Patterns of Intracellular Calcium Oscillations in Horse Oocytes Fertilized by Intracytoplasmic Sperm Injection: Possible Explanations for the Low Success of this Assisted Reproduction Technique in the Horse

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ABSTRACT

In all species studied, fertilization induces intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) oscillations required for oocyte activation and embryonic development. This species-specific pattern has not been studied in the equine, partly due to the difficulties linked to in vitro fertilization in this species. Therefore, the objective of this study was to use intracytoplasmic sperm injection (ICSI) to investigate fertilization induced [Ca\(^{2+}\)]\(_i\) signaling and, possibly, ascertain problems linked to the success of this technology in the horse. In vivo and in vitro-matured mare oocytes were injected with a single motile stallion sperm. Few oocytes displayed [Ca\(^{2+}\)]\(_i\) responses regardless of oocyte source and we hypothesized that this may result from insufficient release of the sperm-borne active molecule (sperm factor) into the oocyte. However, permeabilization of sperm membranes with Triton-X or by sonication did not alleviate the deficient [Ca\(^{2+}\)]\(_i\) responses in mare oocytes. Thus we hypothesized that a step downstream of release, possibly required for sperm factor function, is not appropriately accomplished in horse oocytes. To test this, ICSI-fertilized horse oocytes were fused to unfertilized mouse oocytes, which are known to respond with [Ca\(^{2+}\)]\(_i\) oscillations to injection of stallion sperm, and [Ca\(^{2+}\)]\(_i\) monitoring was performed. Such pairs consistently displayed [Ca\(^{2+}\)]\(_i\) responses demonstrating that the sperm factor is appropriately released into the ooplasm of horse oocytes, but that these are unable to activate and/or provide the appropriate substrate that is required for the sperm factor delivered by ICSI to initiate oscillations. These findings may have implications to improve the success of ICSI in the equine and other livestock species.
INTRODUCTION

In oocytes from all mammalian species studied to date fertilization induces a series of species-specific intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) oscillations [1-3]. These transients are required not only to rescue the oocyte from meiotic arrest, but also for pronuclear formation, the initiation of DNA synthesis and normal progression of embryonic cleavage [2, 4]. Furthermore, recent research suggests that the frequency and duration of [Ca$^{2+}$]$_i$ transients may impact subsequent embryonic development to pre- and post-implantation stages [5].

The exact mechanism(s) by which the sperm initiates [Ca$^{2+}$]$_i$ oscillations in oocytes is not completely understood, but it involves production of inositol 1,4,5-trisphosphate (IP$_3$), which binds to its receptor in the endoplasmic reticulum, the major intracellular Ca$^{2+}$ store, thereby allowing Ca$^{2+}$ efflux into the cytosol [4]. It has been postulated that IP$_3$ production may be initiated through G-proteins or tyrosine kinase signaling pathways linked to receptors present in the oolemma [6-9]. Nonetheless, other studies show that sperm-egg fusion precedes the initiation of [Ca$^{2+}$]$_i$ transients, with a brief lag of time before the first [Ca$^{2+}$]$_i$ spike is detected [10-11], which is consistent with the timing required for diffusion of a sperm-supplied molecule, therefore providing evidence for a sperm factor/fusion theory as responsible for the initiation of oscillations in mammals.

Further support for a sperm factor delivered at the time of gamete fusion comes from findings demonstrating that injection of sperm extracts into oocytes of laboratory and livestock species or fertilization by intracytoplasmic sperm injection (ICSI) of human and mouse oocytes can induce fertilization-like [Ca$^{2+}$]$_i$ responses [12-15]. Additionally,
injection of sperm extracts into frog oocytes was shown to stimulate IP3 production [16], as reported for fertilization in this species [17]. In agreement with the sperm factor hypothesis, a sperm-specific phospholipase C enzyme (PLCζ) has been recently identified and proposed to be the sperm molecule responsible for IP3 production and initiation of [Ca2+]i oscillations during mammalian fertilization [18]. Nevertheless, the possibility that a membrane-linked signaling pathway initiated by the sperm at the time of gamete fusion, either acting upstream of or concomitantly with PLCζ, or that an altogether different mechanism(s) may be involved in oocyte activation in different mammalian species cannot be excluded with the information available so far.

The pattern of fertilization-induced [Ca2+]i transients has not been studied in the equine, partly due to the low rates of sperm penetration that are achieved with in vitro fertilization (IVF) in this species [19-21]. Furthermore, ICSI has so far yielded inconsistent results in the horse, with variable oocyte activation and generally low embryonic development rates [19,20,22]. We have previously shown that equine oocytes display [Ca2+]i transients and become activated when injected with stallion sperm extracts (eSF) [23]. Interestingly, in the same study injection of fresh sperm into horse oocytes (ICSI) did not consistently initiate [Ca2+]i responses. We hypothesized that such failure may be due to inability of injected horse oocytes to promote release of the sperm-borne factor or, alternatively, to a deficient step in the activation of such factor. Therefore, our objective herein was to further investigate which of these possibilities may explain the failure for consistent initiation of [Ca2+]i oscillations following ICSI in horse oocytes.
MATERIALS AND METHODS

Animal Care and Welfare

Experiments performed herein where live animals were used followed NRC animal care and welfare guidelines and were approved by the IACUC committee at the University of Massachusetts.

Retrieval and Culture of Horse Oocytes

Equine oocyte-cumulus complexes (OCCs) for in vitro maturation were obtained by scraping the follicular walls of abattoir-collected ovaries, at the equine reproduction laboratory in Texas A&M University (College Station, TX). Oocytes having expanded cumuli (meiotically competent oocytes) [24] were placed into 1 ml of equilibrated maturation medium (TCM 199 with Earle’s salts; Gibco Life Technologies, Inc., Grand Island, NY) supplemented with 5 μU/ml FSH (Sioux Biochemical Inc.; Sioux Center, IA), 10% fetal bovine serum, and 25 μg/ml gentamycin. The vial containing the oocytes was sealed and packaged into a commercial incubator (Minitube of America, Inc.; Verona, WI) for overnight shipment at 38°C. Once in our laboratory, oocytes were placed in microdroplets of the same medium under light mineral oil in an incubator at 38°C in 5% CO₂ for a total incubation time of 40-42 h. Prior to experiments, oocytes were denuded from their cumulus cells by repeated pipetting in a hyaluronidase solution (0.1% in Dulbecco’s Phosphate Buffered Saline or DPBS; Sigma Chemical Co., St Louis, MO).

In vivo matured horse oocytes were collected from preovulatory follicles in cycling mares 28-32 h post hCG injection, as previously described [25]. Briefly, mares were appropriately restrained in stocks and sedated with Detomedine hydrochloride (5 mg iv; Pfizer, Lees Summit, MO) and Butorphanol tartrate (5 mg iv; Fort Dodge Animal
A 10 x 10 cm site was surgically prepared in the flank ipsilateral to the follicle to be aspirated, and a sterile trocar inserted after blocking the skin with Lidocaine (2 ml; The Butler Co., Columbus, OH). Then, a 14G disposable needle was inserted through the trocar and guided into the preovulatory follicle by holding the corresponding ovary against the abdominal wall with the gloved arm inserted per rectum. An assistant aspirated follicular contents with a 50-ml Air-Tite syringe attached to the aspiration needle through an intravenous extension set, until the follicular wall completely collapsed. Collected OCCs were placed in droplets of medium (as above but containing no FSH) and incubated up to 45-46 h post-hCG prior to ICSI, at 38°C in 5% CO₂ in humidified air. Prior to experiments, oocytes were denuded from cumulus cells as above.

Following ICSI experiments and [Ca²⁺]i monitoring, all horse oocytes were further incubated in culture medium (no FSH) at 38°C in 5% CO₂ in humidified air for an additional 20-24 h prior to activation status evaluation.

Retrieval and Culture of Mouse Oocytes

Metaphase II (MII) mouse oocytes were obtained from the oviducts of 6-16 week-old B6D2F1 euthanized female mice after injection of 5 IU equine chorionic gonadotropin (eCG; Sigma), followed 48 h later by injection of 5 IU human chorionic gonadotropin (hCG; Sigma). Oocytes were retrieved 12-14 h after hCG injection and cumulus cells were removed by incubation in bovine testis hyaluronidase (0.1% in DPBS, 3 min; Sigma). Oocytes showing no evidence of degeneration and that had extruded the first polar body were used for experiments.
Preparation of Stallion Sperm for ICSI Experiments

Stallion semen was collected with an artificial vagina and frozen over liquid nitrogen vapor in a milk-based extender containing egg yolk and glycerol in 2.5 ml straws following conventional sperm freezing methods [26]. Frozen semen was stored in a liquid nitrogen tank until use. Individual straws were thawed in a water bath at 50°C for 40 sec, as recommended for the particular freezing method. ICSI was performed with three different sperm treatments: Frozen-thawed motile sperm (untreated); Triton-X (Sigma) treated sperm; or, sonicated sperm heads. In all instances sperm were washed twice in DPBS (400 g, 12 min) after thawing, and then injected immediately when motile sperm were used, or processed accordingly for the other treatments. Triton-X treatment, which resulted in permeabilization of the membranes, was accomplished by incubating sperm in 5 ml of DPBS containing 0.1% (vol/vol) Triton X for 15 min. Triton-X treated sperm were then washed twice in injection buffer (75 mM KCl, 20 mM Hepes, pH=7.0) prior to ICSI. For injection experiments using sonicated sperm heads, the separation of heads and tails was carried out by a brief sonication (3 min; 4.5 Hz) in injection buffer containing EDTA submerged in ice water. These samples were then washed in injection buffer (no EDTA) and individual sperm heads used immediately for injection.

ICSI Procedure

ICSI was performed as previously described [27, 28]. Briefly, the corresponding sperm suspension (see sperm preparation) was mixed with an equal volume of 12% polyvinylpyrrolidone (PVP; Sigma) and a 50 µl drop of this sample placed on a plastic Petri dish, which served as the microinjection chamber. Manipulations were carried out on an inverted Nikon Diaphot microscope. Injections were performed with a
piezomicropipette-driving unit (Piezodrill; Burleigh Instruments Inc.; Fisher, NY). The sperm was aspirated into the pipette (tail first if intact sperm was used) and several piezo pulses were applied to immobilize/permeabilize the sperm membrane. Then, the tip of the injection pipette containing the sperm/sperm head was moved until it reached the zona pellucida and several piezo-pulses were applied to advance the pipette through the zona. Once in the perivitelline space, the pipette was advanced further against the oolemma until the inner limit of its cortex, while applying light negative pressure. Penetration of the oolemma was accomplished by one or two piezo pulses of lower intensity, and the sperm/sperm head was injected into the ooplasm surrounded by a minimum amount of fluid.

*Fluorescence Recordings and [Ca^{2+}]i Determination*

[Ca^{2+}]i monitoring was carried out with fura-2 dextran (Fura-2D; Molecular Probes; Eugene, OR) loaded horse oocytes as previously described [23,29,30]. In brief, denuded horse oocytes were microinjected using a Nikon Diaphot microscope (Nikon Inc., Garden City, NY) and Narishige manipulators (Medical Systems Corp., Great Neck, NY). Injection pipettes containing 0.5 mM fura-2D were advanced into the ooplasm of each individual oocyte and an appropriate volume of the reagent injected by pneumatic pressure (PLI-100, picoinjector, Harvard Apparatus, Cambridge, MA). The injection volume ranged from 15-20 pl. Mouse oocytes used in these experiments were loaded with 1 µmol/l of fura-2 acetoxymethylene (fura-2 AM; Molecular Probes) by incubation at room temperature for 20 min.
Fluorescence of fura-loaded oocytes subjected to ICSI was monitored as previously described [23,31]. Briefly, the illumination was provided by a 75-W xenon arc lamp on a Nikon Diaphot microscope equipped with a 40X UV objective (Nikon Inc., Garden City, NY). Excitation wavelengths were of 340 and 380 nm and the corresponding emitted light, attenuated 32-fold by neutral density filters, was quantified by a photomultiplier tube that averaged the fluorescence signal over the whole oocyte. Fluorescence values of a group of oocytes were measured simultaneously using the software Image 1/FL (Universal Imaging, Downington, PA). Images were acquired using a SIT camera (Dage-MTI, Michigan City, IN) coupled to an image intensifier (Video Scope International Ltd., Sterling, VA). Values for [Ca\textsuperscript{2+}]\textsubscript{i} were not calibrated in this system and therefore are reported as the ratio of 340/380 nm fluorescence. Fluorescence ratios were obtained every 20 sec, after 1 sec reading at each wavelength.

Monitoring of horse oocyte groups subjected to ICSI started within 30 minutes of sperm injection and were monitored continuously for at least 2 h, and, in some experiments, at 2-4 h intervals thereafter for up to 9 h. [Ca\textsuperscript{2+}]\textsubscript{i} monitoring was always continued for at least 1 h after a [Ca\textsuperscript{2+}]\textsubscript{i} spike was detected. At least three replicates were performed for each experiment.

Assessment of Oocyte Activation Status

At 20-24 h following ICSI, horse oocytes were fixed in buffered formaldehyde with 0.1% Triton X-100 (vol/vol), mounted on a slide with 6.5 µl of 9:1 glycerol:PBS containing 2.5 µg/ml Hoechst 33258 (Sigma), and examined using fluorescence microscopy to determine the chromatin configuration. Oocytes were considered activated if they had extruded the second polar body and two pronuclei were present. Non-
activated oocytes were those in MII or anaphase-telophase configurations, with a condensed or partially decondensed sperm head in the ooplasm. Degenerate oocytes and those in MI were not included in the assessment of activation rates.

**ICSI, Enucleation, and Sperm Head Reinjection**

For the following experiments, sonicated sperm heads were stained with Hoechst 33342 for 15 min in DPBS prior to injection into mouse or horse oocytes and left within the corresponding ooplasm, at room temperature, for a period of time ranging 15-30 min. Enucleation (term used here exclusively to indicate removal of the sperm head) was carried out by using the same pipette and Piezo electric unit used for ICSI, as previously described [32]. Prior to the procedure, oocytes were placed in 5 µg/ml cytochalasin B for 10-20 min to facilitate enucleation. To enucleate, the pipette was brought near the Hoechst-stained sperm head, which was identified by brief pulses of UV light, and the sperm head was aspirated using an IM-55-2 Narishige syringe. The enucleated sperm head was brought out of the enucleating drop, and Piezo pulses were applied to remove any surrounding ooplasm. Then, the withdrawn sperm head was reinjected into a fresh MII oocyte, thus performing mouse-to-horse, horse-to-mouse and mouse-to-mouse (control) reinjection experiments.

**Horse-Mouse Oocyte Electrofusion**

To generate mouse-horse oocyte hybrids that were then used for [Ca\(^{2+}\)]\(_i\), monitoring, pairs of oocytes were electrofused after sperm injection into horse oocytes. The fusion procedure was performed as previously reported for mouse oocytes [31]. Briefly, the zona pellucida of both oocytes was removed by exposure to a 0.25% pronase solution in TL-Hepes for 1-5 min. Egg pairs were then placed in 300 µg/ml of
phytohemagglutinin (Sigma) in DPBS without BSA on a warm plate at 37°C for 10 min. Pairs were then transferred to a pulsing dish consisting of 2 stainless-steel wire electrodes 0.5 mm apart and attached to a tissue culture dish filled with fusion medium containing 0.3 M mannitol, 100 µM MgCl₂, 50 µM CaCl₂, and 0.01 g/L BSA. A BTX Electro-Cell Manipulator 200 (BTX, Inc., San Diego, CA) was used to administer the electrical pulse of 0.8 kV/cm (40 V) for 70 µsec. The fused eggs were then immediately transferred into a M-199 drop under mineral oil in a Petri dish for \([\text{Ca}^{2+}]_i\) monitoring.

Statistical Analyses

Data for \([\text{Ca}^{2+}]_i\) responses of in vivo or in vitro matured oocytes that underwent ICSI (Table 1) were compared by \(\chi^2\)-test using JMP IN software (SAS Institute Inc.; Cary, NC). Statistical significance was assumed at P<0.05.

RESULTS

ICSI Does Not Consistently Result in \([\text{Ca}^{2+}]_i\), Oscillations in In Vitro or In Vivo Matured Horse Oocytes

We have previously shown that horse oocytes are capable of consistently responding with \([\text{Ca}^{2+}]_i\) transients when injected with stallion sperm extracts but not when injected with a single motile stallion sperm [23]. In this experiment we wanted to ascertain whether the ability to display \([\text{Ca}^{2+}]_i\) transients was influenced by the source of oocytes, and thus we tested in vitro and in vivo matured oocytes. The \([\text{Ca}^{2+}]_i\) responses induced by ICSI in horse oocytes where not influenced by oocyte source and exhibited limited duration (Table 1). For instance, no \([\text{Ca}^{2+}]_i\) oscillations were detected in 12/19 oocytes, although 2 of those oocytes displayed one \([\text{Ca}^{2+}]_i\) rise (Fig.1). Only 7/19
oocytes displayed [Ca\(^{2+}\)]_i oscillations (i.e. more than one [Ca\(^{2+}\)]_i spike), and only 3 of
these showed more than three [Ca\(^{2+}\)]_i transients. Two oocytes, one from each source,
showed persistent [Ca\(^{2+}\)]_i oscillations (Fig. 2). Noteworthy is that although some oocytes
showed a near immediate [Ca\(^{2+}\)]_i rise, within 5 min of initiation of monitoring, many
fertilized oocytes did not respond for up to 80 min. In those oocytes that oscillated, the
average interval between spikes was 38.5 min (range 17-79 min) and the mean transient
duration was 2.6 min (range 1.7-4.3 min). When these oocytes were stained and
evaluated 20-24 h after ICSI, 12/19 (63%) exhibited two pronuclei. Hence, 5 oocytes that
did not show [Ca\(^{2+}\)]_i oscillations were activated.

Polyspermy Does Not Increase the Percent of Oocytes Displaying [Ca\(^{2+}\)]_i Responses

A possible explanation for the low [Ca\(^{2+}\)]_i responses of horse oocytes after ICSI is
that injected sperm fail to completely release sperm factor in a timely manner due to
incomplete sperm membrane permeabilization. Therefore, if this was the case, injection
of several sperm should induce more frequent [Ca\(^{2+}\)]_i responses as the result of greater
sperm factor availability. To test this possibility, horse oocytes were injected with 3
sonicated stallion sperm heads and monitored as above. However, once again, only 4/10
(40%) of such oocytes displayed [Ca\(^{2+}\)]_i oscillations, although in this case the [Ca\(^{2+}\)]_i
responses appeared highly variable. For instance, the average spike interval was 92 min,
but ranged from 3 to 168 min (Fig. 3) and the mean [Ca\(^{2+}\)]_i spike duration was 4 min
(range 1-5 min), which appeared to be longer than in previous experiments.
We have previously shown that mouse oocytes injected with stallion sperm readily display \([\text{Ca}^{2+}]_i\) transients of high frequency that last for over 2 h [23]. From our data, we can conclude that the factor responsible for initiating \([\text{Ca}^{2+}]_i\) oscillations is indeed present in stallion sperm. However, the inability of ICSI in this study to consistently initiate \([\text{Ca}^{2+}]_i\) oscillations in horse oocytes may suggest that the factor is not released in these oocytes. Notably, this is in spite of the fact that sperm were immobilized by piezoelectric pulses, which have been shown to facilitate release of the factor in sperm from other species [28,33,34]. Therefore, we adopted more aggressive methods to promote release of the factor from sperm such as with the detergent Triton-X or by sonication, the latter of which resulted in complete sperm head-tail separation. In spite of these treatments, Triton-X did not improve \([\text{Ca}^{2+}]_i\) responses (Fig. 4A) and only 1/8 (13%) of oocytes injected with such sperm displayed \([\text{Ca}^{2+}]_i\) oscillations, albeit short lasting. Additionally, only 25% of these oocytes were activated. Sperm sonication prior to ICSI also did not stimulate \([\text{Ca}^{2+}]_i\) oscillations, and only 5/14 (36%) of horse oocytes displayed \([\text{Ca}^{2+}]_i\) oscillations (Fig. 4B), whereas all mouse oocytes injected with these sperm responded (n = 6; results not shown). The average spike interval for oscillating horse oocytes injected with sperm treated by sonication was 40 min (range 30-45 min) and the duration of each spike averaged 2.3 min (range 1.25-3.3 min), values that were similar to previous results after injection of a single motile sperm. Despite the low percent of \([\text{Ca}^{2+}]_i\) responses, 5/7 (71%) oocytes that were not degenerated at the time of
evaluation and that had been injected with a sonicated sperm head had two pronuclei when stained at 20-24 h post-ICSI.

*The Ooplasm Specifically and Rapidly Removes Sperm Factor Activity from the Sperm*

Despite the previous attempts to facilitate the release of the sperm factor, it is possible that in the majority of horse oocytes such factor is not fully released into the ooplasm and, therefore, it is unable to initiate and sustain oscillations. To ascertain this, we took advantage of the findings that injection of stallion sperm is able to consistently initiate $[\text{Ca}^{2+}]_i$ responses in mouse oocytes [23]. We reasoned that withdrawing a stallion sperm from the ooplasm of a horse oocyte followed by reinjection into a mouse oocyte would provide an assessment of whether or not the factor had been released into the former. Towards this end, re-injection of a horse sperm withdrawn after 15-30 min of residence in a horse oocyte induced oscillations in only 2/9 mouse oocytes (Figs. 5A-B), presumably due to quick release of the factor into the horse ooplasm. Similarly, when a sperm was withdrawn from a mouse oocyte and injected into a horse oocyte, only 1/7 oocytes displayed $[\text{Ca}^{2+}]_i$ spikes that subsided within the first 120 min after reinjection (Figs. 5C-D). Control mouse-to-mouse reinjection experiments, as expected, quickly depleted the horse’s sperm activity and only 3/7 reinjected oocytes showed some limited $[\text{Ca}^{2+}]_i$ activity that rapidly subsided in 2/3 oocytes (Fig. 5E). An alternative interpretation to the above results is that the factor could be degraded/inactivated during the time needed for injection, withdrawal and reinjection. To show that this is not the case, sperm heads were left in injection medium at room temperature for periods ranging 90-150 min, and then ICSI was performed. In 6/8 mouse oocytes (Fig. 5F) injection of
sperm readily induced high frequency \([\text{Ca}^{2+}]_i\) oscillations, supporting a specific role for the ooplasm in facilitating the release of the \(\text{Ca}^{2+}\) active factor from the stallion sperm. *Fusion of Mouse Oocytes with Sperm Injected Horse Oocytes Readily Facilitates the Initiation of \([\text{Ca}^{2+}]_i\) Transients*

Altogether, the above experiments supported the idea of adequate release of sperm factor following ICSI into both horse and mouse oocytes, excluding this as the reason for the lack of oscillations in horse oocytes subjected to ICSI. To confirm this assumption, we fused ICSI-fertilized horse oocytes with unfertilized mouse oocytes expecting that if indeed the factor was released and stable in the horse ooplasm, it should initiate oscillations upon encountering mouse ooplasm. For this purpose, zona-free mouse and horse oocytes were fused after performing ICSI in the latter (see Materials and Methods for procedure). Prior to initiating the experiment, both oocytes were loaded with the \([\text{Ca}^{2+}]_i\) indicator Fura-2 and this allowed for accurately following the fusion timeline through our imaging system (Fig. 6). At the initiation of monitoring, oocytes were still separated by their corresponding ooplasms. Initially, a high \([\text{Ca}^{2+}]_i\) baseline was observed in mouse oocytes (Fig. 7A), but not in horse oocytes (Fig. 7B), most likely the result of \(\text{Ca}^{2+}\) influx following the electrical pulse. As fusion became evident, at approximately 15 min post-electrofusion, high frequency \([\text{Ca}^{2+}]_i\) oscillations developed in mouse oocytes, but not in horse oocytes, despite the fact that horse oocytes had been fertilized by ICSI. As fusion proceeded and became complete, which was evidenced by the presence of a single large oocyte (approximately at 30-50 min following electrofusion), \([\text{Ca}^{2+}]_i\) oscillations occurred concomitantly in both oocytes (Fig. 7C). Interestingly, 5/5 fused oocytes displayed \([\text{Ca}^{2+}]_i\) oscillations that lasted for as long as
monitoring continued (30-200 min), although over time the oscillations became less frequent. In these mouse-horse oocyte hybrids $[\text{Ca}^{2+}]_i$, spikes occurred an average of every 18.1 min (range 4.7-35.5 min) and lasted for 3 min (range 0.7-4.3 min); spike duration increased as frequency decreased. Mouse-horse oocyte pairs that were fused without previous sperm injection ($n = 2$) as well as horse oocytes submitted to ICSI and electrical pulses without fusion to a mouse oocyte ($n = 3$) did not display $[\text{Ca}^{2+}]_i$ transients (data not shown). Overall, these results implicate a step downstream of sperm factor release as responsible for the inability of horse oocytes to initiate oscillations upon fertilization by ICSI.

**DISCUSSION**

In this report we confirm the inability of horse gametes to consistently initiate $[\text{Ca}^{2+}]_i$ responses when subjected to ICSI, as suggested in a previous report [23]. Seven births by ICSI have been reported in the horse [35-38], however the published oocyte activation rates after ICSI in this species have been largely inconsistent and generally low amongst laboratories (0 – 71%) [19,20,22,39,40]. Whenever reported, very few ICSI embryos develop *in vitro* to the blastocyst stage [19,40], thus limiting the clinical application of the technique in this species. Thus problems with ICSI could emanate from inadequate *in vitro* oocyte maturation, poorly established embryo culture conditions, and suboptimal oocyte activation after sperm injection. Results presented in this study suggest that the failure of generating long-lasting $[\text{Ca}^{2+}]_i$ oscillations during fertilization by ICSI in the horse may be an important cause for the low success of this technique in this species.
The main question is that of ‘why are oscillations not initiated or maintained?’.

Although the possibility that oocytes used in this study were not optimally matured cannot be completely discounted, the fact that similar results were observed in in vitro and in vivo matured oocytes suggests that the quality of the oocytes was not the main factor responsible for the lack of oscillations. Therefore, we evaluated the simple notion that the sperm factor may have not been properly released, since sperm permeabilization has been shown to facilitate release of the factor in other species. Therefore we treated stallion sperm with Triton-X or by sonication, anticipating that this may improve the percent of oocytes responding with \([\text{Ca}^{2+}]_i\) oscillations, since reports in other species have shown this to be the case [28,33,34]. However, neither of the treatments applied stimulated the consistent generation of persistent \([\text{Ca}^{2+}]_i\) oscillations. A first possible interpretation is that, despite membrane permeabilization, the factor was actually not released at all. To test whether this was indeed happening, we performed ICSI, followed by enucleation and reinjection of sperm to ascertain whether sperm that had resided in horse oocytes for a limited time period still retained the ability to induce \([\text{Ca}^{2+}]_i\) oscillations in mouse oocytes. We showed that such sperm were almost completely devoid of activity, suggesting that the sperm factor had indeed been released into the ooplasm of horse oocytes.

Having eliminated the possibility that the factor is not released, it was still possible that, despite appropriate release, the sperm factor was inactivated. To assess this possibility, we fused ICSI-fertilized horse oocytes, which were not oscillating, with unfertilized mouse oocytes. Interestingly, once fusion occurred, oscillations were persistently observed. Notably, the \([\text{Ca}^{2+}]_i\) rises started in the mouse oocyte, despite the
The fact that the horse oocyte contained the sperm, thus supporting two key points: a) that the factor is highly soluble, and b) that the factor is nearly completely released and able to remain active for prolonged periods of time. We therefore suggest that a step involved in the activation and/or provision of the factor is responsible for the inability to initiate oscillations in horse oocytes.

The precise nature of the mechanism(s) responsible for the inactivity of the sperm factor in horse oocytes was not revealed in these studies. However, two possibilities are worth considering. First, the factor may need to be activated/processed before it can become fully functionally active in this species. In this regards, it is plausible that, under natural fertilization conditions, the molecular changes that are associated with sperm capacitation and acrosome reaction and/or sperm-oocyte fusion may, at least in part, serve this purpose. In this regard, it is worth noting that proteolytic processing by proteases activated/released during the acrosome reaction may serve/contribute to activation. The sperm is known to posses several proteases, although the function of these remains to be elucidated (41,42). Second, the appropriate substrate, i.e. PIP$_2$, may not be present in adequate amounts or may not be accessible to the sperm factor following ICSI in horse oocytes. In this regard, it is possible that events initiated by fusion at fertilization may induce structural or biochemical changes in the oocyte that are responsible for stimulating production and/or presentation of the substrate. In this context, it is worth noting that cortical granule exocytosis, which entails fusion, has been associated with increased PIP$_2$ production [43]. Nevertheless, our previous results showing that injection of stallion sperm extracts into horse oocytes consistently induces [Ca$^{2+}$]i oscillations makes the unavailability of the substrate as a plausible explanation less likely [23]. An
obvious question that arises from this discussion and that deserves further investigation is why mouse oocytes can process such unprepared sperm whereas horse oocytes cannot? Several additional interesting points emerged from our mouse-horse fusion studies. First, our results showing that oscillations only started in mouse oocytes after membrane fusion, which made possible the diffusion of the highly soluble sperm factor, was clearly evident. This finding further supports the notion that a sperm-supplied molecule, which we show to be highly soluble and diffusible, is involved in the initiation of \([\text{Ca}^{2+}]_i\), oscillations at the time of fertilization, as previously suggested by others [13, 18]. Second, the evidence that despite the apparent rapid diffusion of the sperm factor into the mouse oocyte, global \([\text{Ca}^{2+}]_i\), oscillations were not immediately present in horse oocytes, even though they were the source of the factor, highlights possible differences between mammalian oocytes regarding mechanisms of oscillations and sensitivity to agonists. For instance, it is most likely that upon diffusion into mouse oocytes, the sperm factor initiated oscillations by stimulating IP3 production [16]. Notably, and since IP3 readily diffuses [44], it would have been expected that IP3 would have dispersed into horse oocytes immediately generating simultaneous \([\text{Ca}^{2+}]_i\) transients in both oocytes. However, this was not the case, and a significant delay preceded the presence of global oscillations in horse oocytes. An explanation to these results is that the time required to achieve threshold IP3 concentrations in horse oocytes may be significantly greater than for mouse oocytes. Although we have not tested the sensitivity of the IP3 receptor in these oocytes, it is well established that bovine oocytes are less sensitive to IP3 than mouse oocytes [3, 45]. Therefore, if this was also the case in horse oocytes, it may explain the protracted initiation of the rises in the latter. Additionally, it is also possible that \([\text{Ca}^{2+}]_i\)
oscillations in the horse oocyte are only initiated once an activated sperm factor, which in our experimental conditions was performed by mouse oocytes, is available for activation of the [Ca^{2+}]_{i} release mechanisms in horse oocytes. Therefore, the lag time to initiate oscillations in horse oocytes may represent the time needed to activate/process the factor.

The frequency of ICSI induced [Ca^{2+}]_{i} oscillations in this study, which generally occurred at 20-40 min intervals, is strikingly similar to what has been reported for the bovine species at fertilization [1,3]. Since the pattern of [Ca^{2+}]_{i} oscillations at fertilization is species-specific for the oocyte, we can only speculate that the patterns observed herein may represent what is physiological for the horse at fertilization. Furthermore, in other species it has been shown that the pattern of [Ca^{2+}]_{i} transients observed at fertilization can be closely mimicked by ICSI [14,46]. With this in mind, it appears that with some improvements, ICSI may provide a good model to further characterize fertilization-induced responses in the equine species.

It is also worth stating that despite the low percentages of horse oocytes responding with [Ca^{2+}]_{i} oscillations after ICSI in this study, oocyte activation rates ranged from 25-85%, which are comparable and generally higher to those obtained in previous reports [19,20,22]. Furthermore, it is important to keep in mind that oocytes herein were injected with Fura-2 and exposed to UV light for prolonged periods of time for [Ca^{2+}]_{i} monitoring, all of which may have affected the events leading to pronuclear formation. In any case, our activation rates also bring up the point that in certain proportion of oocytes where [Ca^{2+}]_{i} oscillations were not detected, activation still occurred. Since [Ca^{2+}]_{i} monitoring rarely started immediately after sperm injection, it is possible that an initial [Ca^{2+}]_{i} rise resulting from sperm injection itself may have been
sufficient to activate some of these oocytes, although mock injected oocytes (results not shown) never displayed $[\text{Ca}^{2+}]_i$ responses nor activated.

In summary, our results show that horse oocytes fertilized by ICSI inconsistently display $[\text{Ca}^{2+}]_i$ oscillations, and that this failure is not due to inadequate sperm factor release but to inability of the oocyte to activate/process and/or provide the adequate substrate for the factor released by the sperm. These results may partially explain the inconsistencies reported for oocyte activation and embryonic development for this technique in the horse, and suggest that improving the ability of horse gametes to initiate $[\text{Ca}^{2+}]_i$ responses after ICSI may improve the success of this technique in the horse. Therefore, future studies should address sperm and oocyte treatments to improve $[\text{Ca}^{2+}]_i$ responses after ICSI in the horse, and thus to more closely characterize the signaling mechanisms governing fertilization-induced oocyte activation in this species.
REFERENCES


37. McKinnon AO, Lacham-Kaplan O, Trounson AO. Pregnancies produced from fertile and infertile stallions by intracytoplasmic sperm injection (ICSI) of single...


**FIGURE LEGENDS**

Figure 1. *In vivo matured horse oocyte displaying a single [Ca\(^{2+}\)]\(_i\) spike after ICSI with a motile stallion sperm.* Altogether, 12/19 oocytes failed to display [Ca\(^{2+}\)]\(_i\) oscillations, although 2 of those oocytes displayed a single [Ca\(^{2+}\)]\(_i\) rise, such as shown here.

Figure 2. **Oscillatory [Ca\(^{2+}\)]\(_i\) responses induced by ICSI with a stallion sperm in horse oocytes.** Selected [Ca\(^{2+}\)]\(_i\) transient profiles of an in vitro (top panel) and an in vivo (lower panel) matured horse oocyte in response to injection of a motile stallion sperm.

Figure 3. *Injection of three stallion sperm is unable to consistently initiate oscillations in horse oocytes.* Selected [Ca\(^{2+}\)]\(_i\) transient profiles in in vitro matured horse oocytes injected with three stallion sperm heads. Only 4/10 oocytes displayed [Ca\(^{2+}\)]\(_i\) oscillations. Profiles A and B represent the range of responses obtained amongst oocytes with polyspermy.

Figure 4. **Permeabilization of stallion sperm does not stimulate additional [Ca\(^{2+}\)]\(_i\) release upon injection into horse oocytes.** A. Representative [Ca\(^{2+}\)]\(_i\) transient profile of a horse oocyte injected with a Triton X-treated sperm; 7/8 oocytes injected with Triton X-treated sperm displayed a similar response. B. A selected [Ca\(^{2+}\)]\(_i\) profile of an in vitro matured horse oocyte injected with a sonicated sperm head; 5/14 of such oocytes displayed [Ca\(^{2+}\)]\(_i\) oscillations.

Figure 5. *Horse and mouse oocytes deplete the stallion sperm’s ability to initiate oscillations upon reinjection.* Representative [Ca\(^{2+}\)]\(_i\) transient profiles elicited in horse and mouse oocytes by injection of stallion sperm withdrawn from either horse or mouse oocytes. Sonicated stallion sperm heads were used for all experiments. A. Mouse oocyte injected with a sperm enucleated from a horse oocyte displaying limited [Ca\(^{2+}\)]\(_i\) oscillations. B. Same as A; this was the most typical response seen with this treatment. C. Horse oocyte injected with sperm enucleated from a mouse oocyte displaying limited [Ca\(^{2+}\)]\(_i\) responses. D. Same as C; most reinjected horse oocytes did not display [Ca\(^{2+}\)]\(_i\) transients. E. Mouse oocyte injected with a sperm previously injected into a mouse oocyte; when present, [Ca\(^{2+}\)]\(_i\) responses were very limited. F. Mouse oocyte injected with a sperm kept in injection medium at room temperature for 90-150 min. Numbers on the right upper corner indicate the proportion of oocytes with the corresponding response for each group.

Figure 6. **Zona-free mouse and horse oocytes can be fused to generate a mouse-horse oocyte hybrid.** Fusion sequence of a mouse (open arrow) and a horse (solid arrow) oocyte after applying an electrical pulse. Time after pulse (range): (A) 0-15 min; (B) 15-30 min; (C) 30-40 min; (D) 40-50 min; (E,F) > 50 min. Magnification x 200.

Figure 7. **Initiation of [Ca\(^{2+}\)]\(_i\) oscillations in the mouse-horse oocyte hybrid.** Fertilization of the horse oocyte was accomplished by ICSI with a stallion sperm. A. Mouse oocyte profile shows an initial [Ca\(^{2+}\)]\(_i\) rise over baseline probably resulting from
the electric pulse. As fusion starts, frequent $[\text{Ca}^{2+}]_{i}$ transients are restricted to the mouse oocyte. B. Horse oocyte profile showing the initiation of $[\text{Ca}^{2+}]_{i}$ oscillations as fusion with the mouse oocyte was nearly completed. C. Mouse and horse $[\text{Ca}^{2+}]_{i}$ response profiles combined. Numbers on the right upper corner indicate the proportion of oocyte pairs responding with $[\text{Ca}^{2+}]_{i}$ oscillations. Please note that the frequency of the rises show a more “horse-like pace”, as the intervals between rises are significantly greater than those observed in mouse oocytes injected with a stallion sperm.
Table 1. [Ca^{2+}]_i responses in ‘in vitro’ or ‘in vivo’ matured horse oocytes injected with one motile frozen-thawed stallion sperm.

<table>
<thead>
<tr>
<th></th>
<th># eggs oscillating &gt; 1 spike (%)</th>
<th>Total No. spikes detected^b</th>
<th>Spike duration (min)</th>
<th>Activation^a 2 PN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro</td>
<td>4/12 (33)</td>
<td>4.5±3.1</td>
<td>2.3±0.5</td>
<td>6/12 (50)</td>
</tr>
<tr>
<td>In vivo</td>
<td>3/7 (44)</td>
<td>2.0±0.5</td>
<td>2.8±0.5</td>
<td>6/7 (85)</td>
</tr>
</tbody>
</table>

^aFor evaluation of activation status oocytes were fixed and stained with Hoechst 33248 at 20-24 h post-ICSI; PN = Pronuclei. Some oocytes that had not displayed [Ca^{2+}]_i oscillations were activated at the time of evaluation.

^bMean±SEM for eggs that exhibited [Ca^{2+}]_i oscillations.

There were no significant differences (P>0.05) in [Ca^{2+}]_i responses between in vitro or in vivo matured horse oocytes.
Non-oscillating horse oocyte
N = 2/19

Figure 1; Bedford et al.
In vitro matured
N = 1/12

In vivo matured
N = 1/7

Figure 2; Bedford et al.
Figure 3; Bedford et al.

A: Polyspermy
N = 4/10

B: F340/F380
Time (min) after ICSI
Figure 4; Bedford et al.

(A) Triton X-treated sperm
N = 7/8

(B) Sonicated sperm
N = 5/14

F340/F380 vs. Time (min) after ICSI
Figure 5; Bedford et al.

A. Horse-to-mouse
N = 2/9

B. Horse-to-mouse
N = 7/9

C. Mouse-to-horse
N = 1/7

D. Mouse-to-horse
N = 6/7

E. Mouse-to-mouse
N = 3/7

F. Sperm at room temperature
N = 6/8
Figure 7; Bedford et al.

Mouse oocyte

Horse oocyte

Mouse and horse oocytes combined

Time (min after applying electropulse for mouse/horse oocyte fusion)

N = 5/5

F340/F380

A

B

C