cause Ca^{2+} oscillations when injected into mouse oocytes [67].

The protocols so far generally involve using a single electrical pulse, or else a series of pulses within a short time (< seconds), and hence there is still only one large Ca^{2+} increase [30]. There have been no studies carried out on human oocytes exposed to multiple, low frequency stimulation in a microfluidic device of the type used for mouse and rabbit oocytes by Ozil and colleagues. This is essentially for technical reasons since the microfluidic electroporation device is complex and requires specialist knowledge to build. It is not commercially available for clinical trials.

7. Potential alternatives for activating human oocytes.

Whilst most clinical treatment for failed fertilization has involved the use of ionophores there are some other possible treatments that have been, or might be tested. One agent that is obvious to consider is PLCζ. Injection of PLCζ RNA has been shown to stimulate Ca^{2+} oscillations in human oocytes, that failed to fertilize in IVF or ICSI, and these oocytes can develop to form blastocysts [10,68]. PLCζ RNA injection has also been shown to be more effective in stimulating development to the blastocyst stage than ionophore treatment or electrical activation [68]. However, the use of RNA injection is problematic. The main technical problem is that there is variable expression of PLCζ protein from the injected RNA. This means that the amount of PLCζ protein can vary considerably from one oocyte to another and excess PLCζ expression in the oocyte leads to cleavage stage arrest in mice, cow and human embryos [10,30,40]. The second issue with RNA is that it is genetic material and is many parts of the world it would not be permissible to inject it into an oocyte as a therapy for clinic treatment. A better prospect for PLCζ is to use the recombinant protein. PLCζ recombinant protein can be made in bacteria as a fusion tagged protein and can cause prolonged Ca^{2+} oscillations when injected into mouse or human oocytes [32,69,70]. It could potentially be injected at the same time as the sperm during ICSI and offer a simple way of activating the oocyte without the need

for further specific protocols. However, there are still issues to resolve before such recombinant PLCζ can be used in clinical IVF. PLCζ protein readily loses enzyme activity and its activity will need to be stabilized and calibrated before regular use. It is currently not available commercially for use in clinical trials by IVF clinics.

Another activation stimulus mentioned above is incubation in media containing Sr^{2+} which reliably causes Ca^{2+} oscillations in mouse oocytes. There are some studies showing human oocyte activation, subsequent pregnancies and live births after ICSI followed by incubation in Sr^{2+} medium [71-73]. The protocols used involved incubating human oocytes for 10 min or 60 mins in 10mM Sr^{2+} [71-73], which is shorter than the standard treatment of 2 hours used for mouse oocytes. The studies of Sr^{2+} in human oocytes are compilations of case reports where all oocytes within a collection cycle were treated, with no use of sibling control oocytes. Consequently, whilst pregnancies and live births have been achieved after ICSI and incubation of human oocytes in Sr^{2+}, it is not known whether the Sr^{2+} treatment was inducing oocyte activation.

In my lab, Sr^{2+} media has been tested on human oocytes many times with no detection of Ca^{2+} oscillations, using the same 10mM Sr^{2+} (Ca^{2+} free) media used regularly to cause Ca^{2+} oscillations in mouse oocytes. The only time Ca^{2+} oscillations have been seen is with some oocytes after >10 hours of incubation in 20mM Sr^{2+}, which leads to oocyte degeneration (Elgmati and Swann, unpublished data). Other labs, who regularly use Sr^{2+} to activate mouse oocytes, have also reported a similar lack of Ca^{2+} responses, or activation in human oocytes exposed to Sr^{2+} [74,75]. It is worth noting that despite Sr^{2+} being in widespread use in mouse oocytes for over 20 years there are still no published papers showing Sr^{2+} media causing Ca^{2+} oscillations in human, pig or cow oocytes. The reasons why Sr^{2+} does not appear to cause Ca^{2+} release in non-rodent oocytes is unclear. It could be that Sr^{2+} does not cross the plasma membrane as effectively in some oocytes. Sr^{2+} influx in mouse oocytes is mediated via plasma membrane TRPV3 channels which can be stimulated to open by chemicals such as 2-APB and carvacrol [76]. However, TRPV3 channels also exist in human oocytes and in the presence of Sr^{2+} there are still no Ca^{2+} oscillations with incubations of 6 hours [75]. It seems unwarranted to offer a Sr^{2+} media 'activation' to patients as a therapy for failed fertilization until it has been shown that it can cause Ca^{2+} oscillations in human oocytes.

Apart from Sr^{2+} there are only a small number of chemicals that can cause Ca^{2+} oscillations in mature mouse oocytes. One such chemical is $InsP_3$ itself, which of course has to be microinjected. However, this only leads to a short lasting series of Ca^{2+} oscillations in mouse oocytes, and whilst it can trigger some exocytosis , $InsP_3$ injection is unable to cause full activation (pronuclear formation) in mouse oocytes [42]. Injection of the IP_3R agonist adenophostin causes a longer lasting series of Ca^{2+} oscillations and it has been used to activate mouse oocytes after spermatid injection [77]. However, we have found that adenophostin only causes high frequency, low amplitude Ca^{2+} oscillations in human oocytes (Sanders and Swann, unpublished data).

Another effective trigger for large amplitude Ca^{2+} oscillations in both mouse, pig and human oocytes is thimerosal [6,78]. However, thimerosal is a sulfhydryl reagent which causes damage to the MII spindle [79]. One protocol that has proved effective in pig oocytes is to apply thimerosal and then reverse its effects by adding dithiothreitol to reduce the sulfhydryl groups on proteins. This protocol allows thimerosal to cause a large Ca^{2+} increase and activate pig oocytes [79]. However, the dithiothreitol has to be added 10mins after thimerosal which means that there is probably only time for a single Ca^{2+} increase before its actions are terminated. Consequently, thimerosal is not usable in a way that triggers low frequency Ca^{2+} oscillations.

Although Ca^{2+} oscillations are clearly the physiological trigger for oocyte activation, it has been known for many years that some chemicals can activate oocytes without causing a Ca^{2+} increase. The latest such chemical to be shown to do this is TPEN which is a Zn^{2+} chelator that strips Zn^{2+} from the cell cycle-arresting protein EMI2, thus leading to meiotic resumption [80-81]. TPEN is effective in activating development in mouse oocytes and can support development after ICSI with inactivated sperm heads [80]. One study has suggested that it is able to activate human oocytes, but this was just from meiotic metaphase to anaphase, rather than pronuclear formation [82]. In pig oocytes TPEN was found to be effective in causing activation, but it was more effective when used at lower concentrations in combination with Ca^{2+} ionophore, which is similar to that of agents such as puromycin [81]. Again artificial stimuli often appear to be more effective when used in combinations.

8. **Future Prospects.**

The role of Ca^{2+} in animal oocyte or egg activation first came to light in the 1970s. We now know that there is a role for $InsP_3$ and PLCζ in triggering Ca^{2+} oscillations. Data on mouse and other animal oocytes also suggests that Ca^{2+} oscillations are probably a more effective way of activating development than a single large Ca^{2+} rise We also know that failed oocyte activation is a common cause of failed fertilization after ICSI. Currently, the only agent available for clinical use for activating oocytes is the Ca^{2+} ionophore A23187. The data thus far suggests that Ca^{2+} ionophores may be effective in improving activation rates for failed fertilization after ICSI, and that it may exert beneficial effects upon later development. Nevertheless, Ca^{2+} ionophores alone have not previously been found to be the optimal way of causing parthenogenetic oocyte activation in mammals. They could perhaps be regarded as a first generation treatment. It is likely that an improvement in human oocyte activation is possible if a stimulus can be provided that causes low frequency, large amplitude Ca^{2+} oscillations similar to those seen at fertilization. The only candidate methods that might do this so far, in human oocytes, are recombinant PLCζ and multiple electroporation.

Another potential innovation for oocyte activation is monitor the occurrence of Ca^{2+} oscillations in human zygotes after ICSI. It has been shown that there are brief and subtle movements, or spasms, in the cytoplasm of mouse and human zygotes during sperm, or PLCζ induced Ca^{2+} transients [83-85]. These spasms occur immediately following each Ca^{2+} transient and provide a relatively non- invasive way of monitoring how many Ca^{2+} spikes have occurred during fertilization. The pattern of such spasms has been linked to developmental potential in mice [83,85]. This type of technology could be developed to allow clinicians to assess whether any extra Ca^{2+} stimuli are required after poor fertilization in a treatment cycle. It could also be a new way to assess the effectiveness of any oscillatory stimulus in promoting further Ca^{2+} transients in the oocyte.

Conflict of interest

The author declares he has no conflict of interest.

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Figure Legends.

Fig.1. Ca^{2+} oscillations during fertilization in a mouse oocyte. A typical pattern of oscillations is shown (10-20 Ca^{2+}) spikes from a single oocyte. The oocyte was microinjected with the Ca^{2+} sensitive dye Rhod dextran and fluorescence monitored at 10 second intervals for ~10 hours. The fluorescence of the dye is plotted as a ratio relative to the starting fluorescence signal. Further experimental details are given in elsewhere [18,32].

Fig.2. A schematic representation of the known methods of generating Ca^{2+} increases in human oocytes. Electroporation in the presence of Ca^{2+} causes Ca^{2+} influx into the cytosolic compartment and, as used so far, leads to a single large Ca^{2+} increase. Ca^{2+} ionophores, A23187 and ionomycin, cause Ca^{2+} release principally from the endoplasmic reticulum (ER) and a single large Ca^{2+} rise. The sperm introduces PLCζ which generates $InsP_3$ and a series of Ca^{2+} oscillations. The general pattern of Ca^{2+} transients induced by each stimulus is represented in red.

Fig 1. Ca^{2+} oscillations during fertilization in a mouse oocyte

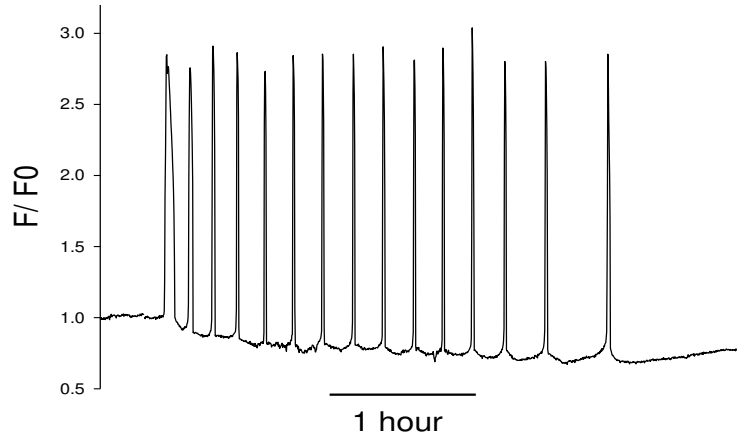

Fig 2. Schematic representation of mechanisms for generating Ca^{2+} increases in human oocytes.