Intracellular calcium oscillations and activation in horse oocytes injected with stallion sperm extracts or spermatozoa

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In oocytes from all mammalian species studied to date, fertilization by a spermatozoon induces intracellular calcium ($[Ca^{2+}]_i$) oscillations that are crucial for appropriate oocyte activation and embryonic development. Such patterns are species-specific and have not yet been elucidated in horses; it is also not known whether equine oocytes respond with transient $[Ca^{2+}]_i$ oscillations when fertilized or treated with parthenogenetic agents. Therefore, the aims of this study were: (i) to characterize the activity of equine sperm extracts microinjected into mouse oocytes; (ii) to ascertain in horse oocytes the $[Ca^{2+}]_i$ -releasing activity and activating capacity of equine sperm extracts corresponding to the activity present in a single stallion spermatozoon; and (iii) to determine whether equine oocytes respond with $[Ca^{2+}]_i$ transients and activation when fertilized using the intracytoplasmic sperm injection (ICSI) procedure.

The results of this study indicate that equine sperm extracts are able to induce $[Ca^{2+}]_i$ oscillations, activation and embryo development in mouse oocytes. Furthermore, in horse oocytes, injection of sperm extracts induced persistent $[Ca^{2+}]_i$ oscillations that lasted for > 60 min and initiated oocyte activation. Nevertheless, injection of a single stallion spermatozoon did not consistently initiate $[Ca^{2+}]_i$ oscillations in horse oocytes. It is concluded that stallion sperm extracts can efficiently induce $[Ca^{2+}]_i$ responses and parthenogenesis in horse oocytes, and can be used to elucidate the signalling mechanism of fertilization in horses. Conversely, the inconsistent $[Ca^{2+}]_i$ responses obtained with sperm injection in horse oocytes may explain, at least in part, the low developmental success obtained using ICSI in large animal species.

Introduction

Assisted reproduction technologies have gained wide acceptance in humans and in domestic animal species. In horses, the success of technologies such as intracytoplasmic sperm injection (ICSI) has been limited and this is thought to be due primarily to inadequate oocyte activation and *in vitro* embryo culture systems (Kato *et al.*, 1997; Li *et al.*, 2000, 2001). With respect to oocyte activation, two reports have specifically addressed parthenogenetic methods of activation in horses (Hinrichs *et al.*, 1995; Choi *et al.*, 2001), but the underlying signalling mechanisms leading to activation of equine oocytes have not been characterized.

In oocytes from all mammalian species studied to date, fertilization by a spermatozoon induces a series of species-specific increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that are crucial for oocyte activation (Fissore *et al.*, 1992; Kline and Kline, 1992; Nakada *et al.*, 1995). These fertilization-associated oscillations are responsible for downregulation of maturation-promoting

factor (MPF), which allows resumption of meiosis, cortical granule exocytosis and block to polyspermy, pronuclear formation, and initiation of embryonic cleavage, all of which are events in egg activation (Kline and Kline, 1992; Carroll, 2001). Furthermore, recent research indicates that the initiation and modulation of these $[Ca^{2+}]_i$ oscillations may affect not only preimplantation, but also postimplantation, embryonic development in mammalian species (Ozil and Huneau, 2001).

The exact mechanism by which a spermatozoon triggers $[Ca^{2+}]_i$ oscillations is the subject of intense research, and although both membrane-mediated (receptor) and fusion-mediated (sperm factor) pathways have been postulated as initiators of $[Ca^{2+}]_i$ oscillations, there is more evidence for a role of an activating factor introduced by the spermatozoon (for reviews, see Swann, 1996; Fissore *et al.*, 1998). Despite the uncertainty of how oscillations are initiated, it is widely accepted that fertilization results in activation of the phosphoinositide pathway, with production of inositol 1,4,5-triphosphate (IP_3) by the enzymatic action of phospholipase C (PLC), and the subsequent release of $[Ca^{2+}]_i$ from the endoplasmic reticulum (Carroll, 2001). Further investigation into the role of PLC isoforms as the

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initiators of $[Ca^{2+}]_i$ oscillations has led to the recent identification of a sperm-specific PLC (PLC ξ) as the putative sperm-derived oocyte activating factor (Saunders *et al.*, 2002). Furthermore, a homologue of PLC ξ has been found in mouse, human and monkey spermatozoa (Cox *et al.*, 2002).

As well as fertilization, oocyte activation can also be initiated parthenogenetically. The most commonly used treatments, electroporation, Ca^{2+} ionophores and ethanol, are all known to induce a single $[Ca^{2+}]_i$ increase in oocytes (Fissore and Robl, 1992, 1993; Collas *et al.*, 1993; Nakada and Mizuno, 1998) and thus are unlikely to support activation and embryonic development at rates similar to those observed at fertilization. Therefore, ethanol and ionomycin are often used in combination with inhibitors of protein phosphorylation (for example, 6-DMAP) or synthesis (for example, cycloheximide) to optimize rates of oocyte activation for assisted reproduction in cows and horses (Presicce and Yang, 1994; Hinrichs *et al.*, 1995; Chung *et al.*, 2000; Choi *et al.*, 2001; Knott *et al.*, 2002). Other parthenogenetic treatments, such as thimerosal, strontium chloride, IP_3 or adenophostin, induce multiple $[Ca^{2+}]_i$ responses in mammalian oocytes and yield rates of activation more comparable to those observed at fertilization (Fissore and Robl, 1993; Fissore *et al.*, 1995; Nakada and Mizuno, 1998; Sato *et al.*, 1998; Jones and Nixon, 2000), although the difficulty of administration and some undesirable side effects have limited their use.

A more novel and possibly more physiological procedure for activation of mammalian oocytes is the injection of cytosolic sperm extracts, also referred to as sperm factor (SF). Previous reports show that injection of SF stimulates the phosphoinositide pathway resulting in a pattern of $[Ca^{2+}]_i$ oscillations similar to that observed at fertilization (Wu *et al.*, 1997, 1998a, 2001; Knott *et al.*, 2002), thereby further supporting the theory that a molecule introduced by the spermatozoon at the time of gamete fusion is the trigger for oscillations and subsequently for oocyte activation (Fissore *et al.*, 1998). Furthermore, injection of either homologous or heterologous SF supports oocyte activation and embryonic development in mouse, rabbit, cow and pig oocytes (Stice and Robl, 1990; Wu *et al.*, 1997, 1998b; Machaty *et al.*, 2000; Knott *et al.*, 2002), indicating that a highly conserved mechanism or molecule is probably responsible for the initiation and persistence of $[Ca^{2+}]_i$ oscillations in mammalian species.

Recently, crude sperm extracts obtained from the supernatant of frozen-thawed stallion sperm lysates have been used to activate equine oocytes subjected to nuclear transfer (Choi *et al.*, 2002a). However, the $[Ca^{2+}]_i$ responses produced by these fractions have not been characterized, and it has not been demonstrated whether equine oocytes respond with $[Ca^{2+}]_i$ transients to parthenogenetic treatments or fertilization. Therefore, the aims of the present study were: (i) to determine the

$[Ca^{2+}]_i$ -releasing and parthenogenetic activity of stallion sperm extracts microinjected into mouse oocytes; (ii) to ascertain whether stallion sperm extracts would induce $[Ca^{2+}]_i$ oscillations and activation in metaphase II horse oocytes; and (iii) to determine whether horse oocytes would undergo fertilization-like $[Ca^{2+}]_i$ oscillations after ICSI.

Materials and Methods

Recovery and culture of mouse oocytes

Two strains of mice, CD1 and B6D2F1, were used for microinjection and ICSI experiments, respectively, as oocytes of the B6D2F1 strain are known to sustain higher rates of survival after sperm injection (Kimura and Yanagimachi, 1995). Ovaries were superstimulated by injection of 5 iu eCG (Sigma Chemical Co., St Louis, MO), followed 48 h later by injection of 5 iu hCG (Sigma). Metaphase II mouse oocytes were obtained from the oviducts of 4–16-week-old female mice that were killed at about 14 h after hCG administration. After retrieval of the oocytes, cumulus cells were removed by incubation for 3–5 min in bovine testis hyaluronidase in Dulbecco's PBS (DPBS; 0.1% (w/v); Sigma). Oocytes showing no evidence of degeneration and that had extruded the first polar body were used for microinjection or ICSI. Oocyte incubation was performed in microdroplets of potassium simplex optimized medium (KSOM; Specialty Media, Phillipsburg, NJ) under paraffin oil at 36.5°C in a humidified atmosphere with 5% CO_2 .

Recovery, in vitro maturation (IVM) and culture of equine oocytes

Equine cumulus–oocytes complexes (COCs) for IVM were obtained by scraping the follicular walls of ovaries collected from an abattoir. Oocytes with expanded cumuli (meiotically competent oocytes; Hinrichs and Schmidt, 2000) were placed into 1 ml equilibrated maturation medium (TCM199 with Earle's salts; Gibco Life Technologies, Inc., Grand Island, NY) supplemented with 5 μ U FSH ml^{-1} (Sioux Biochemical Inc., Sioux Center, IA), 10% FBS and 25 μ g gentamycin ml^{-1} . 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. Upon arrival, the oocytes were placed in microdroplets of the same medium under light mineral oil in an incubator at 38°C in 5% CO_2 in humidified air to continue maturation as required for individual experiments (that is, 24 or 42 h after the time at which the oocytes had been placed in the incubator for shipment). After the appropriate maturation period, the oocytes were denuded from their cumulus cells by repeated pipetting in bovine testis hyaluronidase in DPBS (0.1% (w/v); Sigma). Oocytes with intact cytoplasmic membranes with no signs of degeneration or

fragmentation and with a visible perivitelline space were selected for micromanipulation. After microinjection and monitoring, equine oocytes were incubated in culture medium but without FSH at 38°C in 5% CO₂ in humidified air for an additional 20–24 h before evaluation of activation status.

Preparation of equine sperm extracts (eSF)

Pooled ejaculates from two stallions of proven fertility were used to prepare eSF following a procedure similar to that previously described for pig sperm extracts (pSF; Wu *et al.*, 1997, 1998a). In brief, pooled semen samples were washed twice with TL-Hepes medium and the sperm pellet was resuspended in a solution containing 75 mmol KCl l⁻¹, 20 mmol Hepes l⁻¹, 1 mmol EDTA l⁻¹, 10 mmol glycerophosphate l⁻¹, 1 mmol dithiothreitol l⁻¹, 200 µmol phenylmethylsulphonyl fluoride (PMSF) l⁻¹, 10 µg pepstatin ml⁻¹ and 10 µg leupeptin ml⁻¹, pH 7.0. The sperm suspension was subjected to a freeze–thaw cycle by submerging it in liquid nitrogen at –196°C for 5 min and then thawing it at room temperature, and the suspension was sonicated (XL2020; Heat Systems Inc., Farmingdale, NY) at 4°C for 10–20 min (or until head–tail separation was evidenced by evaluation of a sample by contrast microscopy). The lysate was centrifuged at 10 000 *g* and the supernatant was collected for ultracentrifugation at 100 000 *g* for 1 h at 4°C. Ultrafiltration membranes (Centriprep-30; Amicon, Beverly, MA) were used to wash the supernatant obtained from ultracentrifugation and to concentrate the protein extracts. Crude extracts were slowly mixed with ammonium sulphate to 50% saturation and the precipitate was collected by centrifugation (10 000 *g*, 15 min, 4°C). The pellets were stored at –80°C to resume sperm extract preparation at a later time before use.

For microinjection purposes, the stored precipitate was resuspended and washed in injection buffer (75 mmol KCl l⁻¹, 20 mmol Hepes l⁻¹, pH 7.0) and protein was concentrated in ultrafiltration membranes (Centriprep-30 and Centricon-30; Amicon). Final protein concentrations were assessed using a bicinchoninic acid protein assay kit (Sigma) for protein determination. In brief, samples with known protein concentrations and eSF aliquots were incubated at 37°C for 30 min in a reagent containing 1 part copper sulphate pentahydrate (4% (w/v) solution) and 50 parts bicinchoninic acid solution (B-9643). Absorbance of the samples was determined in microplates (562 nm) using MRX^{TC} revelation software (Dynex Technologies, Inc., Chantilly, VA). Protein concentrations in eSF aliquots were extrapolated from a plot obtained with standard samples.

Microinjection procedure

Microinjection was performed as described by Wu *et al.* (1998a,b). In brief, denuded metaphase II mouse

or equine 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 mmol fura-2 dextran l⁻¹ (fura-2D; Molecular Probes; Eugene, OR) or appropriate concentrations of pSF, eSF or adenophostin were advanced into the cytoplasm of each individual oocyte and an appropriate volume of the reagent was injected by pneumatic pressure (PLI-100 Picoinjector; Harvard Apparatus; Cambridge, MA). The injection volume ranged from 5 pl to 10 pl in mouse oocytes and from 15 pl to 20 pl in equine oocytes.

For microinjection of mouse oocytes, the concentration of eSF (0.25 or 0.50 µg µl⁻¹) was chosen on the basis of results from previous research in our laboratory, in which use of similar concentrations of equally prepared pSF provided fertilization-like [Ca²⁺]_i responses and optimal rates of activation and embryonic development in mouse oocytes (Wu *et al.*, 2001).

ICSI procedure

ICSI was performed as described by Kimura and Yanagimachi (1995). For ICSI into mouse oocytes, suspensions of washed frozen–thawed or sonicated stallion sperm heads in microinjection buffer were used. Horse oocytes were injected with freshly collected motile spermatozoa washed in DPBS and immobilized by a piezoelectric pulse immediately before injection. Sperm suspensions were mixed with an equal volume of 12% (w/v) polyvinylpyrrolidone (PVP) and a droplet of this sample was placed on a plastic Petri dish, which served as the injection chamber. Manipulations were performed on an inverted Nikon Diaphot microscope. Injections were performed with a piezomicropipette-driving unit (Piezo drill; Burleigh, Inc., Rochester, NY). The spermatozoon was aspirated into the pipette (tail first for motile spermatozoa) and the tip of the injection pipette was put into contact with the zona pellucida. Several piezo-pulses were applied to advance the pipette through the zona pellucida. Once the pipette passed through the zona, it was advanced against the membrane of the oocyte to the opposite side of its cortex while applying light negative pressure; one or two piezo pulses of lower intensity were applied to penetrate the oocyte membrane, and the sperm head was injected into the cytoplasm surrounded by a minimal amount of fluid.

Fluorescence recordings and determination of [Ca²⁺]_i

Fluorescence of fura-2D-loaded oocytes subjected to microinjection or ICSI was monitored as described by Wu *et al.* (1997). In brief, the illumination was provided by a 75 W xenon arc lamp on a Nikon Diaphot microscope equipped with a × 40 UV oil immersion objective (Nikon Inc.). Excitation wavelengths were 340 and 380 nm, and the corresponding emitted light (attenuated 32-fold

by neutral density filters) was quantified by a photomultiplier tube, which averaged the fluorescence signal over the whole oocyte. The rotation of a filter wheel and a shutter apparatus was controlled by a modified Phoscan 3.0 software program (Nikon Inc.) on a 486 IBM-compatible computer to alternate wavelengths. Fluorescence ratios were obtained every 4 s and $[Ca^{2+}]_i$ concentrations were extrapolated from these values in a calibrated system as described by Wu *et al.* (1998a).

For some experiments, fluorescence values of a group of oocytes were measured simultaneously using the software Image 1/FL (Universal Imaging, Downingtown, PA). Images were acquired using a SIT camera (Dage-MTI, Michigan City, IN) coupled to an image intensifier (Video Scope International Ltd., Sterling, VA). Fluorescence ratios were obtained every 10–20 s, after 1 s reading at each wavelength. Data are presented as the ratio of 340:380 fluorescence, which correlates with $[Ca^{2+}]_i$.

Assessment of oocyte activation status

For assessment of activation, as well as for experiments investigating *in vitro* embryonic development, mouse oocytes were observed under a phase contrast microscope for extrusion of the second polar body (2 h), pronuclear formation (4–6 h), cleavage (24–96 h), and development to the morula and blastocyst stages (96–144 h). At day 6 after microinjection, in selected experiments assessing rates of development, blastocysts were stained with Hoechst 33342 and the number of blastomeres was counted under fluorescence microscopy.

At 20–24 h after microinjection or ICSI, horse oocytes were fixed in saline-buffered formaldehyde with 0.1% (v/v) Triton-X100, mounted on a slide with 6.5 μ l of 9:1 glycerol:PBS containing 2.5 μ g Hoechst 33258 ml^{-1} , and examined using fluorescence microscopy to determine the chromatin configuration. Oocytes were considered activated if one pronucleus was observed. Non-activated oocytes were those in metaphase II or anaphase–telophase configurations. Degenerate oocytes and those in metaphase I were not counted in the assessment of rates of activation.

Protein kinase assays

Histone H1 and mitogen-activating protein (MAP) kinase assays were performed as previously described by Fissore *et al.* (1996) for microinjected mouse oocytes cultured to the pronuclear stage and contemporary non-injected metaphase II oocytes (controls). Myelin basic protein (MBP) was assumed to measure mostly MAP kinase activity as shown by Fissore *et al.* (1996). Groups of mouse oocytes ($n = 5$ per group) were lysed by several repeated cycles of freezing and thawing in H1 kinase buffer (80 mmol glycerophosphate l^{-1} , 5 mmol EGTA l^{-1} , 15 mmol $MgCl_2$ l^{-1} , 1 mmol dithiothreitol l^{-1} , 10 μ g aprotinin ml^{-1} , 10 μ g leupeptin ml^{-1} , 10 μ g pepstatin

ml^{-1} and 500 nmol cAMP-dependent protein kinase inhibitor l^{-1}) and stored at $-80^\circ C$ until use. Kinase reactions were started by adding 5 μ l of a solution containing 2 mg histone H1 ml^{-1} (type II-S; Sigma), 1 mg MBP ml^{-1} (Sigma), 0.7 mmol ATP l^{-1} and 50 μ Ci $[\gamma\text{-}^{32}P]\text{ATP}$ (Amersham, Arlington Heights, IL) to 5 μ l of the crude oocyte lysates. The reaction was performed for 30 min at $30^\circ C$ and terminated by the addition of 5 μ l SDS sample buffer (Laemmli, 1970). Samples were boiled for 3 min and loaded into 15% (w/v) SDS–polyacrylamide gels. Control samples contained all the components required for the reaction but without the addition of oocytes. Phosphorylation of histone H1 and MBP was visualized by autoradiography using DuPont's Cronex intensifying screens at $-70^\circ C$ (DuPont, Wilmington, DE). Autoradiographs were scanned and quantified using Adobe Photoshop (Mountain View, CA) and plotted using SigmaPlot software (Jandel Scientific Software, San Rafael, CA). Multiple gel exposures were used to avoid saturation of the quantification system. Kinase activity in microinjected oocytes was normalized to the kinase activity in metaphase II oocytes, which were arbitrarily given the value of 1.

Statistical analysis

Values from kinase assays were compared by chi-squared analysis, whereas means for activation, embryonic development and $[Ca^{2+}]_i$ transient data were analysed by one-way ANOVA, followed by comparisons between means applying Student's *t* test using JMP Inc. software (SAS Institute, Gary, NC). Means were considered significantly different at $P < 0.05$.

Results

Effect of stallion sperm extracts (eSF) on $[Ca^{2+}]_i$ oscillations in CD1 mouse oocytes

Wu *et al.* (1997) showed that injection of pSF into mouse oocytes induces $[Ca^{2+}]_i$ oscillations similar to those observed at fertilization in this species. The aim of the present study was to investigate whether eSF would have a similar $[Ca^{2+}]_i$ -releasing activity in mouse oocytes. Representative $[Ca^{2+}]_i$ oscillation profiles for microinjection of 0.25 and 0.50 μ g eSF μ l $^{-1}$ are shown (Fig. 1) and the results are also summarized (Table 1). More mouse oocytes showed $[Ca^{2+}]_i$ responses when injected with 0.50 rather than 0.25 μ g eSF μ l $^{-1}$. Furthermore, in half of the oocytes injected with the lower concentration of eSF, $[Ca^{2+}]_i$ oscillations lasted for < 30 min, whereas in all oocytes injected with 0.50 μ g eSF μ l $^{-1}$, transients lasted for > 30 min and, in most oocytes, for up to 60–120 min. Spike frequency was higher for oocytes injected with the higher concentration of eSF ($P < 0.05$). Overall, $[Ca^{2+}]_i$ responses obtained with

Fig. 1. Representative intracellular Ca²⁺ concentration ([Ca²⁺]_i) profiles for metaphase II mouse oocytes microinjected with (a) 0.25 (*n* = 7) or (b) 0.50 μg (*n* = 10) equine sperm factor (eSF) μl⁻¹. Arrows indicate times of injection.

0.5 μg eSF μl⁻¹ were similar to those previously reported with pSF in our laboratory and were similar in duration to those reported in mouse oocytes at fertilization (Fissore *et al.*, 1998; Wu *et al.*, 2001). Therefore, 0.5 μg eSF μl⁻¹ was chosen as the working concentration for subsequent experiments. As expected, injection of buffer alone did not induce [Ca²⁺]_i oscillations.

Effect of microinjection of eSF on MPF and MAP kinase downregulation in mouse oocytes

Fertilization in mammalian oocytes results in the resumption of meiosis, which is accomplished by downregulation of MPF and mitogen-activated protein (MAP) kinases, both of which are responsible for the metaphase II arrest (Whitaker, 1996; Carroll, 2001). Therefore, to test the activity of the stallion sperm extracts further, mouse oocytes were microinjected with 0.5 μg eSF μl⁻¹ and incubated until the pronuclear stage.

Table 1. Calcium-releasing activity of 0.25 or 0.50 μg equine sperm extracts (eSF) μl⁻¹ microinjected into metaphase II mouse oocytes

	eSF	
	0.25 μg μl ⁻¹	0.5 μg μl ⁻¹
Number of eggs oscillating		
> 5 min	7/8	10/11
> 30 min	4/8	10/11
Amplitude (nmol l ⁻¹)		
First spike	372.1 ± 57.6	607.1 ± 57.2*
Spike > 10 min	251.0 ± 11.7	279.4 ± 26.6
Duration (min)		
First spike	5.43 ± 0.33*	4.25 ± 0.25
Spike > 10 min	1.33 ± 0.11	0.92 ± 0.04
Frequency (number of spikes)		
10–20 min	2.3 ± 0.50	5.2 ± 0.50*

Values are mean ± SEM; results are from at least three replicates.

*Significant difference between groups (*P* < 0.05).

Fig. 2. (a) Kinase assay showing decline of histone H1 (H1K; MPF) and MAP kinase (MAPK) activities in pronuclear (PN) stage mouse oocytes activated with 0.5 μg eSF μl⁻¹ compared with uninjected metaphase II (MII) oocytes. (b) Plot for H1 and MAP kinase activities averaged for five replicates. Asterisks indicate significant differences (*P* < 0.05).

Oocytes were collected for kinase assays and the results were compared with metaphase II (uninjected) oocytes incubated under the same conditions. A decrease in MPF and MAP kinase activity was observed in oocytes microinjected with eSF, indicating that eSF is able to promote this decrease in activity and thus support resumption of meiosis in mouse oocytes (Fig. 2a,b).

Table 2. Activation of mouse oocytes injected with sperm buffer, pig sperm extracts (pSF), adenophostin and equine sperm extracts (eSF)

	Number of oocytes	Extrusion of second polar body (2–4 h)	Pronuclear stage (6–8 h)	Two-cell stage (24 h)
Buffer	10	1 (10 ± 0) ^c	3 (30 ± 0) ^b	0 (0.0 ± 0) ^b
pSF				
0.2 µg µl ⁻¹	27	26 (96 ± 2.2) ^{ab}	25 (92 ± 7.5) ^a	25 (92 ± 7.5) ^a
0.5 µg µl ⁻¹	28	25 (89 ± 7.2) ^{ab}	27 (96 ± 5.4) ^a	26 (93 ± 3.5) ^a
1.0 µg µl ⁻¹	45	40 (88 ± 3.1) ^b	40 (88 ± 3.1) ^a	40 (88 ± 5.3) ^a
Adenophostin				
10 µmol l ⁻¹	33	28 (85 ± 6.5) ^b	28 (85 ± 5.0) ^a	27 (82 ± 3.5) ^a
100 µmol l ⁻¹	37	35 (95 ± 4.8) ^{ab}	34 (92 ± 7.2) ^a	34 (92 ± 4.2) ^a
eSF				
0.5 µg µl ⁻¹	42	41 (98 ± 1.2) ^a	38 (90 ± 4.0) ^a	35 (83 ± 3.5) ^a

All data are shown as *n* (% ± SEM).

Results are from at least three replicates.

^{ab}Different superscripts within a column indicate significant differences (*P* < 0.05).

Table 3. Activation and development of mouse oocytes injected with pig sperm extracts (pSF), adenophostin and equine sperm extracts (eSF), and diploidized by incubation in cytochalasin B

	Number of oocytes ^a	Second pronuclear stage (6–8 h)	Two-cell (24 h)	Morulae (72 h)	Blastocysts (day 6)	Number of cells (range) ^b
pSF						
0.5 µg µl ⁻¹	29	29 (100 ± 0) ^c	29 (100 ± 0)	23 (79 ± 11.4)	19 (66 ± 16.7)	49 (29–93)
Adenophostin						
10 µmol l ⁻¹	34	31 (91 ± 6.8)	30 (88 ± 6.8)	28 (82 ± 9.3)	21 (62 ± 15.4)	45 (27–72)
eSF						
0.5 µg µl ⁻¹	33	31 (94 ± 5.9)	25 (76 ± 8.0)	24 (73 ± 4.2)	24 (73 ± 8.8)	41 (33–59)

All data for development are shown as *n* (% ± SEM).

Results are from at least three replicates.

^aNumber of diploidized oocytes after incubation in 5 µg cytochalasin B ml⁻¹ for 4 h.

^bMean number of cells in blastocysts after 6 days of culture.

There were no significant differences between means.

Effect of microinjection of eSF on activation and development to the blastocyst stage in CD1 mouse oocytes

Metaphase II mouse oocytes were microinjected with 0.5 µg eSF µl⁻¹ and monitored for pronuclear formation and cleavage to the two-cell stage to characterize further the activity of eSF. Mouse oocytes were also activated by injection of pSF (0.2, 0.5 or 1.0 µg µl⁻¹) and adenophostin (10 or 100 µmol l⁻¹), a potent non-hydrolysable agonist of the IP₃ receptor, which has been shown to induce [Ca²⁺]_i oscillations and activation in mouse oocytes (Sato *et al.*, 1998). There were no differences in the ability of the different agents or concentrations to induce cleavage of mouse oocytes to the two-cell stage (Table 2). In contrast, mouse oocytes injected with microinjection buffer alone did not activate.

Subsequently, metaphase II mouse oocytes were microinjected with eSF (0.5 µg µl⁻¹), pSF (0.5 µg µl⁻¹) and adenophostin (10 µmol l⁻¹), and development to the blastocyst stage was monitored. The oocytes were

diploidized by incubation in the presence of cytochalasin B (5 µg ml⁻¹) for 4 h, thereby preventing extrusion of the second polar body, to optimize results. There were no differences in the ability of the different agents to support development of mouse oocytes to the blastocyst stage (Table 3).

[Ca²⁺]_i-releasing activity in a single stallion spermatozoon

As we wished to use eSF as a parthenogenetic agent in horse oocytes, but these are not readily available, the concentration of eSF that would provide [Ca²⁺]_i-releasing activity that most closely resembled that of a single stallion spermatozoon was first determined. B6D2F1 mouse oocytes at metaphase II were injected with one frozen-thawed sonicated stallion sperm head or eSF (1 or 5 µg µl⁻¹), and the resulting [Ca²⁺]_i profiles were compared. All mouse oocytes injected with a stallion spermatozoon displayed frequent [Ca²⁺]_i oscillations that

Table 4. Calcium-releasing activity of equine sperm extracts (eSF) and one stallion spermatozoon when microinjected into mouse oocytes

	<i>n</i>	Frequency (number of oscillations in 20 min)*	Average duration of transient (min)	Number of eggs oscillating	
				≥ 40 min	≥ 120 min
eSF					
1 µg µl ⁻¹	6	5 ± 1.0 ^a	0.97 ± 0.2	4/6	1/3
5 µg µl ⁻¹	6	11 ± 1.7 ^b	1.02 ± 0.1	6/6	4/4
ICSI	8	11 ± 1.7 ^b	1.09 ± 0.1	8/8	5/5

*Spike frequency was measured from 10 min to 30 min after microinjection or intracytoplasmic sperm injection (ICSI).

ICSI: mouse oocytes were microinjected with the head of a single sonicated, frozen-thawed stallion spermatozoon by intracytoplasmic sperm injection.

Values are mean ± SEM.

^{ab}Means within a column with different superscripts are significantly different (*P* < 0.05).

Fig. 3. Representative intracellular Ca²⁺ concentration ([Ca²⁺]_i) profiles for metaphase II mouse oocytes injected with (a) a single stallion sperm head (intracytoplasmic sperm injection; ICSI; *n* = 8) or with (b) 1 µg equine sperm extract (eSF) µl⁻¹ (*n* = 6) or (c) 5 µg eSF µl⁻¹ (*n* = 6). Arrows indicate times of injection.

lasted for > 120 min. The concentration of eSF that initiated oscillations with similar duration and frequency to those induced by a stallion spermatozoon was 5 µg µl⁻¹ (Fig. 3 and summarized in Table 4), a concentration ten times greater than the concentration needed to induce oscillations comparable to those initiated by a mouse spermatozoon. Therefore, 5 µg eSF µl⁻¹ was thought to most closely represent the amount of SF in a stallion spermatozoon and was used for subsequent microinjection of horse oocytes.

Effect of microinjection of eSF on [Ca²⁺]_i oscillations and activation in horse oocytes

The ability of eSF to induce [Ca²⁺]_i oscillations and activation in horse oocytes was investigated by using oocytes matured *in vitro* for 24 or 42 h, as the optimal duration of maturation before fertilization in horse oocytes has not been well defined. Therefore, horse oocytes in both maturation groups (24 or 42 h) were injected with 5 µg eSF µl⁻¹ and the resulting [Ca²⁺]_i responses were compared. Overall, 22 of 25 injected oocytes responded with [Ca²⁺]_i oscillations (13 of 14 and 9 of 11, for the 24 and 42 h maturation groups, respectively). Representative [Ca²⁺]_i profiles for 24 and 42 h *in vitro* matured oocytes are shown (Fig. 4) and the pattern of [Ca²⁺]_i responses is summarized (Table 5). No differences in [Ca²⁺]_i responses were observed between the two groups of oocytes. The initial [Ca²⁺]_i spikes occurred every few minutes but then spikes occurred at longer intervals (Fig. 4); in most oocytes that were monitored for a prolonged period, [Ca²⁺]_i oscillations lasted for between 60 min and 120 min.

The [Ca²⁺]_i oscillations initiated by injection of 5 µg eSF µl⁻¹ induced normal activation of development. For example, when evaluated at 20–24 h after microinjection, eight of 13 (61%) and 13 of 14 (81%) oocytes matured for 24 and 42 h, respectively, showed pronuclear formation (Fig. 5).

As for our previous results with mouse oocytes, microinjection of buffer alone or of sperm extracts with protein concentrations < 0.1 µg µl⁻¹ into mare oocytes did not result in the initiation of [Ca²⁺]_i oscillations or induce activation.

Effect of ICSI performed with one progressively motile fresh stallion spermatozoon on [Ca²⁺]_i transients in *in vitro* matured horse oocytes

As eSF was able to induce consistent [Ca²⁺]_i responses in horse oocytes, the pattern of sperm-induced [Ca²⁺]_i

Fig. 4. Representative intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) profiles for horse oocytes matured *in vitro* for (a) 24 ($n = 13$) or (b) 42 h ($n = 9$) and microinjected with $5 \mu\text{g}$ equine sperm extract μl^{-1} . Arrows indicate times of injection.

oscillations in horse oocytes was evaluated. In this experiment, $[\text{Ca}^{2+}]_i$ monitoring was started within 30 min of sperm injection. ICSI did not consistently initiate fertilization-like $[\text{Ca}^{2+}]_i$ transients, as only five of 13

(38%) of the sperm-injected oocytes showed $[\text{Ca}^{2+}]_i$ oscillations, and only two of these oocytes displayed responses that lasted for > 2 h (Fig. 6). In oocytes that showed Ca^{2+} responses, the average spike interval was approximately 40 min and the mean duration was 3 min. Despite the small percentage of oocytes displaying long lasting $[\text{Ca}^{2+}]_i$ oscillations, six of ten (58%) oocytes were activated when evaluated at 20–24 h after ICSI.

Discussion

The results of the present study demonstrate for the first time that horse oocytes respond to the injection of eSF with $[\text{Ca}^{2+}]_i$ oscillations and activation. Furthermore, and more importantly, the ability of horse oocytes to display $[\text{Ca}^{2+}]_i$ oscillations after sperm injection is demonstrated, strongly indicating that sperm-induced $[\text{Ca}^{2+}]_i$ responses are probably an important event leading to oocyte activation in horses, as in all other mammalian species studied to date. The activity of eSF was initially tested using mouse oocytes as a model, as it is well recognized that heterologous sperm extracts can induce fertilization-like $[\text{Ca}^{2+}]_i$ responses and embryonic development in several species (Fissore *et al.*, 1998; Knott *et al.*, 2002), and partly also due to the difficulty in obtaining horse oocytes. Equine sperm extracts are capable of inducing $[\text{Ca}^{2+}]_i$ oscillations in mouse oocytes similar to those initiated by fertilization and injection of pSF (Fissore *et al.*, 1992, 1998). Furthermore, injection of eSF was able to induce activation and development to the blastocyst stage in mouse oocytes at rates similar to those obtained with pSF and adenophostin. These results support the contention that the molecule responsible for oocyte activation is highly conserved among species.

The results of the present study demonstrate that a higher concentration of eSF than is needed to activate mouse oocytes was required to simulate the $[\text{Ca}^{2+}]_i$ -releasing activity of one stallion spermatozoon. Therefore, a novel approach to estimate the SF activity present in one spermatozoon is presented. Previous studies have approximated the physiological concentrations of SF, referred to as one spermatozoon equivalent, on the basis

Table 5. Intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) transient data for metaphase II horse oocytes matured *in vitro* for 24 or 42 h and microinjected with $5 \mu\text{g}$ equine sperm extracts (eSF) μl^{-1}

Duration of <i>in vitro</i> maturation	n^a	Frequency (number of oscillations in 20 min) ^b	Transient duration (min)	Spike amplitude	Number of eggs oscillating ≥ 60 min ^c	Activation to pronuclear stage (%) ^d
24 h	13/14	3.5 ± 0.9^c	1.3 ± 0.1	386.4 ± 134.4	8/8	8/13 (61)
42 h	9/11	3.2 ± 0.5	1.1 ± 0.3	306.6 ± 99.8	7/8	13/16 (81)

Data are mean $\pm$ SE.

^aNumber of oocytes displaying $[\text{Ca}^{2+}]_i$ responses out of number of oocytes microinjected with eSF.

^bNumber of spikes from 10 min to 30 min after injection.

^cNumber of oocytes displaying $[\text{Ca}^{2+}]_i$ responses out of number of oocytes monitored for ≥ 60 min.

^dActivation data include oocytes in which $[\text{Ca}^{2+}]_i$ responses were not monitored due to insufficient fura-2 or technical difficulties with the monitoring system.

Fig. 5. Horse oocyte parthenogenetically activated by injection of stallion sperm extracts ($5 \mu\text{g eSF } \mu\text{l}^{-1}$) as evidenced by the presence of two polar bodies (open arrows) and one pronucleus (solid arrow). (a) Phase contrast microscopy. (b) After fixation and staining with Hoechst 33248.

Fig. 6. Intracellular Ca²⁺ concentration ([Ca²⁺]_i) profile for a horse oocyte injected with a single progressively motile stallion spermatozoon immobilized by piezoelectric pulses before injection. Only two oocytes of 13 oocytes displaying persistent [Ca²⁺]_i oscillations had long lasting [Ca²⁺]_i oscillations. Arrow indicates time of intracytoplasmic sperm injection (ICSI). Fluorescence changes representing [Ca²⁺]_i are given as ratios of 340 nm:380 nm fluorescence.

of the amount of protein obtained from a certain number of spermatozoa in the ejaculate at the time of sperm extract preparation (Wu *et al.*, 1998a). However, it is likely that part of the active protein is lost or inactivated during the preparation process and thus such comparisons may be largely inaccurate. In the present study, the amount of eSF capable of inducing oscillations in mouse oocytes equivalent to the responses induced in mouse oocytes by injection of one stallion spermatozoon was used; in the conditions in the present study this concentration of eSF was $5 \mu\text{g } \mu\text{l}^{-1}$. The concentration of eSF needed to induce [Ca²⁺]_i oscillations comparable to those initiated by a mouse spermatozoon was $0.5 \mu\text{g } \mu\text{l}^{-1}$, indicating that a stallion spermatozoon may contain at least ten times more SF than a mouse spermatozoon.

On the basis of the above results, horse oocytes were microinjected with $5 \mu\text{g eSF } \mu\text{l}^{-1}$ and thus it was presumed that the [Ca²⁺]_i responses obtained closely resemble those at fertilization in horses. This assumption is of particular importance in this species, for which the pattern of [Ca²⁺]_i oscillations at fertilization has not been ascertained, and in which the low availability of oocytes for investigation and the technical difficulties linked to low rates of sperm penetration during *in vitro* fertilization have made the gathering of such data very challenging (Palmer *et al.*, 1991; Hinrichs *et al.*, 2002). The frequency of [Ca²⁺]_i responses observed in equine oocytes on eSF microinjection closely resembled that reported for bovine oocytes at fertilization or when injected with appropriate concentrations of pSF (Fissore *et al.*, 1992; Nakada *et al.*, 1995; Nakada and Mizuno, 1998; Knott *et al.*, 2002), thereby suggesting that the observed responses were physiological.

The [Ca²⁺]_i transients observed in the present study by eSF-injected horse oocytes matured *in vitro* for 24 or 42 h were very consistent in amplitude, frequency and overall duration. This observation is particularly important as the optimal time for IVM of horse oocytes has not been well defined. In most reports addressing assisted reproductive techniques in horses, the oocytes are matured for a period ranging from 24 h to 44 h (Grøndahl *et al.*, 1995; Hinrichs *et al.*, 1995; Dell'Aquila *et al.*, 1997; Li *et al.*, 2000; Choi *et al.*, 2001). Horse oocytes must be matured *in vitro* for at least 24 h to achieve significant rates of metaphase II stage oocytes (Hinrichs *et al.*, 1993), but the optimal time for reaching full developmental competence is unknown. Studies in laboratory species demonstrate that immature oocytes, either at the germinal vesicle stage or before the metaphase II stage, show lower [Ca²⁺]_i responses to

parthenogenetic activation or fertilization (Shiraishi *et al.*, 1995; Jones and Nixon, 2000). Similarly, only 16% (two of six; data not shown) of horse oocytes found in metaphase I at the time of staining responded with $[Ca^{2+}]_i$ transients in the present study. In other species, this appears to be the result of changes in distribution and sensitivity of the IP_3 receptor that occur during oocyte maturation (Shiraishi *et al.*, 1995; Fissore *et al.*, 1999), although whether this is the case in horse oocytes is not known.

Ageing of oocytes also limits responsiveness. Recent evidence in mice shows that oocytes aged *in vitro* for prolonged periods lose their ability to display long lasting $[Ca^{2+}]_i$ oscillations after microinjection of sperm extracts or fertilization, and may undergo apoptosis instead of the expected normal progression of embryonic development (Gordo *et al.*, 2000, 2002). The results of the present study indicate that metaphase II horse oocytes have already gained the ability to respond with $[Ca^{2+}]_i$ transients after 24 h, and that this ability is not reduced after 42 h of IVM. Future studies should investigate the relationship between the ability of horse oocytes to display $[Ca^{2+}]_i$ oscillations, time of maturation and developmental fate.

In the present study, 88% of eSF-microinjected horse oocytes responded with $[Ca^{2+}]_i$ transients, and 71% of these oocytes showed pronuclear formation within 20–24 h after eSF injection, a clear sign of activation. These results may actually underrepresent the true rates of activation, as the oocytes in the present study had been injected with fura-2 and were exposed to UV light for prolonged periods, all of which compromise pronuclear formation. Notably, the high rates of $[Ca^{2+}]_i$ responses and activation obtained with eSF in the present study contrast with the variable, and mostly low, rates of activation and development reported for ICSI (ranging from 0% to 71%; Dell'Aquila *et al.*, 1997; Li *et al.*, 2000; Choi *et al.*, 2002b). Importantly, the result of the present study showing that only a small percentage of horse oocytes are able to respond with $[Ca^{2+}]_i$ oscillations to sperm injection indicates that the low rates of activation and embryonic development may be related, at least in part, to an inadequate activation stimulus caused by the lack of initiation of $[Ca^{2+}]_i$ oscillations. This defect may arise from a presumed inability of horse oocytes to promote release of the factor from the injected spermatozoon, or, alternatively, it may be the consequence of a deficient step in the activation or processing of the factor once released. Moreover, it is possible that species-specific differences in IP_3 receptor sensitivity in oocytes, or decreased ability of *in vitro* matured oocytes, may contribute, at least in part, to the decreased responsiveness of horse oocytes to the injected spermatozoon. All of these possibilities require further investigation.

In conclusion, it is shown for the first time that horse oocytes are capable of displaying $[Ca^{2+}]_i$ transients

in response to injection of sperm extracts, and, to a lesser degree, after ICSI. This finding proves three important points: (i) that stallion spermatozoa contain the molecule or molecules responsible for initiation of $[Ca^{2+}]_i$ oscillations in oocytes; (ii) more importantly, that the mechanism leading to oocyte activation is $[Ca^{2+}]_i$ -dependent in horse oocytes, similar as it is in other species; and (iii) that the pattern of $[Ca^{2+}]_i$ oscillations displayed by oocytes in the present study is comparable to that described in bovine oocytes at fertilization. At present, it is only possible to speculate that horse oocytes may display a similar frequency of $[Ca^{2+}]_i$ transients under conventional fertilization. Further studies should investigate the reasons that preclude consistent $[Ca^{2+}]_i$ responses in horse oocytes submitted to ICSI, which may lead to mechanisms to improve the success rate of this assisted reproduction tool in horses and other domestic species.

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