

Intracytoplasmic sperm injection in the bovine induces abnormal $[Ca^{2+}]_i$ responses and oocyte activation

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Abstract. Fertilisation by intracytoplasmic sperm injection (ICSI), a technique that bypasses the membrane fusion of the gametes, has been widely used to produce offspring in humans and mice. Success with this technique has lent support to the hypothesis that in mammalian fertilisation, a factor from the sperm, the so-called sperm factor, is responsible for oocyte activation and that the fusion process is not involved in the generation of the hallmark $[Ca^{2+}]_i$ signalling seen following fertilisation. However, the success of ICSI has largely eluded large domestic species, such as the bovine, porcine and equine, casting doubt on the current model of oocyte activation at fertilisation in these species. Using Ca^{2+} imagery and a series of treatments to manipulate the chemical structure of the sperm, we have investigated the early events of oocyte activation in response to ICSI in the bovine. Our results demonstrate, for the first time, that following ICSI, the majority of bovine oocytes are unable to mount $[Ca^{2+}]_i$ oscillations, although, in few cases, the initiation of $[Ca^{2+}]_i$ oscillations can occur in a manner indistinguishable from *in vitro* fertilisation. We also show that bull sperm possess a full complement of sperm factor. However, either the release and/or activation of the sperm factor are compromised after ICSI, leading to the delivery of a defective Ca^{2+} stimulus, which results in premature termination of embryo development.

Introduction

In oocytes of all species studied to date, the fertilising sperm evokes a rise in the concentration of intracellular free calcium ions ($[Ca^{2+}]_i$). In mammals, this increase in $[Ca^{2+}]_i$ takes the form of long-lasting oscillations, initiating shortly after sperm–oocyte fusion and terminating around the time of the first mitotic cell cycle. This series of $[Ca^{2+}]_i$ oscillations is necessary and sufficient for completing the events of oocyte activation that, in general terms, comprises the exocytosis of cortical granule material (block to polyspermy), the resumption of meiosis and the initiation of embryonic development (for a review, see Schultz and Kopf 1995). Although the mechanism by which the sperm initiates these $[Ca^{2+}]_i$ transients has not been fully elucidated, evidence supports the idea that shortly after fusion of the gametes, the sperm delivers a factor that then results in the activation of the phosphoinositide pathway. This factor, that nature of which is not yet known, has collectively been referred to as ‘sperm factor’ and is thought to act either as an activator of an egg phospholipase C (PLC) or is itself a PLC. Phospholipase C enzymes catalyse the hydrolysis of phosphatidyl 4,5-bisphosphate (PIP₂) into the two signalling molecules, inositol 1,4,5-trisphosphate (IP₃)

and 1,2-diacyl glycerol (DAG). Whereas DAG plays a role in several signalling pathways, including protein kinase C activation, IP₃ is a direct mediator of intracellular Ca^{2+} release by binding to its receptor, the IP₃R, a tetrameric ligand-gated Ca^{2+} channel located on the smooth endoplasmic reticulum, the Ca^{2+} store of the cell.

Although there is consensus in the field for the need of persistent $[Ca^{2+}]_i$ oscillations to exit meiosis in mammalian fertilisation, controversy remains regarding how the sperm initiates and maintains the signalling cascade responsible for the delivery of such a persistent stimulus. The advent of intracytoplasmic sperm injection (ICSI; Palermo *et al.* 1992), which bypasses fusion of the gametes and is able to induce a normal $[Ca^{2+}]_i$ responses in the mouse (Nakano *et al.* 1997; Kurokawa and Fissore 2003), seemed to have consolidated the view that a soluble sperm factor(s) diffuses from the sperm head into the ooplasm and is responsible for triggering the oscillations (Swann 1990). Intracytoplasmic sperm injection has been used widely for the production of offspring in species ranging from the mouse (Kimura and Yanagimachi 1995) to the human (Devroey and Van Steirteghem 2004). However, success with this technique has been very limited in

large domestic livestock species, namely the bovine, porcine and equine (Suttner *et al.* 2000; Bedford *et al.* 2003; Choi *et al.* 2004). With the exception of the birth of several calves (Goto *et al.* 1990; Horiuchi *et al.* 2002; Wei and Fukui 2002; Galli *et al.* 2003), conventional ICSI techniques have failed to produce physiological rates of embryonic and fetal development in the absence of an exogenous activation stimulus, such as calcium ionophore and an inhibitor of M-phase cyclins (or their respective cyclin-dependent kinases; Rho *et al.* 1998; Chung *et al.* 2000; Suttner *et al.* 2000). Therefore, the shortcomings of this technique in species such as the bovine pose serious questions as to the mechanism of oocyte activation in these species and raise valid concerns regarding the identity of the sperm factor(s) and its mechanism of activation and/or release in large domestic species. Notably, a recent report has demonstrated that bull sperm subjected to ICSI do not undergo comparable dissolution of the sperm perinuclear theca, a dense cytoskeletal matrix contiguous with the nucleus, as is seen during *in vitro* fertilisation (IVF; Sutovsky *et al.* 2003). Because it has been hypothesised that the active sperm factor molecule resides in the perinuclear theca (Kimura *et al.* 1998; Perry *et al.* 2000), the inability of the egg to disassemble critical regions of this structure may prevent the release of the active molecule and preclude oocyte activation. With regard to the identity of the sperm factor, a sperm-specific PLC, namely PLC ζ , has been identified recently in mouse sperm and it is believed to represent the sperm factor (Saunders *et al.* 2002). Phospholipase C ζ is expressed in bull sperm (Malcuit *et al.* 2005) and it is possible that the release or activation of PLC ζ may be compromised during ICSI in large domestic species.

If, indeed, the absence of release of the active sperm factor/PLC ζ is the limiting factor in bovine ICSI, the very early events of egg activation, namely the presence of [Ca²⁺]_i oscillations and the underlying production of IP₃, should not take place. Therefore, in an effort to address the mechanistic failures inherent to bovine ICSI, we made use of Ca²⁺ imaging to determine whether sperm injected into the ooplasm of bovine oocytes are capable of initiating and maintaining [Ca²⁺]_i oscillations, as seen during fertilisation (Fissore *et al.* 1992; Nakada *et al.* 1995). We also sought to determine whether chemical manipulation of the physical properties of the sperm structure would facilitate the initiation of [Ca²⁺]_i release after this procedure. Finally, we investigated whether the rates of preimplantation embryo development after ICSI corresponded with the [Ca²⁺]_i responses triggered by this method in the bovine.

Materials and methods

Animal care and welfare

Animals used for gamete collections for the present studies were handled according to the National Research Council Animal Care and Welfare guidelines and these procedures were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts.

Collection of gametes

Germinal vesicle-stage bovine oocytes were isolated from cow ovaries collected from an abattoir located in the mid-western region of the USA. Oocytes were isolated from follicles 2–8 mm in diameter and only those with several layers of compact cumulus cells were selected. Following isolation, oocytes were placed into vials in 2 mL TCM-199 medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), 0.1 U mL⁻¹ luteinising hormone (LH; Sioux Biochemical, Sioux Center, IA, USA) and 1 μ g mL⁻¹ oestradiol for 20 h. Oocytes were shipped overnight at 39°C in a portable incubator (Mini Tube of America, Verona, WI, USA). At approximately 20 h after the onset of maturation, oocytes for ICSI experiments were denuded of their cumulus cells by brief vortexing in Tyrode's lactate medium (Parrish *et al.* 1988) supplemented with 0.3% Fraction V bovine serum albumin (BSA) and 0.001% hyaluronidase (Sigma, St Louis, MO, USA; all chemicals were obtained from Sigma unless indicated otherwise). Cumulus-free oocytes were held in KSOM (Speciality Media, Phillipsburg, NJ, USA) supplemented with 1/2 \times amino acids (Life Technologies, Invitrogen, Carlsbad, CA, USA) and 0.1% Fraction V BSA. Cumulus-enclosed oocytes to be used for IVF were washed for 5 min in Tyrode's Albumin Lactate Pyruvate (TALP)–HEPES (Eid *et al.* 1994) and were then transferred into fertilisation medium (Eid *et al.* 1994). Bull sperm was graciously donated by Dr Marvin Pace (American Breeder Services, DeForest, WI, USA). Live, motile sperm were separated from cryoprotectant using the Percoll method (Parrish *et al.* 1985).

Mouse oocytes were collected from B6D2F1 female mice between 8 and 12 weeks of age. Mice were superovulated by sequential injections of 5 IU equine chorionic gonadotrophin (eCG) followed by 5 IU human chorionic gonadotrophin (hCG), as described previously (Wu *et al.* 1998). Metaphase II (MII) oocytes were collected from the oviducts of stimulated females 14 h following the administration of hCG into Tyrode's Lactate (TL)–HEPES supplemented with 10% FCS. Cumulus cells were removed by exposure to 0.025% hyaluronidase for 3–5 min followed by washing in TL–HEPES. Mouse sperm was obtained from the cauda epididymis of 12- to 16-month-old CD-1 males and collected into injection buffer (IB; 75 mM KCl and 20 mM HEPES, pH 7.0).

Boar sperm was obtained from a fresh ejaculate at the University of Massachusetts' barn and was washed and stored in IB at –80°C for no longer than 1 week before use.

Sperm treatments

Several treatments were applied to sperm before ICSI. All working solutions were prepared fresh daily and sperm were washed several times in IB after treatment, before injection. Sperm decapitation was accomplished by sonication using an XL2020 sonicator (Heat Systems, Farmingdale, NY, USA; Wu *et al.* 1998) on a setting of 3 for 30 s on ice. Triton X-100 (TX-100), a mild detergent, was used to permeabilise sperm heads. The TX-100 was diluted in IB at a concentration of 1% and sperm were incubated in this solution for 15 min at room temperature. Dithiothreitol (DTT), a reducing agent, was prepared at a concentration of 15 mM in TALP–HEPES and sperm were incubated in this solution for 1 h at room temperature. Capacitation was performed in fertilisation medium containing 6 mg mL⁻¹ fatty acid-free BSA, 25 mM Na₂HCO₃, 10 μ g mL⁻¹ heparin, 1 mM dibutyryl cAMP and 100 μ M 3-isobutyl-1-methylxanthine (IBMX; Galantino-Homer *et al.* 1997) for 2 h at 39°C in 5% CO₂. The acrosome reaction was induced by the addition of 5 μ M ionomycin (Calbiochem, San Diego, CA, USA) for 10 min immediately after the 2 h capacitation incubation (Larson and Miller 1999).

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection was performed as described previously (Kimura and Yanagimachi 1995; Knott *et al.* 2003) with some

modifications. Briefly, either intact or treated sperm (see above) were resuspended in an arbitrary volume of IB, to which an equal volume of 12% polyvinyl pyrrolidone (PVP) was added. The sperm/PVP suspension was placed in a 30- μ L drop onto a 100-mm Petri dish along with 50- μ L drops of either TL-HEPES (for bovine) or Chatot-Ziomek-Bavister (CZB)-HEPES (for mouse), each supplemented with 0.05% polyvinyl alcohol (PVA). Bovine oocytes were centrifuged in 200 μ L TL-HEPES for 8 min at 5000g to polarise lipid droplets to allow visualisation of sperm injection. Intracytoplasmic sperm injection was performed on a Nikon (Tokyo, Japan) TE200 inverted microscope under 200 \times phase-contrast magnification. Sperm were aspirated into a 12- μ m (outside diameter) blunt, glass pipette preloaded with 1 cm Hg. Oocytes were positioned with a fire-polished holding pipette (75 μ m outside diameter) with the first meiotic polar body to either the 12 or 6 o'clock position to avoid disruption of the actin-rich network at the spindle pole. The injection pipette containing sperm was placed against the zona pellucida and a single pulse of a PiezoDrill (Burleigh Instruments, Rochester, NY, USA) was delivered with gentle aspiration to facilitate zona pellucida (ZP) penetration. Once through the ZP, the pipette was placed approximately three-quarters across the oocyte and the oolema was aspirated gently into the injection pipette while applying a second pulse of the PiezoDrill to break the oolema. The sperm was then deposited in the ooplasm and the pipette withdrawn.

In vitro fertilisation

In vitro fertilisation was performed as described previously (Damiani *et al.* 1996; Sutovsky *et al.* 1997; Knott *et al.* 2003). Briefly, cumulus-enclosed oocytes were washed for 5 min in TALP-HEPES, picked up in a 2- μ L volume and then placed into 44- μ L drops equilibrated fertilisation medium under oil. Oocytes were allowed to incubate for 1 h before insemination. Sperm was prepared by the Percoll method, as described above. Briefly, sperm were centrifuged for 20 min at 400g through a discontinuous gradient of 45% and 90% Percoll. Live, motile sperm were collected from the pellet and sperm was diluted appropriately in TALP-HEPES to yield a final concentration of 1×10^6 in the insemination drop. Immediately following insemination, heparin, diluted in TALP-HEPES, was added to the insemination drops to a final concentration of 10 μ g mL⁻¹. For [Ca²⁺]_i-monitoring experiments, inseminated oocytes were removed from the fertilisation medium 8 h after insemination and were denuded of cumulus cells by brief vortexing in warm TALP-HEPES.

Parthenogenetic activation of bovine oocytes

At 26 h after the onset of *in vitro* maturation, bovine oocytes were selected for the presence of a first meiotic polar body. These oocytes were then placed into a solution of TL-HEPES containing 5 μ M ionomycin (Calbiochem) for 4 min. Following several washes in TL-HEPES, ionomycin-treated oocytes were placed into a solution of KSOM (described below) containing 5 μ g mL⁻¹ dihydro cytochalasin B (Calbiochem) and 10 μ g mL⁻¹ cyclohexamide (Calbiochem) for 6 h at 38.5°C in 5% CO₂ in air. After four consecutive washes through TL-HEPES, activated oocytes were placed into culture dishes containing KSOM (see culture methods below).

Embryo culture

Immediately after fertilisation, ICSI and parthenogenetic activation, all zygotes were cultured in KSOM containing 1 mg mL⁻¹ BSA and supplemented with 1/2 \times essential and non-essential amino acids (Life Technologies) until Day 3 of culture. On Day 4, embryos were transferred to the above medium supplemented with 10% heat-treated FCS (Hyclone, Logan, UT, USA) and cultured for an additional 4 days. All culture was performed at 38.5°C in 5% CO₂, 5% O₂ and a balance of 90% N₂.

Measurement of [Ca²⁺]_i

Bovine and mouse oocytes were injected with the fluorescent dye Fura-2 dextran or loaded with 1 μ M Fura-2 acetoxymethylester (Fura-2 AM; Molecular Probes, Eugene, OR, USA) supplemented with 0.02% Pluronic F-127 (Molecular Probes) at room temperature for 20 min. The Ca²⁺ imaging was performed as described elsewhere (Fissore *et al.* 1992). In brief, excitation wavelengths were at 340 and 380 nm and the light emitted was quantified, after passing through a 500-nm barrier filter. Neutral density filters attenuated the intensity of the excitation light and the fluorescent signal was averaged for the whole cell. Oocytes were monitored in groups of six to nine in 40- μ L drops of TL-HEPES on a glass coverslip on the bottom of a Petri dish covered with paraffin oil. Fluorescence ratios were taken every 20–30 s for various time-points depending on the experiment and are reported as the ratios of 340/380 nm fluorescence.

Assessment of oocyte activation

Approximately 16 h after insemination or 12 h after ICSI, the ZP of the zygotes was removed by brief incubation in acid Tyrode's solution, pH 1.8, at room temperature. After removal of the ZP, zygotes were placed into 3% paraformaldehyde containing 0.25% TX-100. Fixation was performed for 30–60 min at room temperature. Embryos were washed extensively in phosphate-buffered saline (PBS) containing 0.3% PVP for 12–14 h. Groups of up to 10 embryos were then mounted on glass slides in 90% glycerol containing 0.5 mg mL⁻¹ Hoechst 33358. Embryos were visualised for the presence of a pronucleus on a Nikon TE300 inverted microscope with ultraviolet (UV) fluorescence at a magnification of $\times 200$.

Western blot technique

To assess the levels of intact IP₃R-1 protein, five oocytes were added to 15 μ L double-strength reducing Laemmli buffer (Laemmli 1970), as described previously (He *et al.* 1999; Jellerette *et al.* 2000). Samples were boiled for 3 min to denature proteins, loaded onto a 5% sodium dodecyl sulfate (SDS) polyacrylamide gel and proteins were separated by electrophoresis followed by transfer onto nitrocellulose membranes (Micron Separations, Westboro, MA, USA). Membranes were blocked in PBS containing 0.1% Tween (PBST) supplemented with 5% non-fat dry milk for 1.5 h and incubated overnight with one of two rabbit polyclonal antibodies raised against the same C-terminal region of IP₃R-1 diluted in PBST (Parys *et al.* 1995). Membranes were subsequently washed in PBST, followed by incubation for 1 h with a goat anti-rabbit secondary antibody coupled to horseradish peroxidase. Membranes were incubated for 1 min in chemiluminescence reagent (NEN Life Science Products, Boston, MA, USA) and developed according to the manufacturer's instructions. Prelabelled molecular weight markers were run in parallel to estimate the molecular weight of the IP₃R-1 bands. The intensities of IP₃R-1 bands were assessed using a Kodak 440 Image Station (Eastman Kodak, Rochester, NY, USA) and plotted using Sigma Plot (Jandel Scientific Software, San Rafael, CA, USA). The IP₃R-1 band from MII oocytes was arbitrarily given the value of 100% and the values from the bands in other lanes were expressed relative to 100%.

Statistical analysis

Statistical analysis was performed for all experiments using a two-tailed *t*-test assuming unequal variances. Only those values within the 5% confidence interval ($P < 0.05$) were considered to be statistically significant. All values presented herein are displayed as the mean + the standard error above the mean.

Results

Bull sperm initiate an incomplete $[Ca^{2+}]_i$ response after intracytoplasmic sperm injection

The $[Ca^{2+}]_i$ oscillations are required to induce oocyte activation in mammals (for reviews, see Schultz and Kopf 1995; Ducibella *et al.* 2002). Coincidentally, a dominant feature of oocytes of large domestic species subjected to ICSI is the lack of activation and cleavage (Suttner *et al.* 2000; Wei and Fukui 2002). Hence, to determine whether the lack of efficiency of ICSI in the bovine could be ascribed, at least in part, to failure to initiate appropriate $[Ca^{2+}]_i$ responses, we compared the $[Ca^{2+}]_i$ oscillations observed during IVF with those induced by ICSI. As expected, the majority of bovine eggs that underwent IVF showed $[Ca^{2+}]_i$ oscillations, which were characteristically separated by prolonged intervals (Fig. 1a; Fissore *et al.* 1992). Importantly, injection of an intact bull sperm was unable to trigger Ca^{2+} release/oscillations in the majority of oocytes monitored (data not shown), although,

in a few cases, a single $[Ca^{2+}]_i$ transient was observed within 1 h of sperm injection (Fig. 1b).

Capacitation and the acrosome reaction of bull sperm do not increase the incidence of $[Ca^{2+}]_i$ increases after intracytoplasmic sperm injection

The demonstrated inability of bull sperm to initiate oscillations upon injection into bovine oocytes could be attributed to the fact that, during ICSI, certain physiological events that occur before sperm entry are bypassed. For instance, it is well known that mammalian sperm must undergo capacitation and the acrosome reaction to penetrate the oocyte vestments and fuse with the oolema. Accordingly, capacitation and the acrosome reaction were induced in bull sperm before ICSI and $[Ca^{2+}]_i$ monitoring. To induce capacitation, sperm were incubated in TALP-HEPES containing dbcAMP, IBMX, 6 mg mL^{-1} BSA, $10 \mu\text{g mL}^{-1}$ heparin and 25 mM NaHCO_3 for 2 h at 38°C (Wu *et al.* 1998). Western blot

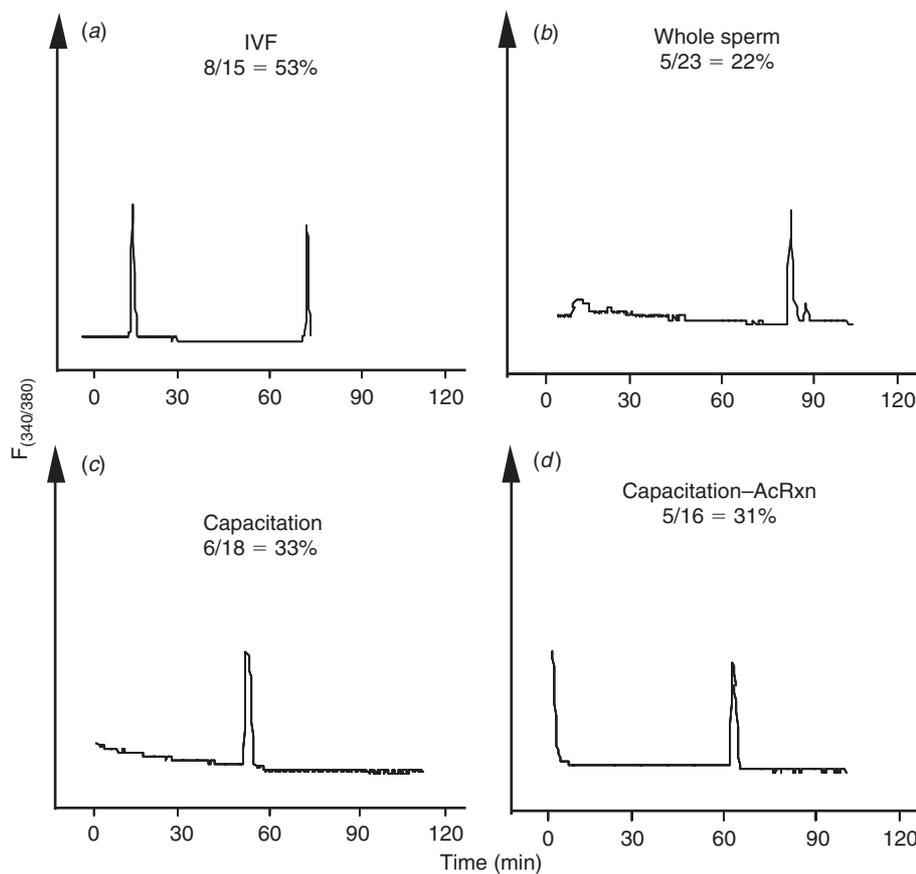


Fig. 1. The $[Ca^{2+}]_i$ responses in bovine oocytes subjected to intracytoplasmic sperm injection (ICSI) cannot duplicate the responses observed following *in vitro* fertilisation (a). Untreated whole sperm (b), capacitated (c) and acrosome-reacted (d) bull sperm were injected into mature metaphase II bovine eggs and the typical $[Ca^{2+}]_i$ response for each group is depicted. Fifty-three per cent of IVF zygotes displayed $[Ca^{2+}]_i$ oscillations. Conversely, none of the ICSI groups tested was able to match either the percentage of oocytes responding or the frequency of oscillations observed. Data show the ratiometric value of 340/380 nm fluorescence ($F_{(340/380)}$; y-axis) over time (min; x-axis).

analysis for tyrosine-phosphorylated proteins, a reliable indicator of sperm capacitation (Visconti *et al.* 1995), confirmed that capacitation was taking place under our conditions and the maximal intensity of this signal was seen by 2 h (data not shown). After exposure to capacitating conditions, sperm were washed several times in TALP-HEPES and were resuspended in IB-PVP for ICSI. To induce the acrosome reaction, capacitated sperm were treated with 5 μM ionomycin for 10 min in TALP-HEPES, washed and prepared as above. Capacitated and acrosome-reacted sperm (Fig. 1*c,d*, respectively) injected into MII oocytes failed to consistently initiate repetitive $[\text{Ca}^{2+}]_i$ oscillations as those observed during IVF. Collectively, these results demonstrate that conventional ICSI techniques are not capable of initiating the $[\text{Ca}^{2+}]_i$ oscillations typical of those seen after fertilisation in the bovine and that neither capacitation nor the acrosome reaction of sperm induced by *in vitro* protocols increase the Ca^{2+} -releasing ability of bull sperm.

Bull sperm contain a full complement of $[\text{Ca}^{2+}]_i$ oscillation-inducing activity

Given the inability of bull sperm to initiate oscillations upon injection into bovine oocytes, it could be thought that, unlike the sperm of other species, bull sperm do not contain the so-called sperm factor. Even though it has been shown that bull sperm has the ability to initiate oscillations upon injection into mouse oocytes (Knott *et al.* 2003), we further extended those results here by showing that injection of decapitated bull sperm did, indeed, initiate high-frequency oscillations in mouse oocytes (Fig. 2*a*). Conversely, injection of mouse sperm into bovine oocytes failed to initiate $[\text{Ca}^{2+}]_i$ responses in this species (Fig. 2*b*), indicating that the inability to induce oscillations after ICSI in large domestic species may be due to a combination of both sperm and oocyte factors.

Polyspermy increases the number of eggs responding to intracytoplasmic sperm injection, but not the frequency of $[\text{Ca}^{2+}]_i$ oscillations

Having demonstrated that bull sperm contain a full complement of sperm factor, a possible interpretation of the failure to initiate oscillations in bovine oocytes is that the factor is either not released and/or activated when fertilisation is accomplished via sperm injection. Hence, to test the first possibility, we injected three bull sperm heads into cow oocytes with the notion that partial release from more than one sperm may restore the normal pattern of oscillations. Sperm heads were injected rather than whole sperm because injection of three whole sperm would have required the injection of large volumes likely to result in cell lysis. The injection of three sperm heads increased the proportion of oocytes showing $[\text{Ca}^{2+}]_i$ responses (12/30 (40%); Fig. 3*b*) compared with oocytes injected with one sperm head (1/33 (3%); Fig. 3*a*). Nonetheless, the frequency of oscillations was not greatly affected

by this treatment and, only in a few occasions, two $[\text{Ca}^{2+}]_i$ transients were observed separated by prolonged intervals (Fig. 3*b*). This result suggests that some of the active sperm factor molecules are released from the sperm, albeit not in the concentrations required to initiate $[\text{Ca}^{2+}]_i$ oscillations; therefore, injection of three sperm was able to compensate somewhat for this inadequacy.

Partial sperm head decondensation and disruption of the sperm plasma membrane increases the number of eggs responding to polyspermic intracytoplasmic sperm injection

Although sperm decapitation may facilitate the contact of the sperm membranes with the ooplasm, and consequently enhance the release of the sperm factor, we sought to further stimulate this interaction by artificially manipulating the physical and chemical structure of the sperm by various chemical treatments before ICSI. We first investigated the effect of reducing the disulfide bonds of sperm

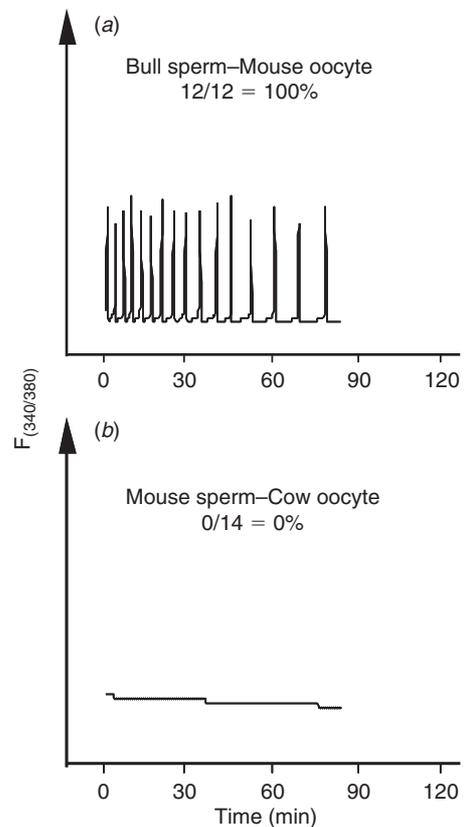


Fig. 2. The Ca^{2+} -releasing ability of bovine gametes. A single bull sperm head injected into a mature metaphase II (MII) mouse oocyte is capable of initiating high-frequency $[\text{Ca}^{2+}]_i$ oscillations in all zygotes monitored (a), whereas the reciprocal experiment of injecting a single mouse sperm head into a mature MII bovine oocyte (b) is not able to initiate even a single $[\text{Ca}^{2+}]_i$ transient in any of the zygotes monitored. $F_{(340/380)}$, 340/380 nm fluorescence.

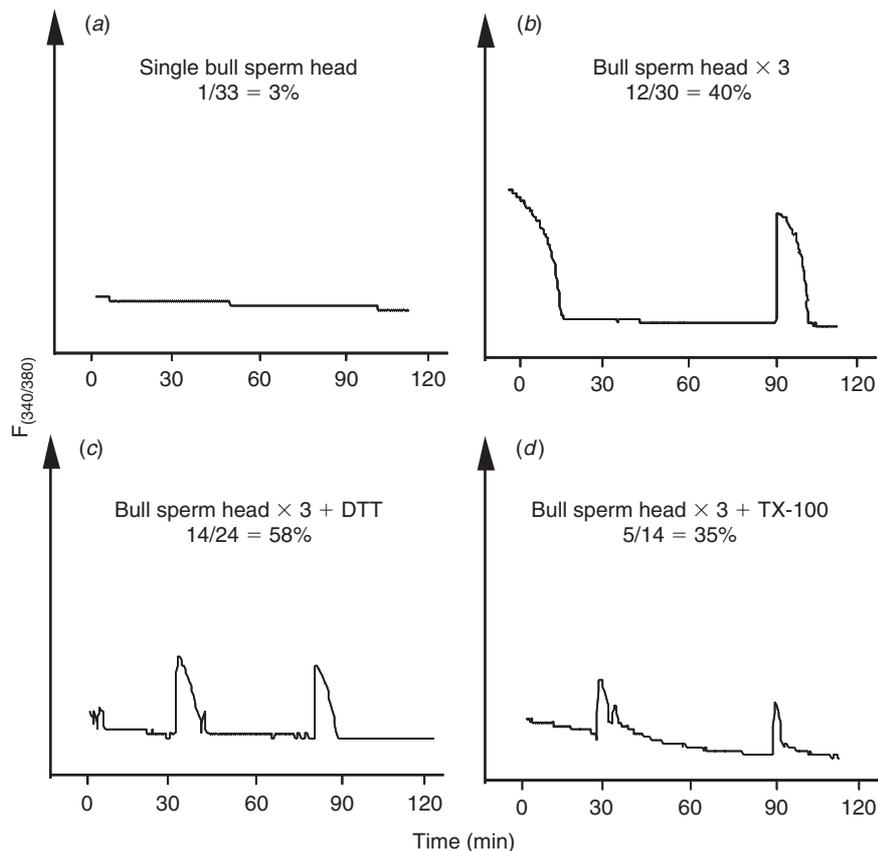


Fig. 3. Injection of three bull sperm heads increases the overall number of bovine oocytes responding to intracytoplasmic sperm injection. Injection of a single bull sperm head (a) is unable to initiate fertilisation-like $[Ca^{2+}]_i$ oscillations in bovine oocytes, whereas injection of three sperm heads increases the number of oocytes responding to 40% (b). Treatment of sperm heads with dithiothreitol (DTT) to facilitate sperm head decondensation (c) further increased the $[Ca^{2+}]_i$ responses to the injection of three sperm, although treatment of sperm heads with Triton X-100 did not do so (d). $F_{(340/380)}$, 340/380 nm fluorescence.

protamines with DTT, because this treatment has been shown to enhance sperm head decondensation (Perreault *et al.* 1998) and development after ICSI (Galli *et al.* 2003). Injection of DTT-treated sperm, which displayed a swelling of the sperm head, as described previously (Kimura *et al.* 1998), increased the overall number of oocytes displaying $[Ca^{2+}]_i$ responses (14/24 (58%); Fig. 3c) but did not consistently initiate oscillations in the injected oocytes. We next chose to permeabilise the sperm's membranes with the hope of increasing the access of the ooplasm to the per-nuclear matrix, which is the structure thought to harbor the sperm's active Ca^{2+} -releasing molecule (Kimura *et al.* 1998; Perry *et al.* 2000). To accomplish this, we exposed sperm to TX-100, followed by polyspermic injection as above. As shown in Fig. 3d, TX-100 treatment of sperm did not appear to increase either the number of responding oocytes or the frequency of $[Ca^{2+}]_i$ oscillations. Collectively, these results suggest that factors downstream of the

release of sperm factor, such as activation and/or substrate availability, may limit the initiation of $[Ca^{2+}]_i$ responses in bovine ICSI.

Porcine sperm are capable of eliciting persistent $[Ca^{2+}]_i$ oscillations in cow oocytes after intracytoplasmic sperm injection

Because boar sperm seemingly contain greater Ca^{2+} -releasing ability than both mouse and bull sperm when injected into mouse oocytes (Fig. 4a), we investigated whether some of the treatments tested previously with bull sperm, if applied to boar sperm, could induce fertilisation-like oscillations upon injection into bovine oocytes. In support of this approach, we have shown previously that injection of boar sperm extracts into bovine oocytes initiated fertilisation-like oscillations (Knott *et al.* 2002). Whereas ICSI with a single boar sperm was unable to increase the

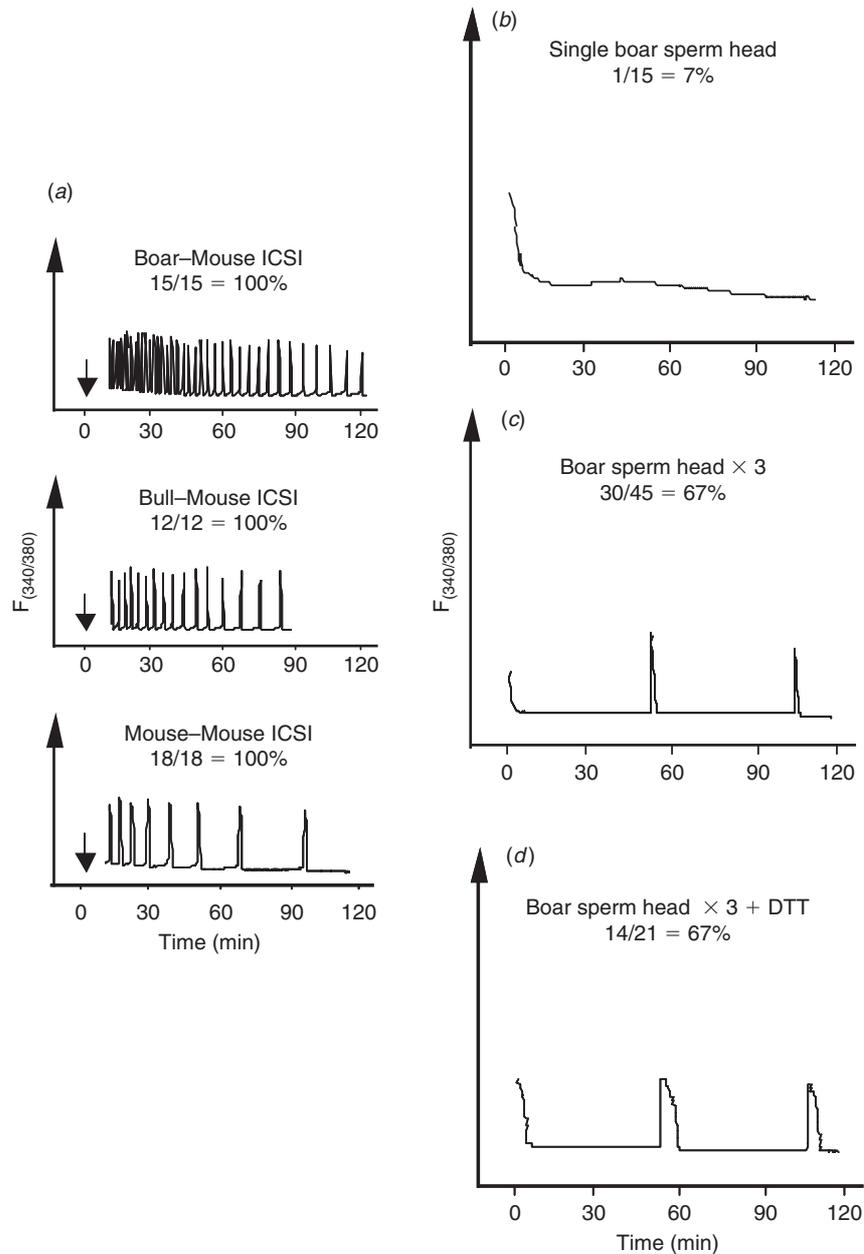


Fig. 4. The $[Ca^{2+}]_i$ responses to intracytoplasmic sperm injection (ICSI) of porcine sperm. Porcine (boar) sperm naturally possess more $[Ca^{2+}]_i$ oscillation-inducing ability than do either mouse or bull sperm when assayed in mouse oocytes (a). Injection of a single boar sperm head (b) was unable to increase the response rate in compared with ICSI with bull sperm (cf. Fig. 3a). Injection of three boar sperm heads, whether (d) treated with dithiothreitol (DTT) or (c) not, substantially increased the $[Ca^{2+}]_i$ responses to ICSI. $F_{(340/380)}$, 340/380 nm fluorescence.

frequency of oscillations or the number of responding oocytes (Fig. 4b) compared with injection of a single monospermic bull sperm (from Fig. 1b), the injection of three boar sperm, whether treated or not with DTT, resulted in an increase in both the number of responding oocytes and the presence

of oscillatory oocytes (Fig. 4c,d). These results suggest that bovine oocytes can mount oscillatory responses upon sperm injection, although it appears that either the release and/or activation of the bull sperm factor is compromised after injection into these oocytes.

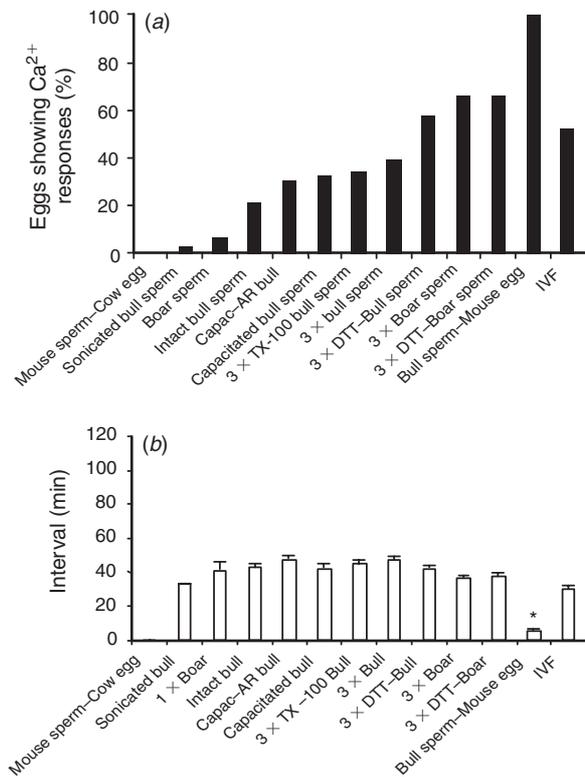


Fig. 5. (a) Percentage of oocytes showing $[Ca^{2+}]_i$ responses to sperm according to treatment. The total number of oocytes responding to intracytoplasmic sperm injection (ICSI) with at least an increase in $[Ca^{2+}]_i$ can be increased by treatments, with polyspermy being the most successful. (b) Average oscillation intervals of responding oocytes. Values represent intervals for those only oocytes showing at least one Ca^{2+} spike within the 120-min monitoring period following ICSI or *in vitro* fertilisation (IVF). The only treatment group statistically significant from bovine IVF is that of bull sperm injected into mouse oocytes. It is interesting to note that, under normal fertilising conditions, polyspermy increases the frequency of oscillations.

Failure of bovine intracytoplasmic sperm injection is due to the inability to induce sustained $[Ca^{2+}]_i$ oscillations

Recent studies have shown that the initiation and completion of the different oocyte activation events have different $[Ca^{2+}]_i$ requirements, with late activation events, such as pronuclear formation and recruitment of maternal mRNAs, requiring many more $[Ca^{2+}]_i$ increases than early activation events (Ducibella *et al.* 2002). Therefore, we investigated whether ICSI-derived bovine zygotes that initially displayed $[Ca^{2+}]_i$ responses were able to sustain these oscillations, as would be expected to occur after IVF. To accomplish this, we used DTT-treated sperm because this is the only treatment that was able to increase the number of responding oocytes after injection of both bovine and porcine ICSI (Fig. 5a). Single bull sperm were injected to provide, as close as possible, a control to IVF and, as mentioned, only ICSI zygotes with an observed $[Ca^{2+}]_i$ response were used for these comparisons. To control for synchronicity, inseminated

eggs were assessed for sperm penetration by staining with Hoechst 33342 at 1 h intervals after IVF. Approximately 80% of the total fertilisation observed occurred between 6 and 8 h after insemination (data not shown). At 8 h after insemination, IVF embryos were denuded of their cumulus cells by brief vortexing in warm TL-HEPES, injected with Fura-2 dextran and transferred immediately to a $[Ca^{2+}]_i$ imaging dish. After sufficient time had elapsed to determine an accurate number of zygotes oscillated for both ICSI and IVF, all oscillating oocytes for either ICSI or IVF were selected and transferred back into the incubator for a period of 2.5 h. After such time, these zygotes were returned to the microscope for further Ca^{2+} imaging. Whereas seven of 10 IVF zygotes continued to oscillate for an additional 2 h (time monitored; Fig. 6a,b), only two of 11 ICSI-derived zygotes showed persistent oscillations (Fig. 6c,d and inset). This result implies that one of the major limiting factors in bovine ICSI lies in the inability of the bull sperm to initiate persistent $[Ca^{2+}]_i$ oscillations. Because IP_3 production underlies the initiation of oscillations in mammalian fertilisation (Jellerette *et al.* 2000), we ascertained whether the concentration of IP_3R-1 in bovine oocytes after ICSI has been reduced. Downregulation of IP_3R-1 , which is mediated by the proteasome (Oberdorf *et al.* 1999), is observed after fertilisation (Jellerette *et al.* 2000) and it is known to be induced exclusively by IP_3 binding to the receptor (Zhu and Wojcikiewicz 2000). As shown in Fig. 6e, only IVF-derived zygotes displayed an observable decrease in the mass of intact IP_3R-1 , whereas ICSI-derived embryos contained similar amounts of IP_3R-1 to both MII eggs and aged-matched control eggs ($n = 2$). This result confirms the hypothesis that bovine ICSI fails to induce robust IP_3 production and that this is responsible for the partial $[Ca^{2+}]_i$ responses observed after sperm injection in this species.

Intracytoplasmic sperm injection-derived bovine zygotes are capable of reaching the blastocyst stage

Notwithstanding the findings that ICSI-derived zygotes are not nearly as efficient as IVF-derived zygotes at producing normal $[Ca^{2+}]_i$ oscillations, there is a small percentage of oocytes treated by ICSI that does manage to initiate persistent $[Ca^{2+}]_i$ responses. In accordance with this observation, it is not surprising, then, that reports in the literature have shown that a small percentage of ICSI-derived zygotes is capable of reaching the blastocyst stage of development (Goto *et al.* 1990; Wei and Fukui 2002; Galli *et al.* 2003). Moreover, to confirm this, bovine ICSI (with DTT-treated sperm) and IVF-derived zygotes (produced by the same methodology described in the preceding section) were cultured for 8 days in KSOM, with 10% FBS supplemented at Day 4. As expected, IVF-derived embryos were capable of reaching the blastocyst stage (Fig. 7b,e,g) at levels comparable to parthenogenetic control embryos (Fig. 7a,d,g), whereas ICSI-derived embryos reached this stage at very low levels

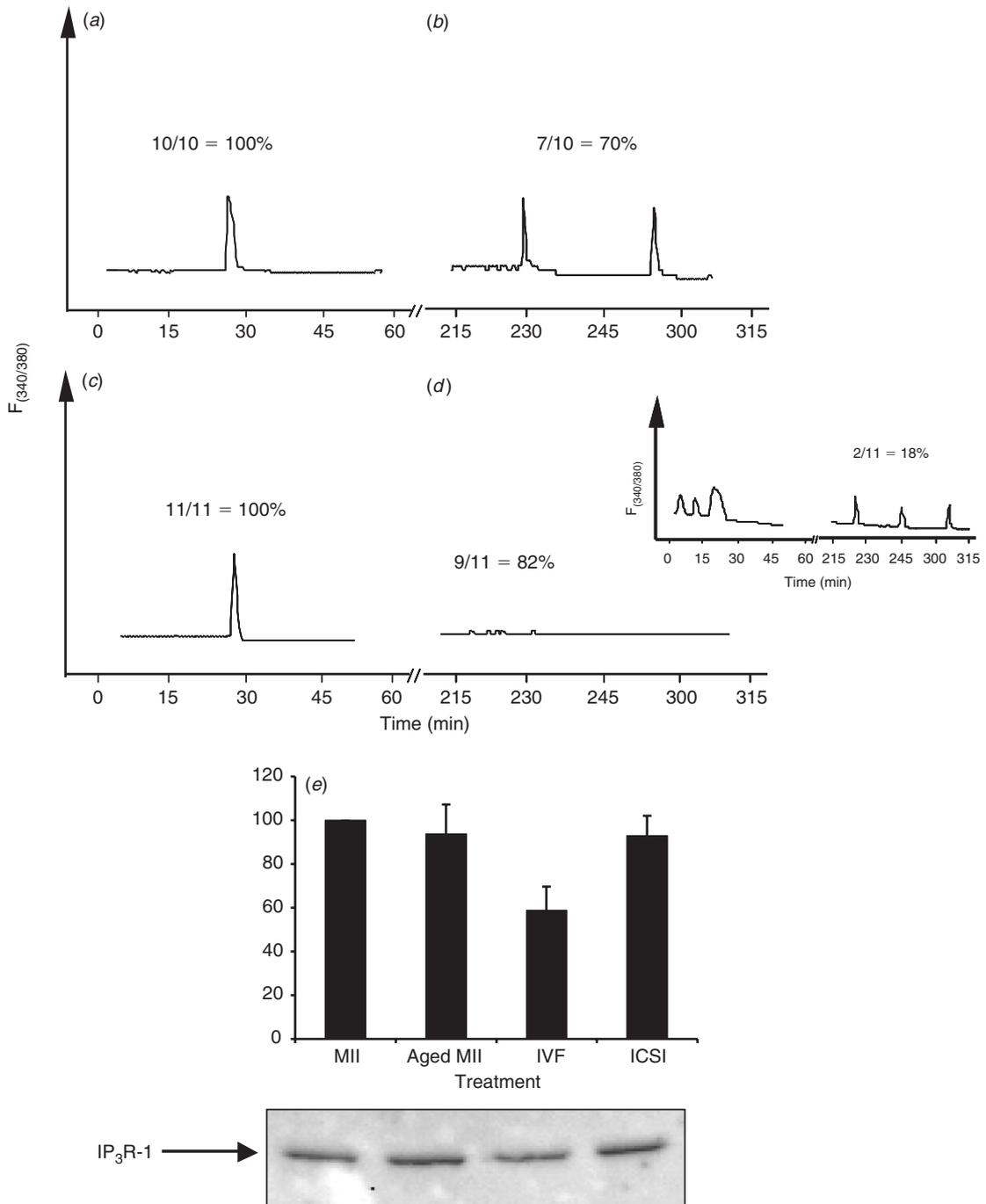


Fig. 6. Bull sperm are unable to mount persistent $[Ca^{2+}]_i$ oscillations in oocytes responding to intracytoplasmic sperm injection (ICSI). Eight hours after insemination (a) or 1 h after ICSI (c), bovine zygotes were monitored for $[Ca^{2+}]_i$ oscillations. Only those zygotes displaying oscillations were chosen and were then incubated for an additional 2.5 h. At this time, *in vitro* fertilisation (IVF)-derived zygotes (b) and ICSI-derived zygotes (d), which had displayed earlier $[Ca^{2+}]_i$ oscillations, were once again monitored for their ability to oscillate over time. Whereas 70% of IVF zygotes (7/10) still displayed continuous $[Ca^{2+}]_i$ oscillations (b), only 18% of ICSI zygotes (d, inset; 2/11) displayed similar responses and the remaining 82% (d; 9/11) failed to show any further response. In accordance with this observation, ICSI-derived zygotes are unable to produce enough inositol 1,4,5-trisphosphate (IP₃), which is evidenced here by the inability to downregulate the IP₃R-1 receptor in bovine oocytes at comparable rates to IVF-produced embryos (e; $P < 0.05$). The ICSI-zygotes, unstimulated metaphase II (MII) and aged MII bovine oocytes exhibit similar rates of IP₃R-1 degradation ($P > 0.05$). Oocytes and/or zygotes were collected for preparation of lysates 12 h after ICSI and 16 h after IVF. F_(340/380), 340/380 nm fluorescence.

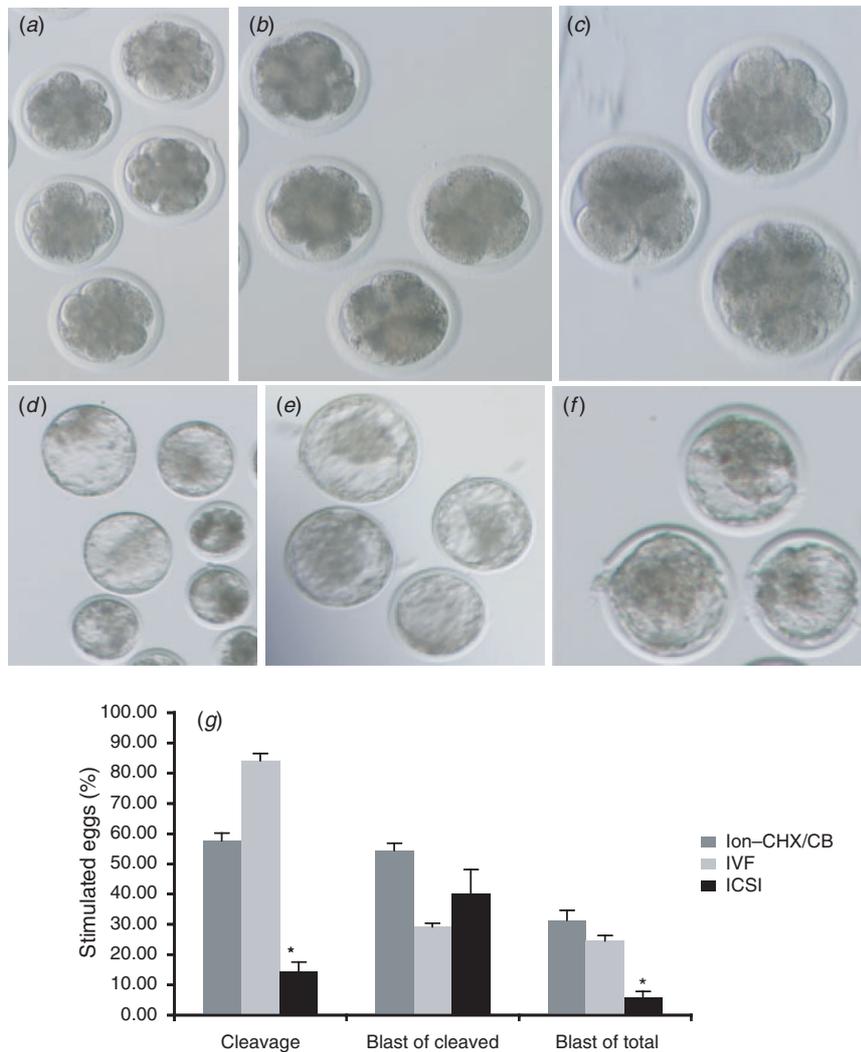


Fig. 7. Intracytoplasmic sperm injection (ICSI) is capable of producing blastocyst-stage bovine embryos. Four- to eight-cell stage embryos (*a–c*) and blastocysts (*d–f*) were produced by parthenogenesis (ionomycin–cyclohexamide + cytochalasin B (Ion-CHX/CB); *a, d*), *in vitro* fertilisation (IVF; *b, e*) and ICSI (*c, f*). (*g*) Although rates of early embryonic cleavage are significantly lower in ICSI-derived embryos compared with parthenogenetic or IVF-derived embryos ($P < 0.05$), the rates of blastocyst development of cleaved embryos were similar among the activation methods used here ($P > 0.05$).

(Fig. 7*c, f, g*). However, the most interesting observation here is that the percentage of blastocysts from cleaved embryos was not significantly dissimilar between IVF and ICSI. It becomes apparent, then, that the most significant limiting factor for successful embryonic development in bovine ICSI is the inability to reach the early stages of development, most likely due to a defective activation stimulus caused by the abnormal $[Ca^{2+}]_i$ responses that follow sperm injection.

Discussion

Intracytoplasmic sperm injection is a very powerful technique in the field of assisted reproduction and has been responsible

for the production of thousands of human infants across the globe. Nevertheless, its application in agriculture and conservation biology has been greatly reduced by the low success rate reported for this method in species of economical importance. Specifically, oocyte activation and zygote cleavage are greatly reduced in ICSI-generated zygotes. Hence, it is of importance from an applied stand-point, as well as for a basic understanding of the mechanisms that underlie fertilisation in mammals, that we identify the signalling pathways and cellular events that occur during normal fertilisation but that fail to be activated during ICSI in large domestic species.

Towards this end, the results of our study indicate that ‘the bovine ICSI problem’ can be ascribed, at least in part,

to one of the very earlier steps of fertilisation, namely the initiation of $[Ca^{2+}]_i$ oscillations. We observed that less than 10% of all oocytes subjected to monospermic ICSI showed any $[Ca^{2+}]_i$ responses. Also revealing were the findings that although injection of multiple sperm in conjunction with chemical treatments increased the overall number of eggs responding to ICSI, such treatments failed to increase the frequency of $[Ca^{2+}]_i$ oscillations, which is expected in cases of polyspermic fertilisation (Figs 3–5). Finally, whereas the majority of oscillations initiated in response to IVF were sustained, as determined by extended monitoring, the $[Ca^{2+}]_i$ responses initiated by ICSI failed to be so. Taken together, the results demonstrate that some of the signalling mechanisms that lead to the activation of the phosphoinositide pathway and generation of oscillations during natural fertilisation are not being replicated by ICSI. The latter is consistent with findings in mouse ICSI, which showed that, in spite of the fact that sperm injection induced normal rates of oocyte activation and embryo development, the pattern of $[Ca^{2+}]_i$ oscillations and IP_3 production, which was evaluated by assessing IP_3R-1 degradation, appeared compromised (Kurokawa and Fissore 2003).

The inability to initiate oscillations and induce oocyte activation appears to be the main reason compromising the success of ICSI in the bovine. To assess the possible association of the presence of $[Ca^{2+}]_i$ oscillations and preimplantation in *in vitro* embryo development, we performed IVF, as described, and ICSI using single sperm heads treated with DTT, which was the only treatment tested herein that increased $[Ca^{2+}]_i$ responsiveness following injection of both bull and boar sperm. Interestingly, although the percentage of ICSI-derived zygotes reaching the blastocyst stage after this treatment was low compared with IVF-generated zygotes, this result was greatly influenced by the low incidence of early embryonic cleavage. However, analysis of the rate of ICSI-derived blastocysts out of cleaved embryos reveals that the rates of development between IVF and ICSI are similar (Fig. 7). Importantly, in both cases, the rates of development to the blastocyst stage closely corresponded to the percentage of oocytes that mounted persistent $[Ca^{2+}]_i$ responses (Figs 1a,b, 6a–d). Moreover, the notion that appropriate activation may be the most important limiting factor in bovine ICSI also emanates from recent findings showing that parthenogenetic activation after ICSI greatly improves preimplantation embryo development and the production of calves (Oikawa *et al.* 2005). The culmination of all these results demonstrates that, if the proper stimulus is delivered following fertilisation, be it IVF or ICSI, the resulting zygote is capable of reaching late stages of preimplantation development.

The question that arises then, is why ICSI fertilisation in the bovine does not induce persistent $[Ca^{2+}]_i$ oscillations? There are several factors that individually, or collectively, could account for oocyte activation failure in this species. The first possibility is that the release of the sperm factor

is compromised after ICSI. Because the sperm undergoes significant changes before fertilisation, such as capacitation and the acrosome reaction, which are bypassed during ICSI, it could be envisioned that some of the molecular underpinning of these events also facilitates the release of the factor upon sperm entry. Our findings that the injection of several sperm partly overcame the faulty $[Ca^{2+}]_i$ responses induced by ICSI support this contention. Nonetheless, polyspermic ICSI did not fully re-establish the oscillatory pattern, nor did any of the chemical treatments intended to facilitate the release of the factor. Moreover, our previous results in horse oocytes, which failed to initiate $[Ca^{2+}]_i$ oscillations after ICSI but did so immediately after fusing to a mouse MII oocyte, suggest the successful delivery of the sperm factor into the ooplasm (Bedford *et al.* 2004). Hence, these results raise the possibility that the function and/or activation of the sperm factor after release may also be compromised after ICSI, thereby leading to incomplete $[Ca^{2+}]_i$ responses. The mechanism(s) that regulates the function of the sperm factor remains to be established, because the identity of the putative molecule responsible for the sperm's $[Ca^{2+}]_i$ oscillatory activity remains to be fully established. The recent identification of a sperm-specific PLC ζ in the mouse and the demonstration that its depletion from hamster sperm extracts abrogates the Ca^{2+} activity of the extract suggest that PLC ζ may be the long sought-after active component of the sperm factor (Saunders *et al.* 2002). In agreement with this concept, our studies in the bovine showed that PLC ζ is expressed in sperm and that injection of mouse PLC ζ mRNA triggers fertilisation-like oscillations in this species (Malcuit *et al.* 2005). How the function of PLC ζ is regulated in the sperm, as well as in the oocyte after its release, and whether the function of PLC ζ is compromised after ICSI is not presently known. However, it is possible that the activity of PLC ζ may be determined, at least in part, by its cellular distribution and/or by interaction with binding partners. With regard to the former, PLC ζ possesses a nuclear localisation signal and nuclear sequestration during zygote formation has been shown to abrogate the $[Ca^{2+}]_i$ oscillations (Larman *et al.* 2004); in the sperm, PLC ζ seems to be localised to the post-acrosomal region (Fujimoto *et al.* 2004). With regard to interacting partners, it has been shown recently that the C2 domain of PLC ζ has great binding affinity for certain phosphoinositide lipids, the binding to which reportedly inhibits *in vitro* PLC ζ activity (Kouchi *et al.* 2004). Therefore, it is possible that the release of these inhibitory interactions may be required for the molecule to attain maximal function and that this 'processing' of PLC ζ may occur more efficiently in sperm that undergo normal fertilisation. Finally, the possibility cannot be discounted that the fusion of gametes, which is circumvented during ICSI, may facilitate the generation of persistent oscillations. Although the success of ICSI in the human and mouse has all but ruled out a role for gamete fusion in the initiation of the signalling cascade leading to activation of the

phosphoinositide pathway, a role for this event in the reorganisation of the oocyte cortex such that the PIP₂ substrate becomes more accessible to PLC ζ warrants further investigation.

In conclusion, our results demonstrate that a significant shortcoming of ICSI in the bovine lies directly with the inability of most injected sperm to mount the persistent and long-lasting [Ca²⁺]_i oscillations that are required for proper embryonic development. Elucidation of the mechanism(s) that fails to be appropriately activated during ICSI not only promises to increase the efficiency of this technique in large domestic species, but will also help in the design of better parthenogenetic methods to be applied in cloning procedures and may aid in the unraveling of the signalling mechanism(s) that underlies mammalian fertilisation.

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