

# Rescue of failed oocyte activation after ICSI in a mouse model of male factor infertility by recombinant phospholipase C $\zeta$

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**ABSTRACT:** Artificial oocyte activation to overcome failed fertilization after intracytoplasmic sperm injection (ICSI) in human oocytes typically employs Ca<sup>2+</sup> ionophores to produce a single cytosolic Ca<sup>2+</sup> increase. In contrast, recombinant phospholipase C $\zeta$  (PLC $\zeta$ ) causes Ca<sup>2+</sup> oscillations indistinguishable from those occurring during fertilization, but remains untested for its efficacy in a scenario of ICSI fertilization failure. Here, we compare PLC $\zeta$  with other activation stimuli in a mouse model of failed oocyte activation after ICSI, in which heat-treated sperm are injected into mouse oocytes. We show that increasing periods of 56°C exposure of sperm produces a progressive loss of Ca<sup>2+</sup> oscillations after ICSI. The decrease in Ca<sup>2+</sup> oscillations produces a reduction in oocyte activation and embryo development to the blastocyst stage. We treated such oocytes that failed to activate after ICSI either with Ca<sup>2+</sup> ionophore, or with Sr<sup>2+</sup> media which causes Ca<sup>2+</sup> oscillations, or we injected them with recombinant human PLC $\zeta$ . All these treatments rescued oocyte activation, although Sr<sup>2+</sup> and PLC $\zeta$  gave the highest rates of development to blastocyst. When recombinant PLC $\zeta$  was given to oocytes previously injected with control sperm, they developed normally to the blastocyst stage at rates similar to that after control ICSI. The data suggest that recombinant human PLC $\zeta$  protein is an efficient means of rescuing oocyte activation after ICSI failure and that it can be effectively used even if the sperm already contains endogenous Ca<sup>2+</sup> releasing activity.

**Key words:** PLC $\zeta$  / ICSI / fertilization / sperm / oocyte

## Introduction

For couples attending *in vitro* fertilization (IVF) clinics, intracytoplasmic sperm injection (ICSI) is a widely used treatment option (Palermo *et al.*, 1992; Johnson *et al.*, 2013) that is remarkably successful, compared with conventional IVF treatments, for treating many cases of male factor infertility (Palermo *et al.*, 1992; Johnson *et al.*, 2013). However, cases of ICSI where all of the available oocytes from a given collection fail to fertilize (Yanagida, 2004) occur in 1–5% of all ICSI treatment cycles (Liu *et al.*, 1995). In such incidences of ICSI failure, the main cause has been shown to be the lack of oocyte activation (Javed *et al.*, 2010; Vanden Meerschaut *et al.*, 2013a; Neri *et al.*, 2014). The options for couples facing total fertilization failure are limited (Yuzpe *et al.*, 2000; Heindryckx *et al.*, 2005). Total fertilization failure cases are relatively rare, partly because typically ~10 oocytes are available for sperm injection (Sunkara *et al.*, 2011). This multiplicity of oocytes masks the fact that fertilization after ICSI is less effective when rated per oocyte injected. For

example, using sperm and oocytes with apparently normal parameters in cases where tubal blockage was the only evident cause of infertility, only 67% of oocytes were activated after sperm injection (Bukulmez *et al.*, 2000), implying that one in three sperm is ineffective in activating the oocyte. This recurrent failure per oocyte becomes a particular concern when oocytes are limited, in mild ovarian stimulation or natural ovulation cycles (Pelinck *et al.*, 2002; Loutradis *et al.*, 2007).

The activation of mammalian oocytes at fertilization involves an extensive series of Ca<sup>2+</sup> transients, known as Ca<sup>2+</sup> oscillations. Each Ca<sup>2+</sup> spike lasts about 1 min and the Ca<sup>2+</sup> transients occur at intervals of 5–30 min (Miyazaki and Ito, 2006; Swann and Lai, 2013). Such Ca<sup>2+</sup> oscillations have been observed in human and mouse oocytes after *in vitro* fertilization and ICSI (Sato *et al.*, 1999). Such Ca<sup>2+</sup> oscillations are both necessary and sufficient for oocyte activation (Kurokawa *et al.*, 2004) and may influence subsequent embryo development (Ducibella *et al.*, 2002; Yu *et al.*, 2008). Accumulating scientific and clinical evidence favours the idea that Ca<sup>2+</sup> oscillations are triggered after sperm oocyte

membrane fusion, which allows the entry of a sperm-specific PLC isoform, termed PLC $\zeta$ , into the oocyte cytoplasm (Saunders et al., 2002; Nomikos et al., 2005). Injection of PLC $\zeta$  cRNA or recombinant protein stimulates parthenogenetic activation of mouse and human oocytes, and subsequent development of mouse embryos up to the blastocyst stage at rates similar to those seen after fertilization (Miyazaki, 1995).

Mouse oocytes have been frequently used as a model system for studying human IVF and ICSI. The mouse oocyte has also been used to directly test the ability of human sperm to cause oocyte activation or Ca<sup>2+</sup> oscillations after cross-species ICSI (Araki et al., 2004; Vanden Meerschaut et al., 2013a). Injection of human sperm into mouse oocytes can trigger oocyte activation, but sperm from failed clinical ICSI cycles generally show a reduced ability to activate mouse oocytes after injection (Araki et al., 2004; Vanden Meerschaut et al., 2013a). Furthermore, injecting human sperm into mouse oocytes also causes a series of Ca<sup>2+</sup> oscillations. The frequency of these human sperm-induced Ca<sup>2+</sup> oscillations is often higher than observed at fertilization in mouse oocytes (Yoon et al., 2012; Nikiforaki et al., 2014). This is probably because human PLC $\zeta$  has a greater intrinsic potency than mouse PLC $\zeta$  and is able to cause the same pattern of Ca<sup>2+</sup> oscillations at ~10 times lower concentrations than mouse PLC $\zeta$  (Yu et al., 2008). It has been reported that sperm from men with repeated fertilization failure, or with mutations in PLC $\zeta$  protein, show a markedly reduced ability to cause Ca<sup>2+</sup> oscillations after ICSI into mouse oocytes (Yoon et al., 2008; Heytens et al., 2009; Kashir et al., 2011, 2012). However, it was noteworthy that even for men who have had successful ICSI cycles, only ~55% of normal sperm cause high-frequency Ca<sup>2+</sup> oscillations in mouse oocytes (Vanden Meerschaut et al., 2013a; Nikiforaki et al., 2014). These data suggest that in cases of human ICSI failure, there might be either a deficiency, or relative lack of Ca<sup>2+</sup> oscillations.

In cases of failed or poor rates of fertilization after ICSI, the only available treatment option is the use of artificial oocyte activation agents. This usually consists of treating oocytes with Ca<sup>2+</sup> ionophores, such as A23187 or ionomycin, a procedure that has been successfully used to overcome fertilization failure in many cases (Kyono et al., 2012). Despite its apparent utility there are few controlled studies on the efficacy of Ca<sup>2+</sup> ionophores as a means of rescuing failed oocyte activation and development. Ca<sup>2+</sup> ionophore application, as currently used in most clinics, only causes a single large Ca<sup>2+</sup> increase that does not mimic the series of Ca<sup>2+</sup> oscillations seen at fertilization (Rinaudo et al., 1997). Stimuli that elicit multiple Ca<sup>2+</sup> transients are known to be a more effective means of activating mammalian oocytes (Ozil and Swann, 1995; Ducibella et al., 2002). Sr<sup>2+</sup> media can be used to cause such repetitive Ca<sup>2+</sup> oscillations in mouse oocytes, but Sr<sup>2+</sup> has not been shown to trigger Ca<sup>2+</sup> release in human oocytes (Rogers et al., 2004). PLC $\zeta$  remains the only physiological agent that has been repeatedly shown to produce a prolonged series of Ca<sup>2+</sup> oscillations in all mammalian oocytes studied, including human oocytes (Ito et al., 2011; Nomikos et al., 2013a, b; Kashir et al., 2014).

In this study, we have investigated whether recombinant human PLC $\zeta$  protein can be used to rescue cases of failed fertilization or to improve poor development rates after ICSI. We use a mouse model of failed oocyte activation after ICSI by applying mild heat treatment of mouse sperm. We find that heat-treated sperm display a reduced ability to generate Ca<sup>2+</sup> oscillations in mouse oocytes, consistent with consequent observations of reduced preimplantation development. We also show

that fertilization failure and poor embryo development after ICSI with heat-treated sperm can be rescued by subsequent microinjection of recombinant human PLC $\zeta$  protein, which along with Sr<sup>2+</sup> media, is more effective than Ca<sup>2+</sup> ionophore. Moreover, we demonstrate that microinjection of PLC $\zeta$  after standard ICSI with normal, untreated sperm does not impair embryo development. These data provide the basis for future studies to examine the potential use of recombinant human PLC $\zeta$  as a biological therapeutic to rescue human oocytes from failed fertilization after ICSI.

## Materials and Methods

All chemicals were obtained from Sigma Aldrich UK unless stated otherwise and were of embryo grade where available. M2 media was used for handling oocytes outside the incubator. KSOM was obtained from Merk-Millipore (Watford, UK), or made as previously described (Summers et al., 1995). The microinjection buffer was 100 mM KCl in 20 mM Hepes, pH 7.2 (Swann, 1990).

### Gametes and embryos

Mouse oocytes were obtained from superovulated female MF1 mice as described previously (Yu et al., 2008). Cumulus-oocyte masses from oviducts were incubated in hyaluronidase in M2 media, and then isolated oocytes were washed in M2 media alone. Mouse embryos were cultured in KSOM media (Summers et al., 1995) under mineral oil in an incubator at 37°C gassed with 5% CO<sub>2</sub>. Mouse sperm were collected from the cauda epididymis of euthanized male mice (C57xCBA F1 hybrid), released into T6 media (Jones et al., 1995) and in some cases, sperm were frozen in the same media (without cryopreservation) in a -80°C freezer. For heat treatment, sperm were incubated for the specified times in a water bath at 56°C then sonicated for ~10 s before being added to drops containing the oocytes. For Sr<sup>2+</sup> activation after ICSI as described by Yoshida and Perry (2007) using heat inactivated sperm for 30 min, oocytes were allowed to recover for 30 min in M2. Oocytes were then incubated afterwards in calcium-free HKSOM supplemented with 5 mM Sr<sup>2+</sup> for 2 h after which they are washed in M2 then cultured in KSOM under 5% CO<sub>2</sub> in air at 37°C.

### Ethical approval

All procedures using animals were approved by Cardiff University Animals Ethics Committee and carried out under a UK Home Office Project Licence.

### Microinjection

Mouse oocytes were microinjected with sperm using custom-made ICSI pipettes (Yoshida and Perry, 2007). The micropipettes containing the sperm were advanced through the oocyte plasma membrane using a piezo-pulse delivered by a Prime Tech piezo manipulation system (Intracel, Royston, UK). Recombinant PLC $\zeta$  was injected using a fine tip micropipette that was inserted into the oocyte using pressure pulses (Picopump, World Precision Instruments, USA), as described previously (Saunders et al., 2002).

### Ca<sup>2+</sup> measurements

Cytosolic free Ca<sup>2+</sup> concentrations were measured in individual mouse oocytes by monitoring the fluorescence of PE3 or Rhod dextran (Takahashi et al., 1999). The PE3 dye was loaded into oocytes by incubating for 30 min in 10  $\mu$ M of PE3-AM (also known as fura2LeakRes-AM) dissolved in M2 media. PE3 is a similar dye to fura2 except that it does not undergo compartmentalization or extrusion from the cytosol and hence can be used for longer-term recordings of Ca<sup>2+</sup> (Takahashi et al., 1999). Oocytes were then washed free of the AM dye and allowed to equilibrate for another ~30 min before making

fluorescence measurements. Rhod dextran was injected into oocytes before the start of experiments. The measurements of fluorescence were made by placing oocytes in drops of HKSOM media on a heated stage of a Nikon TiU epifluorescence microscope. For PE3 based recordings, oocytes were exposed to fluorescence excitation at 380 nm and emission measured at 510–550 nm (Takahashi *et al.*, 1999). The fluorescence ratio of the signals at 350 and 380 nm was then plotted against time as a measure of relative cytosolic Ca<sup>2+</sup> concentrations. In a similar manner for Rhod dextran, the fluorescence was plotted against time with excitation at 550 nm and emission collected at 580–620 nm (Swann and Lai, 2013).

## Recombinant protein expression and purification

Human PLC $\zeta$  was expressed as a NusA-6xHis-tagged fusion protein and purified by affinity chromatography on Ni-NTA resin using standard procedures (Qiagen) and elution with 275 mM imidazole, as described previously (Nomikos *et al.*, 2013b). NusA tagged human PLC $\zeta$  was tested for its ability to cause Ca<sup>2+</sup> oscillations in oocytes prior to use and was injected at a pipette concentration of 0.01 mg/ml (Nomikos *et al.*, 2013a, b; Nikiforaki *et al.*, 2014). Control NusA protein was purified in a similar manner and when injected separately was used at a pipette concentration of 0.1 mg/ml in KCl/Hepes buffer.

## Statistical analysis

Data on the number of Ca<sup>2+</sup> transients and rate of embryo development from control and experimental groups were analysed using ANOVA or pairwise Student's *t*-tests. Differences at *P* < 0.05 were considered significant.

## Results

### Sperm treatment and Ca<sup>2+</sup> oscillations after ICSI

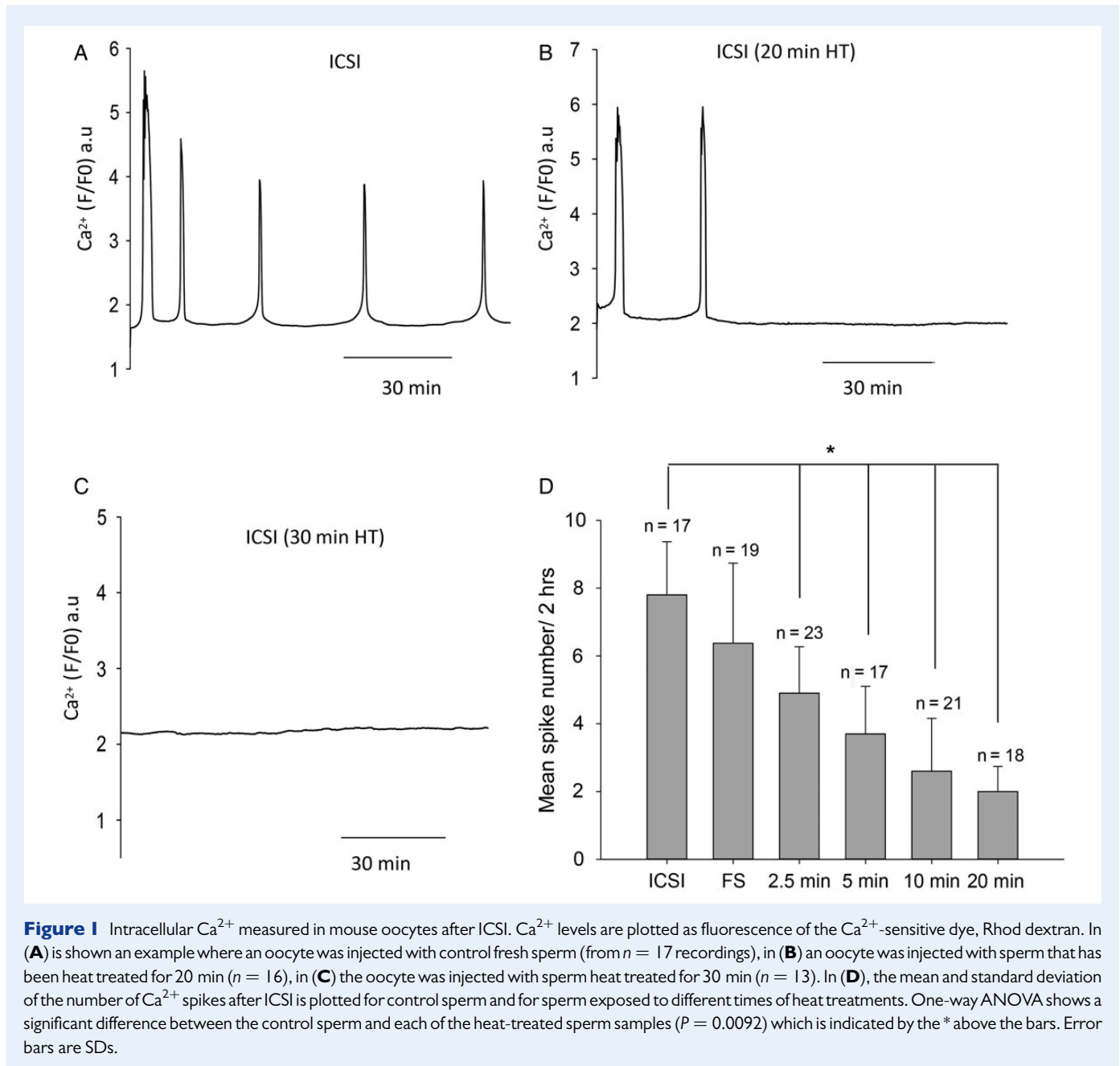
To create a clinical scenario mimicking poor fertilization after ICSI, we exposed mouse sperm to heat treatment since previous studies have shown that the oocyte activating sperm factor is sensitive to mild heat treatment (Perry *et al.*, 1999). We incubated mouse sperm at 56°C for time intervals varying from 2.5 to 30 min. Figure 1A–C shows the recordings of Ca<sup>2+</sup> oscillations observed in mouse oocytes after ICSI with either fresh (control) sperm or with sperm that had been heat treated for various times such as 20 or 30 min. ICSI using fresh sperm results in a series of Ca<sup>2+</sup> oscillations during the first 2 h. In contrast, any heat treatment of sperm resulted in a significantly decreased number of Ca<sup>2+</sup> spikes over the 2 h recording period. ICSI with sperm that had been heat treated for shorter durations such as 2.5 min produced some Ca<sup>2+</sup> spikes (Fig. 1D), but significantly fewer than with control sperm. ICSI with frozen and thawed sperm caused slightly fewer Ca<sup>2+</sup> oscillations in the 2 h recording period than freshly prepared sperm (Fig. 1D). Figure 1D also shows that the mean number of Ca<sup>2+</sup> spikes is progressively reduced as the heat treatment is increased from 2.5 min, with a complete loss of Ca<sup>2+</sup> oscillations after ICSI seen with a 30 min treatment. These data suggest that exposure of sperm at 56°C for increasing time periods is associated with a progressive reduction in the ability of sperm to trigger Ca<sup>2+</sup> oscillations.

We further examined the effect of heat-treated ICSI sperm upon the oocyte activation rate and subsequent development to the blastocyst stage. We used MFI oocytes for this work where control rates of development *in vitro* to the blastocyst stage after fertilization are around 50%

(Ozil and Swann, 1995; Ducibella *et al.*, 2002). Figure 2 and Supplementary Table S1 show that the rate of oocyte activation, as defined by pronuclear formation, was progressively reduced by heat treating sperm for 2.5–30 min. Similarly, embryonic development to blastocysts was also reduced in embryos resulting from ICSI with heat-treated sperm. Figure 2 indicates that an increase in time of sperm exposure to 56°C is consistent with a progressive decrease in the number of ICSI embryos that develop to the 2-cell or blastocyst stage. These data suggest that mild heat treatment of mouse sperm leads to loss in the capacity of sperm to support both Ca<sup>2+</sup> oscillations and preimplantation development after ICSI. The observed thermal impairment of sperm function mimics a clinical scenario of oocyte activation failure and poor development after IVF/ICSI.

The most common treatment for failed fertilization after ICSI is to expose oocytes to Ca<sup>2+</sup> ionophores (Nasr-Esfahani *et al.*, 2010). Figure 3A shows that oocytes injected with sperm heat-treated for 30 min responded with a single large Ca<sup>2+</sup> increase after exposure to ionomycin. For Fig. 3A the ionomycin was washed out after 5 min in order to mimic the duration of the Ca<sup>2+</sup> transient seen in human oocytes exposed to ionophore (Rinaudo *et al.*, 1997). We then wanted to compare these stimuli to those that are known to cause Ca<sup>2+</sup> oscillations. First we used Sr<sup>2+</sup> media which, as shown in Fig. 3B, causes a series of Ca<sup>2+</sup> oscillations (mean of 7.91 Ca<sup>2+</sup> spikes in 2 h, with a standard deviation of 2.64, *n* = 15) after ICSI in a manner previously described (Perry *et al.*, 1999). However, in order to generate the Ca<sup>2+</sup> oscillations in a way that would be effective in human oocytes, we used recombinant PLC $\zeta$  protein. After ICSI with 30 min heat-treated sperm, oocyte Ca<sup>2+</sup> oscillations could be rescued by microinjection of recombinant human PLC $\zeta$  (Fig. 3C). The amount of PLC $\zeta$  protein we used is comparable to that used in previous experiments and was designed to generate a pattern of Ca<sup>2+</sup> oscillations similar to those seen after ICSI with control sperm. Importantly, we also performed double injection experiments by injecting PLC $\zeta$  protein into oocytes that had been through ICSI with control (fresh) sperm (Fig. 4A). For these double-injected oocytes, there were also Ca<sup>2+</sup> oscillations after PLC $\zeta$  injection, although we cannot distinguish the relative contribution to these oscillations from the PLC $\zeta$  injection and the injected sperm. It was, however, notable that the frequency of Ca<sup>2+</sup> oscillations in these experiments was similar to that in control ICSI, or with ICSI using frozen–thawed sperm (see Fig. 4B).

The above experiments show that Ca<sup>2+</sup> signals could be restored by the use of ionomycin, Sr<sup>2+</sup> media, or by introducing recombinant PLC $\zeta$  protein, in oocytes injected with heat-treated sperm. We monitored oocyte activation and subsequent development in such oocytes. Figure 5 shows that following ICSI with sperm treated for 30 min and then incubated with Ca<sup>2+</sup> ionophore, oocytes could be activated but only 13.3% formed blastocysts (see also Supplementary Table S2). In contrast, when we used the same procedure but replaced ionophore activation with Sr<sup>2+</sup> media, or PLC $\zeta$  injection, not only were oocytes activated but more than 34–36% developed to the blastocysts stage. This is significantly more than with ionophore treatment. We found that the use of frozen sperm was less effective than fresh sperm in triggering development up to the blastocyst stage, but that injection of PLC $\zeta$  could effectively rescue these oocytes and give rise to developmental rates similar to those of fresh sperm (Supplementary Table S2). These data suggest that agents that induce Ca<sup>2+</sup> oscillations, such as PLC $\zeta$  protein injection, provide an effective means of rescuing oocyte activation failure or



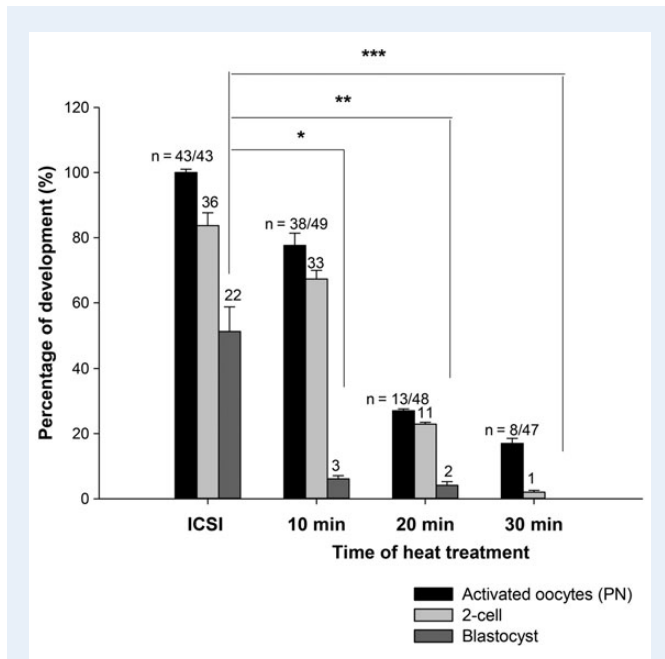
**Figure 1** Intracellular  $\text{Ca}^{2+}$  measured in mouse oocytes after ICSI.  $\text{Ca}^{2+}$  levels are plotted as fluorescence of the  $\text{Ca}^{2+}$ -sensitive dye, Rhod dextran. In (A) is shown an example where an oocyte was injected with control fresh sperm (from  $n = 17$  recordings), in (B) an oocyte was injected with sperm that has been heat treated for 20 min ( $n = 16$ ), in (C) the oocyte was injected with sperm heat treated for 30 min ( $n = 13$ ). In (D), the mean and standard deviation of the number of  $\text{Ca}^{2+}$  spikes after ICSI is plotted for control sperm and for sperm exposed to different times of heat treatments. One-way ANOVA shows a significant difference between the control sperm and each of the heat-treated sperm samples ( $P = 0.0092$ ) which is indicated by the \* above the bars. Error bars are SDs.

poor development after ICSI. We also noted that injecting either PLC $\zeta$  protein after ICSI using control sperm also led to oocyte activation and that the rate of 2-cell and blastocyst formation was similar to that seen with control sperm ICSI alone (Fig. 5 and Supplementary Table S2). These data show that the injection of PLC $\zeta$ , in addition to a normal  $\text{Ca}^{2+}$  oscillations stimulus from a control sperm, does not appear to impair preimplantation development to the blastocyst stage.

## Discussion

Fertilization failure in an oocyte after ICSI occurs both sporadically in many treatment cycles and can sometimes occur in all oocytes. Whilst there may be many reasons for fertilization failure, the most common cause is failure of oocyte activation (Javed et al., 2010; Nasr-Esfahani

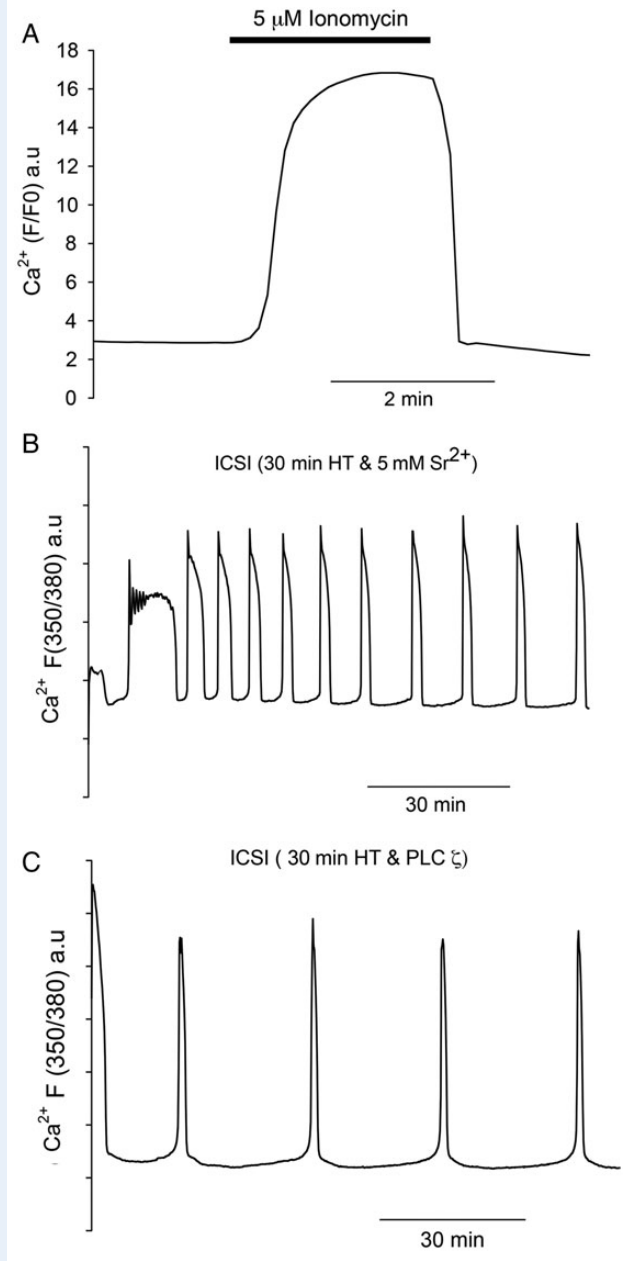
et al., 2010). In this study, we showed that mild heat treatment of mouse sperm leads to a reduction in its ability to trigger  $\text{Ca}^{2+}$  oscillations in mouse oocytes after ICSI. It is known that exposing mouse sperm to temperatures of  $\sim 90^\circ\text{C}$  results in DNA damage, but that heat treatment at lower temperatures ( $56^\circ\text{C}$ ) does not cause DNA damage nor impair embryo development when an independent oocyte activation stimulus is used (Perry et al., 1999). Our present data confirm that sperm contain a heat labile factor that triggers  $\text{Ca}^{2+}$  oscillations and this sperm-borne factor is sensitive to relatively mild heat treatment of  $56^\circ\text{C}$  for 30 min. This heat treatment is similar to that previously used to inactivate SOAF (sperm oocyte activating factor) in earlier reports of mouse ICSI (Perry et al., 1999). Previous ICSI studies also demonstrated that the SOAF, which is present in the sperm perinuclear theca, is PLC $\zeta$  (Perry et al., 1999), as identified by Saunders et al. (2002). PLC $\zeta$  is presently



**Figure 2** The percentage of embryo developmental to the blastocyst stage after ICSI is compared with that after ICSI using sperm that had been heat inactivated for various times. A significant difference for development to blastocyst was seen from control ICSI for the 10, 20, 30 min heat treatments (Student's *t*-test pairwise between the control and each heat treatment,  $P = 0.0038$ ,  $0.0023$ , and  $0.000$ , respectively). Error bars are SEMs.

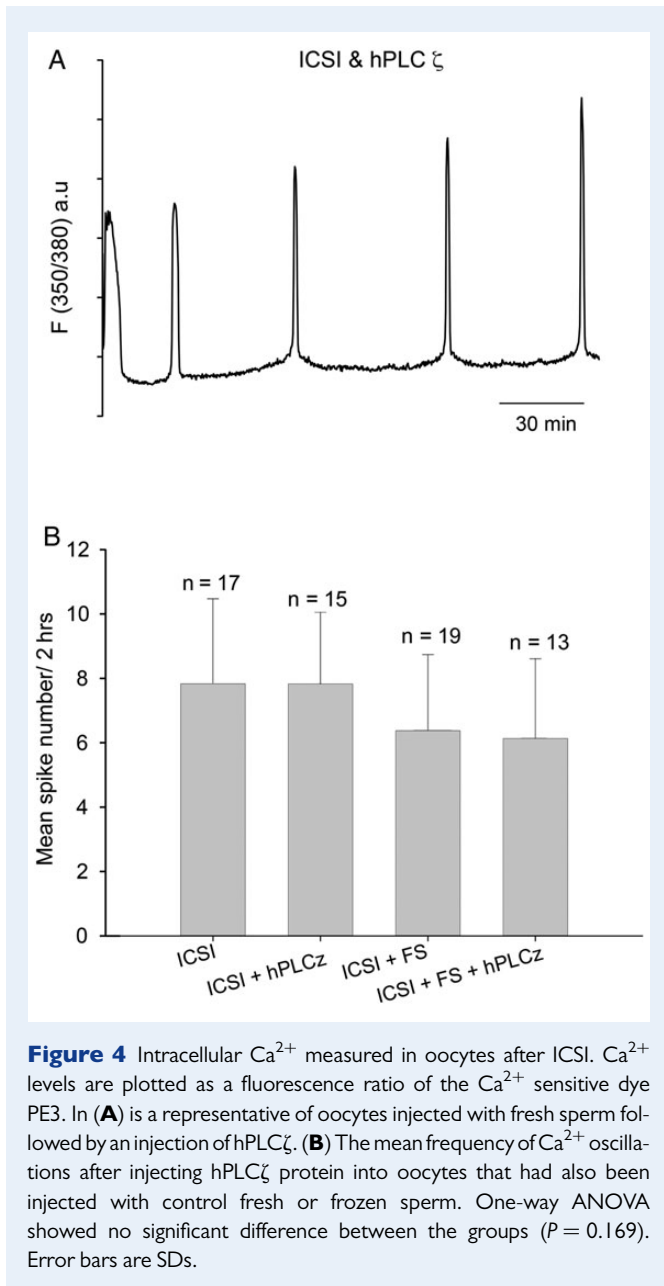
the only sperm-specific protein confirmed to have an intrinsic ability to cause  $\text{Ca}^{2+}$  oscillations in mammalian oocytes by multiple independent research teams (Saunders *et al.*, 2002; Kurokawa *et al.*, 2004; Rogers *et al.*, 2004; Miyazaki and Ito, 2006; Nomikos *et al.*, 2014). Therefore, it is probable that heat treatment leads to sperm PLC $\zeta$  inactivation in our experiments. Interestingly, freeze thawing of sperm reduces the activity and distribution of PLC $\zeta$  (Kashir *et al.*, 2011). This is consistent with our finding that this step slightly reduces the number of  $\text{Ca}^{2+}$  oscillations after ICSI as well as the percentage of embryos developing to the blastocyst stage. This could be due to some PLC $\zeta$  enzyme inactivation due to the freeze thaw cycle, or to loss of PLC $\zeta$  from the sperm. Nevertheless, in our study, defining the exact cause of loss of  $\text{Ca}^{2+}$  oscillations is not critical since we have identified a simple way of mimicking the clinical observation in humans where sperm from subfertile males shows a reduced ability to cause  $\text{Ca}^{2+}$  oscillations in oocytes after ICSI.

One advantage of using  $56^\circ\text{C}$  heat treatment to deplete the ability of sperm to activate oocytes after ICSI is that it enables provision of different time points that creates a range of effects. Indeed we found that varying the duration of sperm heating from 2.5 to 30 min led to progressive loss of  $\text{Ca}^{2+}$  oscillation-inducing activity. Along with the loss of  $\text{Ca}^{2+}$  oscillations, there was a loss of oocyte activation and embryo development up to the blastocyst stage. We found that embryo development to the blastocyst stage fell from  $\sim 50\%$  down to  $0\%$  correlating directly with the reduced number of  $\text{Ca}^{2+}$  spikes caused by increasingly longer heat treatments. It should be noted that we used mouse oocytes from the MFI strain which develop to blastocysts *in vitro* at a rate of  $\sim 50\%$ ,



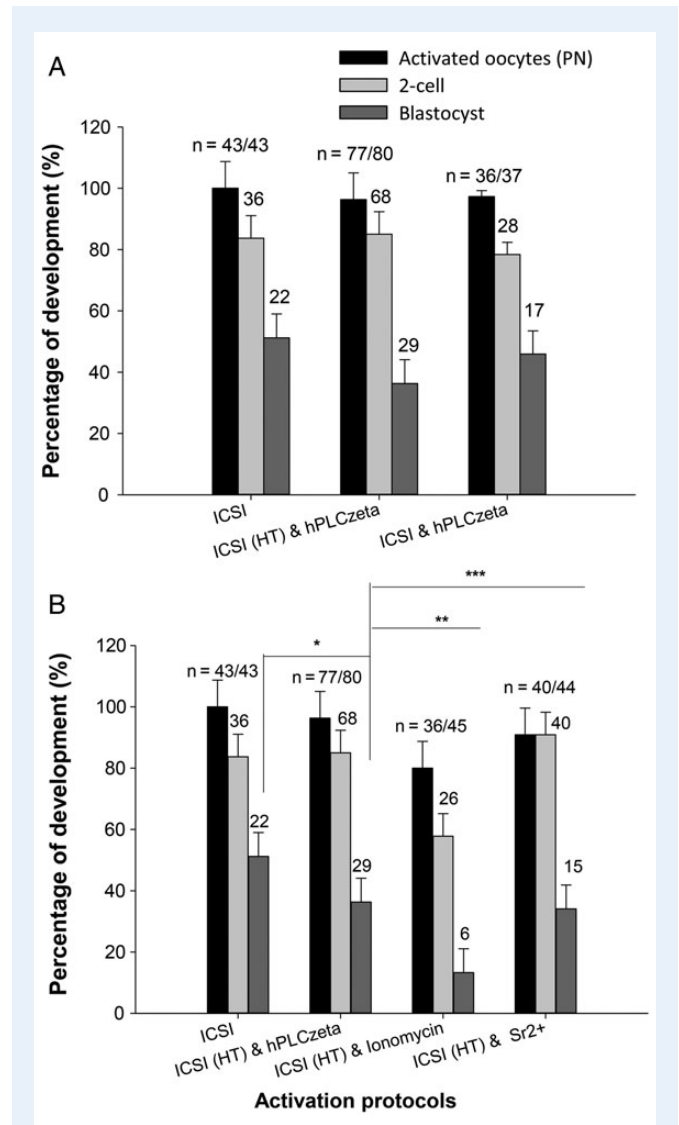
**Figure 3** Intracellular  $\text{Ca}^{2+}$  measured in mouse oocytes treated with artificial stimuli after ICSI. Conditions are the same as in Fig. 1 except that PE3 was used to record  $\text{Ca}^{2+}$  in (B) and (C) where fluorescence is in arbitrary (ratio) units (a.u.). In (A) is shown an example of oocyte treated with ionomycin for 5 min ( $n = 21$  recordings) where the bar indicates the time for which ionomycin was applied. In (B) is an example of an oocyte injected with heat treatment (30 min) inactivated sperm and then treated with  $\text{Sr}^{2+}$  media ( $n = 12$ ). In (C) is shown an example of an oocyte injected with heat treatment (30 min) inactivated sperm and then subsequently injected with purified recombinant PLC $\zeta$  protein ( $n = 15$ ).

which matches clinical scenarios with human embryos. These data suggests that an attenuation of the  $\text{Ca}^{2+}$  oscillations following ICSI leads to decreased rates of oocyte activation and development. This is a



scenario that appears to occur in some cases of subfertility found by practitioners of ICSI (Vanden Meerschaut et al., 2013a; Nikiforaki et al., 2014).

In clinical cases where poor ICSI oocyte activation rates are observed, the current treatment option is the use of  $\text{Ca}^{2+}$  ionophores, which provide an artificial stimulus (Neri et al., 2014). It has been used in many cases of complete activation failure. It can give rise to live births in treatment cycles that would otherwise fail (Yanagida et al., 2008; Nasr-Esfahani et al., 2010), but the efficiency of this activation protocol compared with a physiological stimulus has not been critically examined.  $\text{Ca}^{2+}$  ionophores can only cause one or, in some protocols, two large  $\text{Ca}^{2+}$  increases, which fails to mimic the multiple  $\text{Ca}^{2+}$  oscillations that occur at fertilization (Vincent et al., 1992; Heytens et al., 2008). In our study, we tested the ability of ionomycin to overcome failed oocyte activation after ICSI with heat-treated sperm and found that it could



restore activation. Ionomycin treatment also lead to development to the blastocyst stage, but it was less effective than either  $\text{Sr}^{2+}$  media or recombinant PLC $\zeta$  at producing oocyte activations. PLC $\zeta$  recombinant protein has been previously shown to overcome activation failure after ICSI in human oocytes (Yoon et al., 2012). This set a precedent for the use of PLC $\zeta$  as a means of rescuing oocytes from activation failure.

However, the efficiency of activation was not compared with other protocols. In our current experiments, the higher rates of Sr<sup>2+</sup> and PLC $\zeta$ -mediated oocyte activation versus ionophore-mediated oocyte activation also lead to better rates of embryo development to the blastocyst. Even though it may not be usable for human oocytes, we used Sr<sup>2+</sup> media because it induces Ca<sup>2+</sup> oscillations. Consequently our data indicated that it is because PLC $\zeta$  causes Ca<sup>2+</sup> oscillations that it is a more effective stimulus for rescuing oocyte activation after ICSI failure than Ca<sup>2+</sup> ionophores. This is consistent with previous data showing that multiple Ca<sup>2+</sup> transients are the most effective way to activate mouse or rabbit oocytes (Ducibella *et al.*, 2002, 2006).

In our protocol, we have used a second injection to introduce PLC $\zeta$  and induced Ca<sup>2+</sup> oscillations in the mouse oocyte after ICSI. The use of a second injection has been used in at least one clinic where Ca<sup>2+</sup> is injected after ICSI, and then ionophores are applied, as part of a protocol to rescue oocyte from ICSI failure (Vanden Meerschaut *et al.*, 2013a, b). Human oocytes are more robust than mouse oocytes since mouse oocytes specifically require piezo devices, or electrical oscillation, for pipette insertion (Swann, 1990; Yoshida and Perry, 2007). Hence, a second injection of PLC $\zeta$  protein should be possible in a clinical setting. We additionally anticipate that introducing PLC $\zeta$  into an oocyte should be relatively safe for embryos. So far the treatment of oocytes with Ca<sup>2+</sup> ionophores, which produces an unnaturally large rise in Ca<sup>2+</sup>, has been shown to have no discernible damaging effect on embryos or live young (Yanagida, 2004; Vanden Meerschaut *et al.*, 2013a). PLC $\zeta$  causes Ca<sup>2+</sup> oscillations indistinguishable from those seen at fertilization and so should not lead to any effects upon development other than those arising naturally.

Previous studies have suggested that human sperm contain variable amounts of PLC $\zeta$  and also have a variable ability to cause Ca<sup>2+</sup> oscillations in oocytes (Grasa *et al.*, 2008; Kashir *et al.*, 2013). During ICSI, an embryologist is unable to assess the ability of a chosen sperm to cause Ca<sup>2+</sup> oscillations. If PLC $\zeta$  is injected into ICSI oocytes to overcome cases of suspected activation failure, then it is possible that these oocytes will receive some endogenous Ca<sup>2+</sup> releasing activity from the sperm in addition to that from the injected PLC $\zeta$ . We tested the developmental consequence of this by examining the scenario where control sperm was used for ICSI and then the same oocytes were subsequently injected with the single sperm equivalent dose of PLC $\zeta$  used for rescuing complete activation failure. We also injected PLC $\zeta$  into oocytes that had ICSI with frozen–thawed sperm and have only a slightly reduced number of Ca<sup>2+</sup> oscillations. In both these cases of ICSI + PLC $\zeta$  injection, we found that after oocyte activation, the development rates to the blastocyst stage were similar to that for control ICSI. This benign effect might seem surprising since previous studies have shown that PLC $\zeta$  over-expression can lead to poor development to the blastocyst stage (Yu *et al.*, 2008). However, the previous deleterious effects on development required over-expression of about 10-fold more PLC $\zeta$  than is effective in causing oocyte activation. In the current study, we estimate that oocytes should have received little more than double the normal PLC $\zeta$  level in one sperm. The lack of change in the frequency of Ca<sup>2+</sup> oscillations is consistent with previous studies in mouse oocytes that have found only a marginal difference in Ca<sup>2+</sup> oscillation frequency after fertilization by two versus one sperm in zona-free oocytes (Faure *et al.*, 1999). These data suggest that PLC $\zeta$  can be injected at a dose to successfully activate an oocyte that had not been activated by ICSI, and that this will not lead to detrimental effects on early embryo development.

## Supplementary data

Supplementary data are available at <http://molehr.oxfordjournals.org/>

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## Authors' roles

R.S. and Y.Y. carried out experiments on oocytes and embryos and analysed the data. M.N. purified and characterized the recombinant human PLC $\zeta$  protein. K.S. and F.A.L. planned and supervised the project. All authors prepared the manuscript.

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## Conflict of interest

F.A.L. and K.S. hold patents with Cardiff University on PLC $\zeta$  Sperm factor sequences. Other authors declare no conflict of interest.

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