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Defective sperm head decondensation undermines the success of ICSI in the bovine

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Abstract

The efficiency of intracytoplasmic sperm injection (ICSI) in the bovine is low compared to other species. It is unknown whether defective oocyte activation and/or sperm head decondensation limit the success of this technique in this species. To elucidate where the main obstacle lies, we used homologous and heterologous ICSI and parthenogenetic activation procedures. We also evaluated whether *in vitro* maturation negatively impacted the early stages of activation after ICSI. Here we showed that injected bovine sperm are resistant to nuclear decondensation by bovine oocytes and this is only partly overcome by exogenous activation. Remarkably, when we used heterologous ICSI, *in vivo*-matured mouse eggs were capable of mounting calcium oscillations and displaying normal PN formation following injection of bovine sperm, although *in vitro*-matured mouse oocytes were unable to do so. Together, our data demonstrate that bovine sperm are especially resistant to nuclear decondensation by *in vitro*-matured oocytes and this deficiency cannot be simply overcome by exogenous activation protocols, even by inducing physiological calcium oscillations. Therefore, the inability of a suboptimal ooplasmic environment to induce sperm head decondensation limits the success of ICSI in the bovine. Studies aimed to improve the cytoplasmic milieu of *in vitro*-matured oocytes and to replicate the molecular changes associated with *in vivo* capacitation and acrosome reaction will deepen our understanding of the mechanism of fertilization and improve the success of ICSI in this species.

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Declaration of interest

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

Introduction

Intracytoplasmic sperm injection (ICSI) has become an indispensable tool to overcome infertility in humans (Palermo *et al.* 1992). In addition, ICSI has become an important method to study the mechanisms of fertilization and early events of egg activation in mammals (Yanagimachi 2005). ICSI involves the fertilization of oocytes in metaphase II stage (MII) by direct injection of a spermatozoon (Goto 1997). This procedure is nearly as effective as *in vitro* fertilization (IVF) in producing offspring in humans (Palermo *et al.* 1992) and mice (Kimura & Yanagimachi 1995), and it has been a valuable tool for conservation purposes in species where other assisted reproductive technologies are not available or are not optimized (Perry *et al.* 1999). Nevertheless, the success of ICSI in the bovine is poor with rates of embryo development well below those obtained by IVF (Rho *et al.* 2004, Arias *et al.* 2014). Specifically, in this species, the majority of eggs fail to activate following ICSI (Catt & Rhodes 1995, Malcuit *et al.* 2006) and display delayed and/or incomplete sperm head decondensation (Chen & Seidel 1997, Suttner *et al.* 2000). These defects together conspire to limit success of ICSI in the bovine.

During fertilization the sperm delivers into the ooplasm a sperm-specific phospholipase C (PLCZeta1), PLC ζ (Saunders *et al.* 2002, Knott *et al.* 2005), which is responsible for initiating the intracellular calcium ($[Ca^{2+}]_i$) oscillations that are a hallmark of mammalian fertilization (Wakai & Fissore 2013). The $[Ca^{2+}]_i$ oscillations induce all downstream events of egg activation, which is the first stage of embryo development (Schultz & Kopf 1995). There are early events of activation such as exocytosis of the cortical granules, prevention of polyspermy and exit from the MII stage, which are initiated soon after sperm entry. Late events of egg activation such as sperm head decondensation, progression to the pronuclear (PN) stage and DNA synthesis and recruitment of maternal RNAs unfold over a period of 10 h (h). Remarkably, in species where ICSI is successful, the events of egg activation are closely recapitulated following sperm injection (Kimura *et al.* 1998, Sato *et al.* 1999, Yanagida *et al.* 2001). Nevertheless, this is not the case in the bovine, as whereas bovine eggs are capable of undergoing normal activation following IVF including formation of male PNs with high efficiency, the same cohort of eggs are incapable of supporting similar rates of activation and formation of male PN following fertilization by injection of a spermatozoon.

As noted above, a necessary step for egg activation is the initiation of $[Ca^{2+}]_i$ oscillations. This step is defective in the bovine following ICSI, as oscillations fail to initiate and/or are short lived (Malcuit *et al.* 2006). Another step of egg activation that is defective is the formation of the male PN. Decondensation of the sperm head requires the sperm's nuclear and cytoplasmic contents and its surrounding membranes to mingle with the ooplasm (Sutovsky & Schatten 2000). Further, the transformation into a male PN requires among other things the swapping of the DNA-associated sperm's protamines with maternal histones as well as the incorporation of other chaperone proteins (Florman & Fissore 2014). It is known that IVM oocytes display reduced developmental potential (Rizos *et al.* 2002, Kim *et al.* 2004, Virant-Klun *et al.* 2013), and it is possible that IVM bovine eggs might be unable to process sperm that have not undergone *in vivo* capacitation and the acrosome reaction. Therefore, it is unknown if the defective activation with delayed and incomplete sperm head

decondensation observed after ICSI in the bovine is caused by a deficient stimulus of egg activation or if it is due to 'suboptimal ooplasm' of IVM eggs that are unable to convert the sperm into a male PN.

Thus, to elucidate where the main obstacle for successful bovine ICSI lies, we used homologous and heterologous ICSI and artificial activation procedures to evaluate the ability of sperm exposed to these different conditions to induce $[Ca^{2+}]_i$ responses, undergo sperm head decondensation and PN formation. Our results show that bovine sperm injected into IVM bovine eggs are highly stable and resistant to nuclear decondensation, and exogenous activation by parthenogenetic procedures only partly overcomes this defect. Remarkably, *in vivo*-but not *in vitro*-matured mouse oocytes supported normal $[Ca^{2+}]_i$ oscillations, sperm head decondensation and PN formation. Together, the results suggest that less than optimal ooplasmic conditions present in IVM eggs undermine ICSI in the bovine. Protocols to improve the ooplasmic environment as well as to replicate *in vitro* the molecular changes associated with capacitation and the acrosome reaction will improve ICSI in this species.

Materials and methods

Reagents

Unless stated otherwise, all chemicals were purchased from Sigma Aldrich.

Collection of gametes

All animal procedures were performed in accordance with the Animal Care and Use Committee (IACUC) guidelines of UMass-Amherst. Mouse GV oocytes were collected from the ovaries of 6- to 10-week-old CD-1 female mice 44–46 h after injection of 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma). Cumulus intact GV oocytes were recovered in a HEPES-buffered Tyrode's lactate solution (TL-HEPES) containing 5% heat-treated fetal calf serum (FCS; Gibco) and were matured *in vitro* for 12–14 h in IBMX-free Chatot, Ziomek and Bavister (CZB) (Chatot *et al.* 1989) media supplemented with 3 mg/mL bovine serum albumin (BSA, Sigma) under mineral oil at 37°C in a humidified atmosphere of 5% CO₂, as described before (Lee *et al.* 2016). MII mouse oocytes were collected from the oviducts into a HEPES-buffered solution (TL-Hepes) supplemented with 10% heat-treated calf serum (FCS; Gibco), 12–14 h after administration of 5 IU of human chorionic gonadotropin (hCG), which was administered 46–48 h after PMSG. All animal procedures were performed according to research animal protocols approved by the University of Massachusetts Institutional Animal Care and Use Committee. Bovine MII oocytes were purchased from Boviteq USA (Wisconsin, Madison). 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. Following isolation, oocytes were placed in vials in 2 mL TCM-199 medium (Gibco), supplemented with 10% FCS, 0.1 mL luteinizing hormone (LH, Sioux Biochemical, Sioux Center, IA, USA) and 1 µg/mL oestradiol for 20 h. Oocytes were shipped overnight at 39°C in a portable incubator (MiniTube of America, Verona, WI, USA). Oocytes for ICSI experiments were denuded of their cumulus cells by vortexing in the presence of 1 mg/mL hyaluronidase and selected for the presence of the first polar body. In all cases, mouse and bovine MII oocytes were rinsed

thoroughly and held in KSOM (Specialty Media, Phillipsburg, NJ, USA) supplemented with 0.1% fraction V BSA until initiation of the ICSI procedure.

Sperm preparation

Cauda epididymal sperm from CD1 male mice were collected in 500 μ L of a modified Krebs-Ringer medium (Whitten's HEPES-buffered medium) (Navarrete *et al.* 2015) and allowed to swim out for 10 min at 37°C. The sperm suspension was washed three times and resuspended in 0.5 mL injection buffer. These samples were then washed three times in injection buffer prior to injection. Bovine sperm were obtained from frozen semen samples kindly donated by American Breeder Services (DeForest, WI, USA). After thawing the straws, the sperm were separated using a Percoll gradient. These spermatozoa (5×10^6 spermatozoa/mL) were then washed in Sp-TALP medium (Parrish *et al.* 1988) for 5 min and then used for ICSI.

Microinjection of PLC ζ cRNA

Microinjections were performed as described previously (Kurokawa *et al.* 2005). PLC ζ cRNA was prepared as previously described by us and stored at -20°C (Ross *et al.* 2009). After thawing, the samples were centrifuged, and the top 1–2 μ L was used to prepare microdrops from which glass micropipettes were loaded by aspiration. cRNA was delivered into oocytes by pneumatic pressure (PLI-100 picoinjector, Harvard Apparatus, Cambridge, MA). Each oocyte received 5–10 pL, which is approximately 1–3% of the total volume of the egg.

[Ca²⁺]_i measurements—[Ca²⁺]_i monitoring was performed as previously described (Kurokawa *et al.* 2005). [Ca²⁺]_i changes in mouse oocytes were measured using the Ca²⁺-sensitive dye Fura-2-acetoxymethyl ester (Fura 2-AM, Molecular Probes; Invitrogen). Mouse oocytes were incubated and loaded with 1.25 μ M Fura-2AM supplemented with 0.02% pluronic acid (Molecular Probes) for 20 min at room temperature. For bovine oocytes, [Ca²⁺]_i was measured using Fura-2-dextran (Fura 2-Dextran, Molecular Probes; Invitrogen), which was delivered by injection into the oocytes. Following loading with the dye, oocytes were placed in microdrops of TL-HEPES on a monitoring glass bottom dish (Mat-Tek Corp.) under mineral oil. Eggs were monitored simultaneously using inverted microscopes (Nikon) outfitted for fluorescence measurements. Fura 2-AM and Fura2-Dextran were excited by UV light alternating wavelengths of 340 nm and 380 nm by a filter wheel (Ludl Electronic Products Ltd.), and fluorescence was captured every 20 s. A 75 W Xenon lamp provided the excitation light. The excitation wavelength was alternated between 340 and 380 nm by a filter wheel (Ludl Electronic Products, Hawthorne, NY, USA), and fluorescence ratios were obtained every 20 or 30 s. The emitted light was passed through a 510 nm barrier filter and collected with either a cooled Photometrics SenSys CCD or a cool SNAP ES digital camera (Roper Scientific, Tucson, AZ, USA). SimplePCI software (Compix Imaging Inc., Cranberry, PA, USA) was used to monitor [Ca²⁺]_i and synchronize the rotation of the filter wheel. [Ca²⁺]_i values are reported as the ratio of 340/380 nm fluorescence in the whole egg.

Bovine in vitro fertilization (IVF) and embryo culture

Matured bovine oocytes and sperm were co-incubated for 18–20 h in IVF-TL supplemented with 0.2 mM sodium pyruvate, 3 mg fatty acid-free BSA and 0.025 mg gentamicin sulfate/mL (Parrish *et al.* 1986, Felmer *et al.* 2011). Final IVF-TALP contained PHE (2 mM penicillamine, 1 mM hypotaurine and 0.25 mM epinephrine), 2 µg heparin and 1×10^6 Percoll separated frozen-thawed sperm/mL. Presumptive zygotes were stripped of cumulus cells via vortex and randomly assigned to the different culture systems. *In vitro* maturation and fertilization were conducted in 400 µL drops (50 COCs and/or eggs per well) at 38.5°C and 5% CO₂ in humidified atmosphere, while embryo culture was carried out in 100 µL drops (~45 embryos per drop) under mineral oil of KSOM–0.4% BSA medium (EmbryoMax, Chemicom International, USA) at 38.5°C and 5% CO₂, 5% O₂ and 90% N₂, in a humidified atmosphere.

Intracytoplasmic sperm injection

ICSI was carried out as previously described (Kurokawa & Fissore 2003) using Narishige manipulators (Medical System Corp., Great Neck, NY) mounted on a Nikon diaphot microscope (Nikon). ICSI was performed in CZB medium (Chatot *et al.* 1989) at room temperature using CD1 eggs. One part sperm suspension was mixed with one part injection buffer containing 12% polyvinylpyrrolidone (PVP, M.W. 360 kDa; Sigma). Sperm were delivered into the eggs' cytosol using a piezo micropipette-driving unit (Piezodrill; Burleigh Instruments Inc., Rochester, NY). In certain experiments, the bovine sperm were immobilized by applying a few piezo pulses to the sperm tail prior to ICSI; the immotile sperm still was connected to the tail, and the whole sperm was injected. When sperm heads were injected, sonication was used to decapitate the heads (Kimura *et al.* 1998); sonication (XL2020, Heat Systems Inc., Farmingdale, NY) was carried out for 5 s at 4°C. In certain bovine ICSI experiments, oocytes were washed at least three times in TL-HEPES and then activated as described below.

Collection of mating-derived mouse zygotes

Ovulation was induced in mice by intraperitoneal injection of 10 IU of Pregnant Mare's Serum Gonadotropin Folligon (PMSG; Sigma) followed by 10 IU of human chorionic gonadotropin (hCG; Sigma) after 48 h. The female mouse was mated with a male mouse overnight. The following morning, the mating was confirmed by inspection of the vaginal plug. Mouse with visible copulation plug was killed by cervical dislocation, and then ovary bursae were surgically removed and collected in a HEPES-buffered Tyrode's lactate solution (TL-HEPES) containing 5% heat-treated fetal calf serum (FCS; Gibco) (Lee *et al.* 2016). Under stereomicroscope, zygotes were dissected out from the swollen ampulla and treated with 0.1% bovine testes hyaluronidase (Sigma). The collected zygotes were transferred into 100 µL of TL-HEPES and fixed to determine pronuclear stage.

Parthenogenetic oocyte activation

Bovine oocytes were activated by injection of PLCZ1 cRNA into the ooplasm (concentration as indicated) or chemically. After cRNA microinjection, oocytes were cultured in potassium simplex optimized medium (KSOM; EmbryoMax; Millipore).

Chemical activation was performed with 5 μ M ionomycin (Calbiochem) for 5 min, followed by incubation in KSOM containing 10 mg/mL cycloheximide (CHX) for 5 h (Io + CHX). After activation, oocytes were allocated to 50 μ L culture drops (maximum 20–25 embryos per drop) consisting of KSOM medium. For all experiments, embryo culture was performed at 38.5°C with a gas mixture of 5% CO₂, 5% O₂, 90% N₂ and saturation humidity. Cleavage and Blastocyst rates were assessed at 72 and 192 h, respectively. Pronuclear formation was assessed between 14–18 h post activation (hpa), depending on the experiment.

Determination of Pronuclear (PN) stage

After ICSI or egg activation, oocytes were fixed in paraformaldehyde (4%) at two different times after activation, 14 and 18 h post activation (hpa) and then stained with Hoechst 33342 (10 mg/mL) for 25 min to determine the presence of pronucleus and/or male sperm decondensation. In case of IVF, zygotes were fixed at 14 and 18 h post insemination (hpi). The PN stage and its size were recorded using a Nikon Optiphot microscope equipped with epifluorescence optics ($\times 200$). Image analysis was conducted using the free software ImageJ v1.48, downloaded from the NIH website (<https://imagej.nih.gov/ij/download.html>).

Statistical analysis

Data were analyzed by descriptive statistics based on the mean plus/minus the standard deviation (s.d.) calculated for each of the variables. Differences among treatments were analyzed using one-way ANOVA. Post hoc analysis to identify differences between groups was performed using Scheffe's test. Pronuclear formation was analyzed by a Chi-squared test with Bonferroni correction. Significant differences were considered if $P < 0.05$.

Results

Sperm head decondensation and PN formation after ICSI using bovine gametes

A common defect of bovine ICSI is the inability of eggs to timely decondense the sperm nucleus and form a male PN. Therefore, we first examined if the eggs used in our study were capable of undergoing normal rates of PN formation following *in vitro* fertilization or common parthenogenetic activation procedures. PN formation was evaluated at 14 and 18 h after insemination or activation with ionomycin (Io) + cycloheximide (CHX). Over 80% of the eggs showed 2 PNs by 14 h post IVF, and if observed a few hours later, all fertilized zygotes had cleaved to the 2-cell stage (Table 1 and Fig. 1; also, data not shown). Similar results were observed with the parthenogenetic zygotes. A final control group was used in which the same parthenogenetic activation procedure was performed but instead of a sperm a comparable volume of PVP (5%) was injected. As observed in Table 1 and Fig. 1, the injection procedure did not affect the ability of eggs to undergo PN formation. Therefore, we conclude that the eggs used in our study are capable of undergoing high rates of male and female PN formation and cleavage to the 2-cell stage following IVF. Moreover, the injection procedure does not disturb the capacity of eggs to undergo activation.

We next examined if the same cohort of eggs was capable of undergoing activation and formation of a male PN after injection of a sperm (Table 1; Fig. 1). As expected, without additional parthenogenetic stimulation, ICSI resulted in low rate of PN formation and only 1

of 19 eggs showed a partially decondensed sperm head; there were not marked differences between 14 and 18 h post ICSI. We repeated these studies but accompanied the injection of sperm with exogenous stimulation. In the ICSI-Io + CHX group, 25% (4/16) of the fertilized eggs displayed a fully formed male PN by 14 h, whereas the majority of eggs only showed partially decondensed sperm heads (8/16). Although a slight improvement was observed by 18 h, as 40% (7/17) of the zygotes showed a normal male PN, the rest of the injected zygotes were still unable to form a male PN by this time. We also activated eggs with bovine PLC ζ cRNA, ICSI + bPLC ζ , but this treatment was even less effective, as none of the zygotes formed a male PN by 14 h post injection (0/15), although nearly 53% of the zygotes formed a male PN by 18 h (9/17). Together, these results show that whereas IVM bovine eggs are capable of responding to common parthenogenetic treatments with formation of female PNs, they are unable to efficiently and timely decondense injected bovine sperm.

[Ca²⁺]_i responses after ICSI and supplementary activation treatments

Given the protracted formation of the male PN after bovine ICSI in general and after ICSI combined with bPLC ζ cRNA injection in particular, we examined whether these oocytes/zygotes were capable of mounting [Ca²⁺]_i responses. We also examined whether the delayed male PN formation prevented cleavage to the 2-cell stage. Injection of sperm alone into bovine oocytes induced [Ca²⁺]_i oscillations in only 4/20 oocytes, which were spaced by 80 min intervals (Fig. 2A). These results are consistent with previous studies that showed that in response to sperm injection only a few bovine oocytes can mount persistent oscillations (Malcuit *et al.* 2006). Nevertheless, injection of bPLC ζ cRNA after ICSI induced [Ca²⁺]_i oscillations in nearly all oocytes that were spaced by shorter intervals, which is similar to the responses observed after normal fertilization (Fig. 2B). We also confirmed that all oocytes activated by Io + CHX showed the expected single [Ca²⁺]_i rise of ~5 min duration (Fig. 2C). Together, the results demonstrate that despite replicating the activation stimulus, or bypassing it using Io + CHX, bovine eggs are unable to timely decondense the injected sperm heads and form a male PN. The data therefore suggest that the initial inability to decondense the homologous sperm head is not due to failure of the activation stimulus per se. Remarkably, eggs undergoing ICSI aided with artificial activation were capable of experiencing cleavage at rates similar to those of IVF zygotes and parthenogenetically activated eggs (Table 2), although development to the blastocyst stage was lower for these treatments compared to IVF. Thus, bovine zygotes generated by ICSI are capable of initiating development, although the asynchronous PN formation might undermine the long-term developmental competence of these embryos.

PN formation after heterologous ICSI using mouse oocytes

The inability of IVM bovine eggs to promote male PN formation in a timely fashion after ICSI even after exogenous activation treatments, as shown by us and by many other research groups (Perreault *et al.* 1988, Goto *et al.* 1990, Rho *et al.* 1998), suggests that *in vitro* maturation might impair the ability of eggs to process intact sperm heads such as those delivered by ICSI. To test our hypothesis the logical next step was to perform the same experiments in *in vivo*-matured bovine oocytes. Nevertheless, it is prohibitively expensive, and technically challenging, to obtain enough *in vivo*-matured bovine eggs to carry out meaningful experiments. We therefore resorted to heterologous ICSI, as *in vivo*-matured

mouse eggs are easy to collect. Further, in a previous study we had shown that *in vivo*-matured mouse eggs were capable of responding to injection of bovine sperm with persistent oscillations (Malcuit *et al.* 2006). We therefore examined if these eggs, and also *in vitro*-matured mouse eggs, were capable of inducing PN formation following injection of bovine sperm. PN formation was assessed 5–6 h post sperm injection. Injection of mouse sperm heads into *in vivo* matured, ovulated, eggs (MII_{OY}), as expected, induced PN formation in all injected eggs (Table 3, Figs. 3 and 4), which is consistent with results by many other groups and our own previous data (Kimura & Yanagimachi 1995, Kimura *et al.* 1998, Mizuno *et al.* 2002). *In vitro*-matured mouse eggs (MII_{IVM}) were also efficient at inducing male PN formation following injection of mouse sperm heads (Figs. 3 and 4), although an increasing number of sperm heads remained condensed by the time of observation (Table 3). Remarkably, MII_{OY} eggs were very efficient at inducing male PN formation following injection of bovine sperm heads, although approximately 25% of the sperm heads remained condensed (Table 3). Importantly, MII_{IVM} eggs were largely devoid of capacity to render a bovine sperm into a male PN, as less than 10% of the injected sperm formed a male PN (Table 3; Figs. 3 and 4). Similarly, bovine IVM eggs were largely incompetent to process mouse sperm head regardless of whether or not they received supplementary parthenogenetic activation (Table 3). Altogether, these results indicate that the source of oocytes and eggs and the maturation conditions greatly impact the ability of eggs to process and transform sperm heads into male PNs, especially for sperm that bypass the physiological processes associated with natural fertilization.

The formation of male and female PNs requires several steps the last of which is ‘nuclear swelling,’ which is when the pronuclei attain their final size (Jenkins & Carrell 2012). Given the diminished ability of *in vitro*-matured mouse eggs to convert injected sperm into male PNs, we wondered if differences could be detected between the sizes of the PNs formed following injection of mouse and bovine sperm into *in vivo*- and *in vitro*-matured mouse eggs. We used as a reference point the size attained by the PNs of *in vivo*-fertilized zygotes. Our observations for mouse fertilization revealed, as expected, that the area of the male PN was larger than that of the female PN, $191 \pm 40 \mu\text{m}^2$ and $116 \pm 38 \mu\text{m}^2$, respectively (Figs. 3 and 4). Remarkably, these differences in sizes between males and female mouse PNs were maintained when heterologous ICSI was performed using either ovulated or IVM oocytes, although the average size of the PNs was smaller when IVM eggs were used (Fig. 3) ($P > 0.05$). Injection of bovine sperm into mouse eggs, surprisingly, induced an overall increase in the size of both PNs when it was performed in MII_{OY} eggs, but as it was the case for mouse eggs, the area of the PNs was smaller in IVM oocytes (Fig. 3). For example, the average area of male and female PNs in MII_{OY} eggs was of $338 \pm 78 \mu\text{m}^2$ and $236 \pm 75 \mu\text{m}^2$, respectively, but it was only of $38 \pm 19 \mu\text{m}^2$ and $151 \pm 58 \mu\text{m}^2$ in MII_{IVM} eggs, respectively ($P < 0.05$). It is worth noting that the area of PNs is even greater when the gametes are both bovine. For example, after IVF the PN areas were 1322 ± 411 and $945 \pm 292 \mu\text{m}^2$ and after ICSI the dimensions were 708 ± 269 and $667 \pm 329 \mu\text{m}^2$. For homologous bovine studies, PN formation was always evaluated at 18 hpi and it was not possible to distinguish between male and female PNs. Altogether, our results demonstrate the source of the female gamete and maturation conditions impact the ability to transform sperm heads into male PNs.

[Ca²⁺]_i responses after bovine ICSI into mouse oocytes

The findings that *in vivo*- and *in vitro*-matured mouse eggs display different capacity to transform injected sperm into male PNs led us to examine if these two different source of eggs also influenced the [Ca²⁺]_i responses induced by the injection of sperm. Homologous ICSI using mouse gametes and ovulated oocytes triggered [Ca²⁺]_i oscillations with mean intervals ranging between 15 and 25 min (Fig. 5A), which is in agreement with previous data in the literature indicating that in the mouse the [Ca²⁺]_i responses are influenced by the preparation of the sperm and the strains (Sato *et al.* 1999, Kurokawa & Fissore 2003). Further, these values are similar to those induced by conventional IVF (Knott *et al.* 2003). Remarkably, the responses were less uniform when ICSI was performed into *in vitro*-matured eggs, with 11/18 showing slower oscillations (Fig. 5B) and the rest of the oocytes roughly equally divided between high-frequency responders or non-responders (Fig. 5B and inset, respectively). When heterologous ICSI was performed, bovine sperm caused high-frequency [Ca²⁺]_i responses in 11/16 MII_{OV} eggs, although 5/16 eggs displayed low-frequency oscillations (Fig. 5C and inset, respectively). On the other hand, *in vitro*-matured mouse eggs displayed highly variable responses, and only 7/23 eggs mounted the expected high-frequency [Ca²⁺]_i oscillations (Fig. 5C), whereas 7/23 showed fewer and spaced out responses and 9/23 eggs failed to oscillate (Fig. 5D and inset). Collectively, these results indicate that ICSI failure in *in vitro*-matured eggs is associated with multiple defects in the processing of the sperm heads, including the very early steps of sperm head decondensation and the release of the sperm factor responsible for the [Ca²⁺]_i oscillations that drive egg activation.

Discussion

In this study we examined factors that undermine the success of ICSI in the bovine. We posited that either the inability of bovine sperm to induce [Ca²⁺]_i oscillations and egg activation or the incompetence of the ooplasm to process non-capacitated sperm or a combination of both might explain the low activation rates, asynchronous PN formation and low rates of preimplantation development observed after ICSI in the bovine. Our results show that although bovine eggs in this study can undergo normal activation and cleavage following IVF or parthenogenetic treatment, they are unable to consistently and timely form male PNs despite supplementing the sperm's egg-activating signal with physiological activation stimuli. Nevertheless, following heterologous ICSI we observed that *in vivo*-matured mouse eggs rendered bovine sperm heads into male PNs with high frequency, whereas this was not the case with *in vitro*-matured eggs. We therefore interpret our results to mean that activation failure is not the main factor limiting the success of ICSI in the bovine. Instead, we propose that *in vitro*-matured eggs are developmentally compromised and when confronted with a 'trespassing sperm,' i.e., a sperm that has not undergone 'admission clearance' normally associated with capacitation, acrosome reaction and fusion with the oolema, is unable to timely convert it into a male PN. Improvement of oocyte maturation conditions and better understanding of the molecular mechanisms and cellular changes associated with sperm preparation for fertilization will improve the success of ICSI in the bovine and in other mammalian species.

To ascertain the underlying reasons that limit the success of ICSI in the bovine, we first examined if the gametes used to study this process were competent under optimal conditions. We examined egg activation and embryo development after IVF or parthenogenetic activation. Our results show that the gametes in our study were capable of initiating development, as both procedures induced high rates of cleavage by 72 h along with acceptable rates of development to the blastocyst stage. These results show the bovine gametes used for ICSI are not intrinsically defective. Nevertheless, when performing ICSI with the same gametes, the formation of male PN formation is temporally delayed and greatly reduced. Furthermore, supplementation of ICSI with exogenous activation partially overcomes the deficit, as the rate of male PN formation increases to reach values of near 50% of the injected oocytes, although the formation of the male PN is still delayed. Therefore, as presently performed, the decondensation of the male nucleus appears to be main obstacle to the success of ICSI in the bovine. The difficulty in remodeling the male chromatin following sperm injection is not unique to the bovine, as earlier studies noted impaired and asynchronous chromatin remodeling following ICSI in the mouse (Shoukir *et al.* 1998, Ajduk *et al.* 2006), pig (Kren *et al.* 2003, Lee *et al.* 2003, Nakai *et al.* 2011) and humans (Dozortsev *et al.* 1994, Sousa & Tesarik 1994, Flaherty *et al.* 1995). Several common factors have been suggested to hinder the remodeling of injected sperm, such as the lack of capacitation and acrosome reaction (Sekhavati *et al.* 2012, Zambrano *et al.* 2016, Aguila *et al.* 2017). Nevertheless, unique features of the bovine spermatozoa may further complicate PN formation in this species. For instance, the plasma membrane of bovine spermatozoa is reportedly more stable than the plasma membrane of other mammalian spermatozoa, which may slow down its disintegration and delay the mingling of the sperm's content with the ooplasm (Perreault *et al.* 1988). Bovine spermatozoa also only contain protamine I, unlike the spermatozoa of primates, horses and rodents that have different proportions of protamine II (Balhorn 2007); protamine I contains greater number of disulfide bonds that could hinder sperm nucleus decondensation. In support of this possibility, comparisons of the rates of decondensation of sperm from mammals with different inherent protamine P1/P2 ratios using hamster eggs have shown that the sperm content of protamine P2 appears to regulate the rate with which sperm chromatin decondenses (Perreault *et al.* 1988). In other words, sperm containing a higher proportion of P2 decondense more quickly than sperm containing lower proportions of it (Perreault *et al.* 1988). Therefore, the absence of protamine II in bull and boar spermatozoa may greatly undermine their ability to undergo timely decondensation and PN formation following the injection of sperm, which bypasses all the physiological milestones and checkpoints aimed to ensure fertilization by the fittest sperm.

Questions have persisted as to whether the injection procedure or the abnormal $[Ca^{2+}]_i$ responses contribute to the low success of ICSI in the bovine. Our results here show that the injection procedure, at least using the piezoelectric-actuated methodology, is not detrimental, as sham-injected groups showed similar PN formation and cleavage rates than parthenogenetic derived-zygotes. Regarding $[Ca^{2+}]_i$ responses and despite the highly abnormal oscillations induced by the fertilizing bovine spermatozoon delivered by injection (Malcuit *et al.* 2006), the results of our studies rule this out as an important factor responsible for undermining the success of this technique. First, even though we

supplemented the injection of sperm with injection of PLCzeta1 cRNA, which was capable of inducing nearly identical $[Ca^{2+}]_i$ responses to those induced by fertilization, the rates and timing of male PN formation remained low. It is worth noting that female PNs formed in a timely manner in nearly all of the PLCzeta1 cRNA injected eggs, indicating that MPF activity have decreased as expected following activation induced by this stimulus. These data are also consistent with previous results demonstrating that PLCzeta1 cRNA injected is a very efficient activation stimulus to generate SCNT-derived bovine embryos (Ross *et al.* 2009). Second, another method of activation that is widely used for SCNT, Io + CHX, also was inefficient in our hands to timely decondense and remodel the male chromatin and form a male PN despite inducing 100% female PN formation. Thus, even though we generated optimal $[Ca^{2+}]_i$ responses or bypassed them with efficient, exogenous parthenogenetic activation procedures, male nuclear decondensation was largely defective following bovine ICSI. Here, we therefore propose that the defective $[Ca^{2+}]_i$ responses are not responsible for the poor success of ICSI, but rather are one of the earliest manifestations of flawed male chromatin remodeling, as the male factor, PLCzeta1, cannot be appropriately released/activated following sperm injection, and it is therefore unable to initiate normal $[Ca^{2+}]_i$ oscillations.

It is well established that the female gamete acquires developmental capacity during oocyte maturation (Ferreira *et al.* 2009, Cheon *et al.* 2013, Oqani *et al.* 2016), and that *in vitro*-matured eggs have reduced developmental competence (Kim *et al.* 2004, Gioia *et al.* 2005). In this regard, the great majority of cattle eggs used for experimental purposes are *in vitro* matured, which, as pointed out, have inherently lower developmental capacity (Kim *et al.* 2004, Gilchrist 2011, Ptak *et al.* 2013). Therefore, given that bovine eggs resulting from *in vitro* maturation are unable to timely remodel the male chromatin of sperm delivered by ICSI, even after exogenous activation, we performed experiments using *in vivo*-matured mouse eggs. We have shown that mouse eggs mount normal $[Ca^{2+}]_i$ oscillations when injected with sperm of other species (Knott *et al.* 2003). Therefore, we examined if ovulated mouse eggs were also able to convert the bovine spermatozoa into male PNs. We found that nearly 70% of the bovine sperm were transformed into a male PN within 6 h post injection, which is only slightly lower than what is induced by injection of homologous sperm in the mouse, but considerably higher than what is accomplished by bovine eggs. Furthermore, consistent with the above results, ovulated mouse eggs displayed high-frequency oscillations in response to injection of bovine sperm (Knott *et al.* 2003). Remarkably, *in vitro*-matured mouse eggs were largely unable to decondense bovine spermatozoa and were less efficient at remodeling the mouse sperm head. In agreement with these results, IVM mouse eggs were less able to mount $[Ca^{2+}]_i$ responses to injection of either sperm. Altogether, these results demonstrate that *in vitro*-matured eggs have significantly lower capabilities to reprogram sperm nucleus delivered by ICSI. These results suggest that despite significant progress in improving IVM conditions, IVM eggs do not attain the same developmental competence of *in vivo*-matured eggs. This is agreement with a recent study using *in vivo*- and *in vitro*-matured porcine eggs, where it was shown that the use of *in vitro*-matured eggs compromised the rates of activation and embryo development regardless of the pattern of $[Ca^{2+}]_i$ oscillations (Nakai *et al.* 2016). Further, there is extensive evidence in the literature of multiple methods used by researchers to supplement the 'compromised' remodeling

ability of eggs obtained after *in vitro* maturation or to treat sperm to render them more prone to undergo PN formation, including methods of sperm immobilization (Wei & Fukui 1999, Horiuchi *et al.* 2002), damage to sperm membranes (Arias *et al.* 2014, Zambrano *et al.* 2016), or addition of agents to enhance decondensation of spermatozoa DNA such as dithiothreitol (DTT) (Rho *et al.* 1998, Galli *et al.* 2003) and heparin-glutathione (Sekhavati *et al.* 2012). However, thus far, none of these methods has consistently improved the success of ICSI in this species.

Altogether, our results show that bovine sperm is especially resistant to nuclear decondensation, which is possibly associated with the exclusive expression of protamine I to condense its DNA. Further, the near exclusive reliance on *in vitro*-matured eggs, which contain a suboptimal ooplasm, does not aid in the success of this technique. Considering these results, additional studies are necessary to elucidate the mechanism(s) that underlie the sperm's preparation for fertilization and devise effective methods that render bovine spermatozoa 'remodeling-ready' so that post-fertilizing events after injection can be initiated in a timely and more efficient manner. Such a progress will increase the efficiency of this technique in large domestic species, and will also help in the design of better *in vitro* fecundation methods to be applied to humans and animal models.

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Figure 1.

Representative images of PN formation in bovine oocytes fertilized by conventional *in vitro* fertilization (IVF), chemically activated oocytes without and with sham ICSI injection, or fertilized by ICSI. Upper images were captured after Hoechst staining, whereas the corresponding phase-contrast images are at the bottom. A and B: In vitro fertilization: zygotes examined 14 h post insemination. C and D: Parthenogenetically activated group with Io + CHX (14 h post activation). E and F: Sham-injected group, where injection of comparable volume of PVP (5%) was performed followed by chemical activation with Io + CHX (14 h post activation). G and H: ICSI, bovine zygote generated by ICSI without exogenous activation. White circle in G denotes injected, intact sperm head. I and J: ICSI – Io + CHX 14 hpa, ICSI bovine zygote 14 h post-chemical activation. White circle in I surrounds a partially decondensed sperm head. K and L: ICSI – Io + CHX 18 hpa, ICSI bovine zygote 18 h post-chemical activation. M and N: ICSI + bPLC ζ : ICSI bovine zygote activated with bovine PLC ζ 18 hpa. Arrows indicate PBs and arrowheads PNs. Magnification: 200 $\times$, scale bar in microns.

Figure 2. $[Ca^{2+}]_i$ responses induced by injection of bovine sperm with and without injection of PLC ζ cRNA (0.5 μ g/ μ L) or by the addition of ionomycin. (A) Injection of sperm-induced $[Ca^{2+}]_i$ responses in the minority of injected oocytes. (B) Co-injection of bull sperm with bovine PLCzeta1 cRNA (0.5 μ g/ μ L) caused $[Ca^{2+}]_i$ responses in all injected oocytes. (C) Addition of Io caused a single $[Ca^{2+}]_i$ rise in all exposed oocytes and caused the baseline remain high as long as Io was in the media.

Figure 3.

Representative fluorescent (upper) and phase-contrast (bottom) images of mouse oocytes following IVF or ICSI using mouse or a bovine sperm. A and B: *IVF*. C and D: Homologous ICSI using ovulated oocytes. E and F: Homologous ICSI using IVM oocytes. G and H: Heterologous ICSI using ovulated oocytes. I and J: Heterologous ICSI using IVM oocytes. M: Male pronuclei. FM: Female pronuclei. MP: Metaphase plate. Arrows indicate PBs and red circles denote PNs. Magnification 200 $\times$. Scale bar in microns.

Figure 4. Source of sperm and oocytes affect the size of PNs following homologous and heterologous ICSI using ovulated and IVM mouse oocytes. (A) Female PN. (B) Male PN. Values represent the mean + s.d. Bars with different letters are significantly different ($P < 0.05$).

Figure 5. $[Ca^{2+}]_i$ responses after homologous and heterologous ICSI in mouse oocytes. (A and B) Injection of mouse sperm into MII_{OV} induced consistent and expected $[Ca^{2+}]_i$ responses, although injection into IVM oocytes induced variable responses $[Ca^{2+}]_i$ (B). (C) Injection of bull sperm into MII_{OV} induced mostly high-frequency oscillations, although a few oocytes showed low-frequency oscillations (inset). (D) Injection of bull sperm into IVM induced high-frequency oscillations in half of the oocytes, although in the other half, the oscillations were less frequent or absent (inset).

Table 1

PN formation in bovine oocytes following *in vitro* fertilization, common parthenogenetic activation procedures, ICSI, ICSI + chemical activation or PLC cRNA injection.

Group	Io + CHX	Pronuclear Formation 14 hpa				Pronuclear Formation 18 hpa			
		n	PDSH (%)	PN (%)	UF/NA	n	PDSH (%)	PN (%)	UF/NA
Bovine IVF	–	22	0 (0%) ^a	18 (82%) ^a	4 (18%) ^a	23	0 (0%) ^a	20 (87%) ^a	3 (13%) ^a
Parthenothes	+	23	0 (0%) ^a	17 (74%) ^a	6 (26%) ^a	25	0 (0%) ^a	20 (80%) ^a	5 (20%) ^a
Sham injection	+	11	0 (0%) ^a	9 (82%) ^a	2 (18%) ^a	18	0 (0%) ^a	13 (72%) ^a	5 (28%) ^a
ICSI	–	19	1 (5%) ^a	0 (0%) ^b	18 (95%) ^b	14	1 (7%) ^a	1 (7%) ^b	12 (86%) ^b
ICSI	+	16	8 (50%) ^{ab}	4 (25%) ^b	4 (25%) ^a	17	5 (30%) ^a	7 (40%) ^b	5 (30%) ^a
ICSI + bPLCz	–	15	12 (80%) ^b	0 (0%) ^b	3 (20%) ^a	17	5 (30%) ^a	9 (53%) ^{ab}	3 (17%) ^a

Data within the same column with different superscripts are significantly different ($P < 0.05$)

*These oocytes had already cleaved and were 2 cells.

ICSI, Injection of bovine sperm; ICSI + bPLCz, Injection of sperm followed by injection with bovine bPLCz cRNA (0.5 µg/µL); Io + CHX, Ionomycin plus cycloheximide; PDSH, Partially decondensed sperm head; PN, Pronucleus; 2PB, Two polar bodies; UF/NA, unfertilized or non-activated oocytes.

Table 2

ICSI-generated bovine zygotes initiate embryo development.

Group	<i>n</i>	Cleavage at 72 h	Blastocysts at 192 h
IVF	29	23 (79%) ^a	9 (31%) ^a
ICSI	28	3 (11%) ^b	0 (0%) ^b
ICSI – Io + CHX	27	20 (74%) ^a	4 (15%) ^c
ICSI + bPLCZeta1	28	19 (68%) ^a	3 (11%) ^c
Parthenogenetic activation	32	24 (75%) ^a	3 (9%) ^c

Parthenogenetic activation: oocytes chemically activated with Io + CHX. Data followed by different superscripts are significantly different ($P < 0.05$).

Table 3
PN formation in mouse and bovine oocytes after homologous and heterologous ICSI.

Group	Io + CHX	n	Pronuclear Formation				
			1 FPN + 2PB				
			PDSH (%)	MPN (%)	ISH (%)	ISH + MII (%)	
MIIOV + mouse sperm	–	11	0 (0%) ^a	11 (100%) ^a	0 (0%) ^a	0 (0%) ^a	
MIIVVM + mouse sperm	–	10	0 (0%) ^a	7 (70%) ^b	2 (20%) ^b	1 (10%) ^a	
MIIOV + bovine sperm	–	23	5 (22%) ^c	17 (74%) ^b	1 (4%) ^b	0 (0%) ^a	
MIIVVM + bovine sperm	–	24	2 (8%) ^d	0 (0%) ^c	0 (0%) ^c	22 (92%) ^a	
bMIIOV + mouse sperm	–	25	0 (0%) ^a	0 (0%) ^c	0 (0%) ^c	25 (100%) ^c	
bMIIVVM + mouse sperm	+	24	0 (0%) ^a	0 (0%) ^c	24 (100%) ^c	0 (0%) ^c	

PN formation in mouse oocytes was evaluated at 6 h post sperm injection, and in bovine oocytes at 18 hpi. Data within the same column followed by different superscripts are significantly different ($P < 0.05$).

bMIIVVM: *in vitro*-matured bovine oocyte; FPN, female pronucleus; ISH, intact sperm head; MII, oocyte arrested at metaphase II; MIIOV: mouse oocytes collected from oviduct; MIIVVM: *in vitro*-matured mouse oocytes; MPN, male pronucleus.