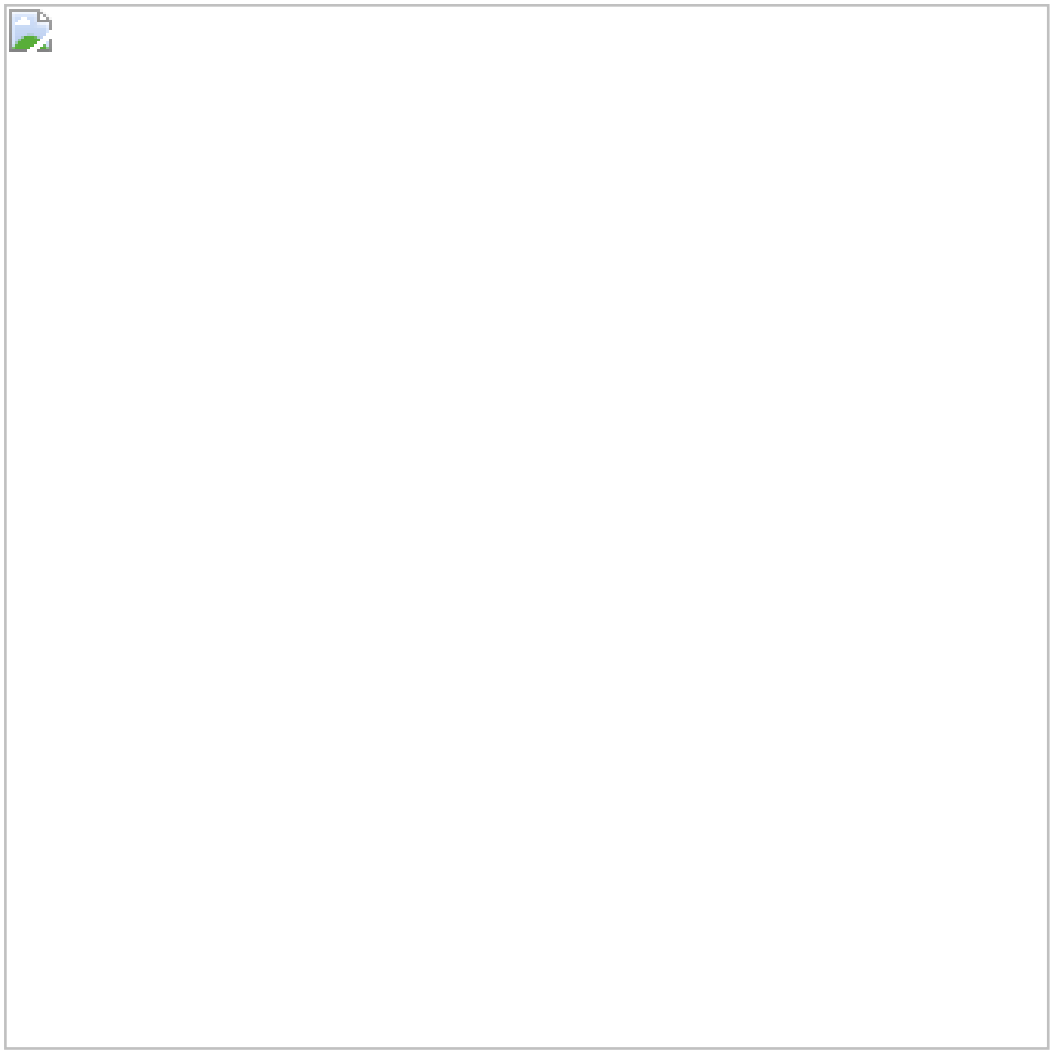

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Research Article

Successful in vitro fertilization in the horse: production of blastocysts and birth of foals after prolonged sperm incubation for capacitation†

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Abstract

Methods for standard in vitro fertilization have been difficult to establish in the horse. We evaluated whether prolonged sperm pre-incubation would support subsequent fertilization. Fresh sperm were pre-incubated with penicillamine, hypotaurine, and epinephrine (PHE) for 22 h. Co-incubation of cumulus-oocyte complexes (COCs) for 6 h yielded 43% fertilization; culture of presumptive embryos yielded 21% blastocysts. Sperm incubated similarly, but without PHE, did not fertilize oocytes. Use of extended semen in the system yielded 54% blastocysts and was applied in subsequent experiments. Transfer of three in vitro fertilization-produced blastocysts to recipient mares resulted in birth of three normal foals. When sperm were pre-incubated for 22 h, 47–79% of oocytes were fertilized after 1 h of co-incubation. Sperm pre-incubated for 15 min or 6 h before co-incubation yielded no fertilization at 1 h, suggesting that capacitation in this system requires between 6 and 22 h. Sperm assessed after 15 min, 6 h, or 22 h pre-incubation showed increasing protein tyrosine phosphorylation of the midpiece, equatorial band, and apical head; this pattern differed from that induced by high pH conditions and may denote functional equine sperm capacitation. Use of the final devised system, i.e., extended semen, with 22 h of sperm pre-incubation and 3 h of COC co-incubation, yielded 90% fertilization with a blastocyst rate of 74%. This is the first report of efficient and repeatable standard in vitro fertilization in the horse and the first report of in vitro production of blastocysts and resulting foals after in vitro fertilization.

Summary Sentence Pre-incubation of fresh equine sperm for 22 h in the presence of penicillamine, hypotaurine and epinephrine supports high rates of in vitro fertilization and production of viable blastocysts.

Keywords: horse, in vitro fertilization, sperm capacitation, protein tyrosine phosphorylation, fertilization, oocyte activation

Introduction

In vitro fertilization (IVF) resulting in birth of live offspring has been reported in a wide variety of species. In the horse, in vitro embryo production is a valuable clinical and research tool but is currently only feasible via intracytoplasmic sperm injection (ICSI) [1, 2]. The necessity for ICSI limits both the clinical use of the procedure and the ability to investigate the biology of sperm capacitation and fertilization in this species. Despite numerous attempts to establish methods for standard IVF, results are not repeatable and, even when fertilization is achieved, the efficiency is poor (review, [3]). In contrast to bovine IVF, in which about 30% fertilization can be achieved in a basic medium without capacitating additives [4], the basal rates of fertilization in the horse are essentially zero [5–8]. Only one laboratory, that of Palmer and coworkers, has reported the birth of live foals after IVF, using in vivo-matured oocytes recovered from the dominant stimulated follicle and sperm treated with the calcium ionophore A23187; two foals were born after surgical transfer of two-cell embryos to the oviducts of recipient mares [8, 9].

Attempts to establish more efficient equine IVF protocols have not been successful despite use of sperm treatments effective in other species, such as heparin [5, 7, 10], caffeine [5, 6], lysophosphatidyl serine [5], combinations of penicillamine, taurine, hypotaurine, and epinephrine [10–13], A23187 [5, 6, 12–16], 8-brcAMP, and ionomycin [17]. Use of procaine to stimulate sperm hyperactivated motility was reported to be effective for equine IVF [18, 19]; however, procaine was later found to directly induce cytokinesis of equine oocytes [20] calling these results into question, and fertilization with this method has not been confirmed. The highest rate of pronucleus (PN) formation that has been reported after equine IVF is 33% [12].

A major impediment to developing an efficient IVF protocol in the horse is the lack of information on sperm capacitation in this species. The time required for capacitation of equine sperm in vivo is not known. Enders et al. [21] found that pronuclear formation was first seen in equine oviductal oocytes 12 h after postovulatory insemination. In comparison, in the mouse, pronuclear formation is seen 4.5 h after postovulatory mating [22]. Thus, it is possible that equine sperm could require a relatively prolonged period to achieve capacitation. Unfortunately, evaluation of physiological changes in equine sperm over time in culture is problematic, as motility decreases

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quickly in culture, dependent on both medium and container [23].

The only research laboratory to publish two separate studies reporting relatively high rates (~30%) of equine IVF is that of Alm and Torner in Germany [13, 24]. These authors used an IVF technique modified from that developed for cattle [25] and incorporating Tyrode's Albumin Lactate Pyruvate (TALP)-based media and using either A23187 or heparin for sperm treatment. The fertilization medium included penicillamine, hypotaurine, and epinephrine (PHE), a combination of a metal chelator, amino acid, and catecholamine. In bovine IVF, PHE decreases the time to oocyte penetration [4] and maintains sperm motility over time [26]. The methods described by Alm and Torner have been used in our laboratory to achieve equine IVF but with low fertilization rates (0–15%; using heparin [7] and using A23187 [16]). However, in considering whether longer incubation times may support capacitation, we were intrigued with the possibility that presence of PHE in the media may allow the maintenance of equine sperm motility during extended culture.

The purpose of this study was to evaluate the fertilizing ability of equine sperm incubated for a prolonged period (overnight) before IVF. We were successful in developing a repeatable and effective procedure for equine IVF and went on to evaluate the treatment parameters associated with fertilization with this protocol, the kinetics of sperm penetration, chromatin remodeling and oocyte meiotic resumption, and changes in capacitation-related processes within the sperm. We also evaluated the ability of the fertilized oocytes to develop to blastocysts in vitro and of these blastocysts to produce foals after transfer to recipient mares.

Materials and methods

All procedures involving live animals were performed according to the United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training and were approved by the Institutional Animal Care and Use Committees at Texas A&M University (Preliminary Studies 1 and 2 and a portion of Experiment 1) and the University of Pennsylvania (all subsequent studies).

General methods

Detailed methods for oocyte recovery, oocyte in vitro maturation, semen collection, media preparation, sperm preparation, co-incubation of sperm and oocytes, zygote culture, embryo culture, staining and evaluation of oocytes, staining of sperm for protein tyrosine phosphorylation (PY), and evaluation of sperm for acrosome status and viability via flow cytometry are provided in a Supplemental File (Supplemental Materials and Methods).

Briefly, cumulus-oocyte complexes (COCs) were collected by transvaginal ultrasound-guided follicle aspiration (TVA) or from ovaries postmortem and were held overnight at room temperature (~22°C), or for one or two nights at 15°C. The COCs were placed in culture for oocyte maturation for ~30 h before being added to the sperm droplet for sperm–oocyte co-incubation.

Semen was collected from one of two stallions and was either used immediately or extended with a milk protein-based extender (INRA96, IMV Technologies, Maple Grove, MN) as outlined for each experiment.

A PHE solution [27] as modified by Alm et al. (H. Alm, personal communication, 2003) was prepared as described in the Supplemental Materials and Methods. The solution contained 154 mM NaCl, 0.93 mM $Na_2S_2O_5$, 5.2 mM sodium lactate, 0.23 mM hypotaurine, 0.46 mM penicillamine, and 0.046 mM epinephrine. This stock solution was frozen and aliquots stored at −20°C.

For the preparation of fertilization droplets, an aliquot of PHE was thawed at room temperature, protected from light, and 120 μL of PHE solution was added to 3 mL of a modified TALP medium [28] (FERT-TALP, see Supplemental Materials and Methods), resulting in final concentrations of 9 μM hypotaurine, 18 μM penicillamine, and 1.8 μM epinephrine. Then, 45 μL droplets of this medium (FT-PHE) were prepared in a 35-mm Petri dish under light mineral oil. The dishes were equilibrated at 38.2°C in a humidified atmosphere of 5% CO_2 in air for at least 4 h and usually overnight before addition of sperm.

Sperm preparation and sperm–oocyte coincubation

Raw or extended semen (200 μL) was layered under 1 mL of GMOPS medium (Vitrolife, Englewood, CO, USA) without BSA in a 5-mL polypropylene tube, which was incubated at a 45° angle at 38°C in air for 20 min. After incubation, the uppermost 750 μL of the medium was collected and was placed in a 1.5-mL Eppendorf tube and centrifuged at 700 × *g* for 5 min.

In some experiments, sperm mitochondria were labeled by addition of MitoTracker Red CMXRos (Invitrogen, final concentration 100 nM) to the swim-up medium, as previously described for visualization of sperm tails after equine ICSI [29]. All other aspects of the sperm preparation procedure remained unchanged. After the first two replicates using this label, the concentration was reduced to 10 nM.

In preliminary studies, A23187 was utilized. After centrifugation of the collected swim-up medium, the sperm pellet was resuspended in 495 μL of Hanks Balanced Salt Solution (HBSS) with calcium and magnesium (ThermoFisher Scientific, Waltham, MA) without additions. Then, 5 μL of an A23187 stock solution (100 μM A23187 in 5.2% DMSO) was added to the HBSS-sperm suspension to a final concentration of 1 μM A23187. One minute after A23187 addition, 25 μL of HBSS containing 100 mg/mL BSA was added to quench the A23187 action (final concentration of BSA 4 mg/mL) [30, 31]. The suspension was centrifuged, the supernatant was removed, and the sperm pellet (20–30 μL) was combined with a modified TALP (TALP-R, see Supplemental Materials and Methods), previously equilibrated at 5% CO_2 in air, to achieve a final volume of 60 μL. In later studies in which A23187 was not used, all the above steps were similarly conducted with the exception that the BSA was added to the HBSS before it was used to resuspend the sperm and the A23187 stock solution was not added.

After resuspension with TALP-R, the sample was held in an incubator at 38°C in 5% CO_2 in air for 15 min while the concentration was determined on an aliquot. After this, a volume of the sperm suspension providing 50 000 sperm was added to the 45-μL FT-PHE droplet. If the volume of sperm suspension added was <5 μL, TALP-R medium was added to the droplet so that the total volume added was 5 μL. The final sperm concentration in the 50-μL droplet was 1×10^6/mL. The droplets containing sperm were incubated for various times (this period was designated "sperm pre-incubation"), as outlined for each experiment, at 38.2°C in a humidified atmosphere of 5% CO_2 in air, before addition of oocytes or COCs.

After sperm had been pre-incubated in the FT-PHE droplets for the designated period, COCs were removed from maturation culture and washed through four 100-μL droplets of FERT-TALP without PHE. One to three cumulus-denuded oocytes (Preliminary study 1) or COCs, in 1 μL of FERT-TALP medium, were added to each 50-μL FT-PHE droplet containing the pre-incubated sperm suspension. The dishes holding the droplets were placed back in the incubator under the same conditions (38.2°C in a humidified atmosphere of 5% CO_2 in air). The sperm and oocytes were co-incubated for the duration designated in each experiment.

After the period of sperm–oocyte co-incubation, the oocytes were transferred to droplets of commercial human embryo culture medium (Global medium, LGGG-050, LifeGlobal, Guilford, CT, USA) supplemented with 10% FBS (GL-FBS) with one oocyte or COC per 15-μL droplet. The time of transfer of the oocyte to the embryo culture droplet was designated as Time 0 for reference to embryo age. The culture dishes were incubated at 6% CO_2, 5% O_2 and the reminder N_2 for 36 h or as described for each experiment. This period was designated as the zygote culture period.

In some experiments, after the zygote culture period, presumptive embryos were denuded of cumulus, and those that had cleaved were transferred individually to new droplets of GL-FBS (5 μL medium per embryo). On Day 5, cleaved embryos were transferred to droplets of DMEM/F-12 with 10% FBS at a ratio of 5 μL medium per embryo and were cultured at 6% CO_2, 5% O_2, and the reminder N_2 until blastocyst formation was recognized or until Day 10 of culture.

For determination of fertilization status, or of presence of normal nuclei in cleaved embryos, oocytes/embryos were fixed, stained with DAPI, and imaged using a fluorescence microscope (Zeiss AxioObserver) with a 365-nm excitation filter. Fertilization rates were expressed as (fertilized + embryos)/(metaphase oocytes + fertilized + embryos). In experiments in which sperm had been stained with MitoTracker during swimup, oocytes were counter-stained with DAPI as described above and were evaluated under fluorescence microscopy with both 365- and 545-nm excitation filters. Confocal microscopy was performed on a subset of intact COCs after co-incubation with MitoTracker-labeled sperm for assessment of chromatin (Hoechst 33342), sperm tail (MitoTracker as described above), and acrosome status via staining with Alexa Fluor 488-conjugated lectin from *Arachis hypogaea* (peanut agglutinin, PNA; Thermo Fisher).

For the assessment of protein tyrosine phosphorylation (PY) in sperm under conditions that support IVF, sperm were fixed and stained with an anti-phosphotyrosine antibody (clone 4G 10, EMD Millipore Sigma) and then were exposed to a secondary antibody (Alexa Fluor 633 goat anti-mouse IgG (H + L), ThermoFisher) before imaging. Sperm were also evaluated for the acrosome status and viability (membrane integrity) via flow cytometry after staining with FITC-conjugated PNA (Sigma) and propidium iodide (PI).

Experimental design

Preliminary study 1: exposure of cumulus-intact or denuded oocytes to sperm treated with A23187 then pre-incubated 24 h

In this study, we assessed whether sperm treated briefly with A23187, then pre-incubated 24 h before oocyte addition, were capable of fertilization. Oocytes recovered by TVA were used. Because it is unclear whether the cumulus hinders or aids equine IVF [13, 32], we assessed fertilization rates with both cumulus-intact and denuded oocytes. Freshly collected raw semen from one stallion (FS) was prepared as described above and then pre-incubated in FT-PHE droplets for 24 h. Then, COCs were removed from maturation culture (30–35 h maturation) and either denuded of cumulus by pipetting in 0.3 mg/mL hyaluronidase or were not denuded. For denuded oocytes, only those with polar bodies were used; cumulus cells removed during denuding were washed in FERT-TALP and were added to the fertilization droplet with the denuded oocytes. Oocytes or COCs were co-incubated with sperm for 6 h, followed by a 32-h zygote culture period and then oocytes were fixed, stained, and evaluated as described above.

Preliminary study 2: requirement for treatment with A23187

Based on the results of Preliminary Study 1, we used cumulus-intact oocytes for all remaining studies. In Preliminary Study 2, we evaluated whether A23187 treatment of sperm was required in this system. Raw semen from one stallion (FS) was used. Sperm were prepared as described above, or prepared with the same protocol but without addition of A23187. We opted not to use vehicle (5.2% DMSO) in the No-A23187 group to evaluate the fertilizing ability of sperm in the absence of DMSO. Oocytes recovered by TVA were used. Co-incubation (6 h), staining, and evaluation after a 36-h zygote culture period were performed as described above.

Experiment 1. Effect of supplementation of the fertilization droplet with embryo culture medium on development to blastocyst

From the results of Preliminary Study 2, A23187 was not used in any further experiments. Experiment 1 was conducted to determine whether the embryos produced by this method could develop to the blastocyst stage in vitro. Because FERT-TALP is a relatively simple salt solution that might lack components necessary for oocyte metabolism, we compared blastocyst development between oocytes co-incubated with sperm in FT-PHE versus a mixture of FT-PHE with 10% or 50% embryo culture medium (Global with 6 mg/mL BSA, GL-BSA). The GL-BSA was equilibrated in 5% CO_2 in air for at least 3 h before use. For this experiment, two stallions (FS and SM) were used as semen donors, as available. Oocytes recovered both by TVA and postmortem were used.

Sperm and oocyte preparation and 24-h sperm pre-incubation were performed as described for the "No-A23187" procedure in Preliminary Study 2, with the exception that BSA was added to the HBSS before sperm resuspension (final BSA concentration 4 mg/mL). After the 24-h pre-incubation and immediately before the COCs were added, the sperm-containing fertilization droplets were adjusted to create three treatments: Control (0GL), no change; 10GL, 5 μL was removed from the droplet, and 5 μL of GL-BSA was added; and 50GL, 25 μL was removed from the droplet and 25 μL of GL-BSA added. To compensate for the dilution of sperm occurring in the 50GL treatment, this treatment had been prepared initially with twice the number of sperm (100 000 sperm per 50 μL droplet).

The 6-h co-incubation and 36-h zygote culture and subsequent culture of cleaved embryos were performed as described above. Oocytes that were uncleaved after zygote culture were

denuded, fixed, and stained. Cultured embryos were evaluated daily from Days 7–10 for evidence of blastocyst formation. One Day-7 blastocyst in the control group was transferred transcervically to the uterus of a recipient mare. All other blastocysts produced were vitrified.

As noted in the Results, two of the six replicates performed yielded no blastocysts, associated with the collected raw semen sitting for ≥30 min before processing. To verify that the procedure was still repeatable when semen was appropriately handled, a seventh replicate was performed with semen processed immediately after collection. As only seven oocytes were available, the 0GL treatment alone was performed and data were not included in the analysis for Experiment 1. The resulting blastocyst was transferred transcervically to the uterus of a recipient mare.

Preliminary study 3: use of extended semen for IVF

Based on the results of Experiment 1, that the 0GL treatment provided the highest numeric fertilization rate and resulted in viable blastocysts, all further studies were performed using FT-PHE, without added embryo culture medium, for co-incubation.

As detailed in the Results, two of the six replicates in Experiment 1 showed low fertilization rates and no blastocyst development, associated with the collected raw semen sitting for ≥30 min before processing. We therefore performed two replicates to evaluate whether semen extended with a commercial semen extender, INRA96, immediately after collection, could be used in the developed IVF system. Semen was collected (Stallion SM) and extended 2:1 or to 50 million/mL with INRA96. Because of the lower sperm number per tube due to dilution, typically eight swimup tubes were prepared. Each supernatant (750 μL) was collected into an individual Eppendorf tube for centrifugation, and the resulting sperm pellets pooled. To this approximate 200 μL volume, 700 μL of HBSS containing 4.7 mg/mL BSA was added. All other procedures were performed as for the control (0GL) treatment in Experiment 1, except that the sperm pre-incubation period was reduced to 20–22 h (termed "22 h" for simplicity) to aid scheduling. In the first replicate, all oocytes and embryos were fixed and stained with DAPI after the 36-h zygote culture period to assess fertilization. In the second replicate, embryos were continued in culture to blastocyst. Oocytes recovered both by TVA and postmortem were used. One Day-7 blastocyst was transferred transcervically to the uterus of a recipient mare; all other blastocysts produced were vitrified.

Experiment 2. Effect of removal of PHE on fertilization rates

In this experiment, we explored whether presence of PHE was required for the successful capacitation of equine sperm in this system. Oocytes were recovered by TVA or postmortem. Semen was collected from Stallion SM and was immediately extended with INRA96 extender. For this and subsequent experiments, the sperm was extended to 25–50 million sperm/mL, maintaining a minimum ratio of extender:semen of 3:1 (v:v). After extension, the semen was processed as described for Preliminary Study 3, above. All procedures were the same between treatments, with a 22 h sperm pre-incubation period and a 6-h co-incubation period, with the exception that in the "no-PHE" treatment, no PHE was added to the FERT-TALP before preparation of the fertilization droplets. All oocytes and embryos were fixed and stained for evaluation immediately after the 36-h zygote culture period.

Experiment 3. Time of sperm penetration of oocytes after initiation of co-incubation

This experiment was conducted to determine, for sperm that had been pre-incubated for 22 h, how quickly penetration of oocytes occurred after co-incubation was initiated. Extended semen from Stallion SM was processed as for the above experiments, with the exception that MitoTracker was added to the swim-up medium as described above. Sperm were pre-incubated in droplets of FT-PHE for 22 h before addition of COCs. Two separate studies were performed.

In Study 1, the COCs were placed in the fertilization droplets and were removed 3 or 6 h later, or were transferred at 6 h to zygote culture and then removed after 3 h (i.e., 9 h after first exposure to sperm). Based on the findings of Study 1, to better define the time to fertilization, in Study 2, the COCs were removed 1, 2, or 3 h after being placed in the fertilization droplet.

For both studies, immediately after being removed from the fertilization droplets, the oocytes were denuded of cumulus, fixed, and stained with DAPI as described above and then were evaluated for chromatin and MitoTracker staining indicating the presence of a sperm tail in the oocyte cytoplasm.

Three COCs that had been removed 3 h after placement in the fertilization droplet were stained with the cumulus intact for confocal microscopic evaluation of chromatin, sperm tail, and acrosome status, as described in the Supplemental Materials and Methods.

Because the assessment of oocytes removed after 1–3 h of co-incubation with sperm revealed polyspermy in a small proportion of fertilized oocytes (see Results), we wished to evaluate blastocyst production using the developed system both to assess the blastocyst development rate and to perform genetic analysis on blastocysts produced. Three additional replicates were performed with a 22-h pre-incubation and 3-h coincubation period, as described for this experiment with the exception that sperm were not stained with MitoTracker. After the 3-h coincubation period, COCs were transferred to zygote culture and then were placed into embryo culture (three oocytes per 15 μL droplet) and were cultured to blastocyst. The blastocysts produced were vitrified. These were later warmed and the zona pellucida was removed by treatment with pronase [33] and the zona-free embryo was cultured an additional 24 h to increase the cell number. Embryos were then placed individually in Eppendorf vials and were frozen at −20°C and were then shipped for genetic analysis to the Veterinary Genetics Laboratory at the University of California, Davis. Multiplex PCR of the extracted DNA was performed to amplify 13 equine microsatellite identification markers (*AHT4*, *AHT5*, *ASB17*, *ASB2*, *ASB23*, *HMS2*, *HMS3*, *HMS6*, *HMS7*, *HTG10*, *HTG4*, *LEX3*, *LEX33*, and *VHL20*) and two sex-related genes (equine sex determining region Y (e*SRY*) and amelogenin (*AMEL*)). Results were evaluated to determine whether more than two alleles were present for any marker, which is considered to be an indicator of polyspermy.

Experiment 4. Effect of duration of sperm pre-incubation (15 min, 6 h or 22 h) on fertilizing ability and capacitation-related changes

Results of Experiment 3 showed that sperm pre-incubated for 22 h could penetrate oocytes within 1 h of co-incubation,

indicating that these sperm were likely capacitated after 22 h pre-incubation, i.e., at the time of COC addition. In Experiment 4, we investigated the duration of pre-incubation required for sperm capacitation, as measured by oocyte penetration after 1 h co-incubation in this system. Extended semen from Stallion SM was processed with MitoTracker as for Experiment 3 and sperm were placed in the FT-PHE droplets for pre-incubation. After 15 min, 6 h, or 22 h pre-incubation, COCs were added to the fertilization droplet. To standardize the maturity of the oocytes used, groups of COCs were removed from 15°C holding and were placed into maturation culture on a schedule so that all COCs were cultured for maturation for 30 h before being added to the fertilization droplet. After 1 h co-incubation, the COCs were removed from the fertilization droplet, then denuded and evaluated to determine the fertilization status.

For the determination of sperm PY, duplicate sperm droplets were made simultaneously with the droplets used for pre-incubation but without MitoTracker staining. At the time that COCs were added to the fertilization droplets (i.e., after 15 min, 6 h, or 22 h pre-incubation), sperm from the duplicate droplets were removed and were stained for PY as described above. In one replicate, samples of the original extended semen that had been placed in an Equitainer immediately after extension were also processed and were evaluated for PY at the same times as a control. Sperm from duplicate droplets at 15 min and 22 h were also evaluated for viability and acrosome status by flow cytometry.

Because the PY staining pattern appeared to be different from that previously observed for sperm incubated in high pH or in calcium-deficient media [34, 35], a trial was performed to compare the staining patterns between IVF-capable sperm (pre-incubation droplets) and sperm incubated in high pH medium. Semen was collected and extended as described and then aliquots of sperm were washed and incubated in either FT-PHE as for the developed IVF system or in modified Whitten's medium with 25 mM bicarbonate incubated at 38.2°C in air (high pH medium, [35]) for 0, 6, or 22 h. Extended cooled semen was used as a control at the same time points. Photomicrographs were taken with standard wide-field fluorescence microscopy so that images from the high pH treatment could be compared with those previously published using this treatment.

Statistical analysis

Differences in the fertilization rate and embryo development between treatments were evaluated by Fishers Exact test (https://www.graphpad.com/quickcalcs/contingency1/). Flow cytometric data were analyzed between treatments using a paired T-test (SPSS Statistics v 28.0.1.0, IBM, Armonk, NY, USA) after arc-sine transformation. Differences with a $P < 0.05$ were considered to be significant.

Results

Preliminary study 1: exposure of cumulus-intact or denuded oocytes to sperm treated with A23187 then pre-incubated 24 h

Upon staining after the zygote culture period, of 10 oocytes that were denuded before co-incubation with sperm, all 10 were in metaphase with no sperm chromatin, for 0 fertilization. Of nine oocytes that were cumulus-intact during co-incubation with sperm, six were immature or degenerated, one was in metaphase, and two were fertilized (one three- to four-cell embryo and one five-cell embryo) for a fertilization rate of 2/3.

Preliminary study 2: requirement for treatment with A23187

One replicate was performed comparing A23187 with No-A23187. On evaluation after the zygote culture period, of 22 oocytes in the A23187 treatment, 13 were degenerated, 8 were in metaphase, and 1 was a cleaved embryo with 2 nuclei for a fertilization rate of 1/9 (11%; Table 1). Of 23 oocytes in the No-A23187 treatment, 5 were degenerated, 11 were in metaphase, and 7 were fertilized (2 with 2 nuclei, and 5 with 4–6 nuclei) for a fertilization rate of 7/18 (38.9%; Figure 1A). To determine if the fertilization rate without A23187 was repeatable, two additional replicates of the No-A23187 treatment were performed. In these two replicates, the fertilization rates were 5/10 (50%) and 3/7 (42.9%).

Experiment 1. Effect of supplementation of the fertilization droplet with embryo culture medium on development to blastocyst

Six replicates were performed. The cleavage rates after the zygote culture period (cleaved embryos/metaphase oocytes + cleaved embryos) for the 0GL, 10GL, and 50GL treatments were 54.5, 46.4 and 36.7%, respectively (Supplemental Table S1). The blastocyst development rates per estimated MII oocyte for the three treatments were 21.2, 14.3, and 6.7%. There was no significant difference in any parameter between the oocytes recovered by TVA and those recovered postmortem. The blastocyst development rate per estimated MII oocyte for the two stallions individually (all treatments combined) was 8/33 (24.2%; two replicates) for Stallion FS and 6/58 (10.3%; four replicates) for Stallion SM, respectively.

In two of the four replicates performed with Stallion SM, we noted that sperm motility was poor in the fertilization droplets after the 24-h pre-incubation, that cleavage was low (3/20, 15.0% vs. 20/38, 42.9% for the other two replicates with SM), and no blastocysts were produced. In these two replicates with poor motility and fertilization, the semen had been collected ≥30 min before being transported to the laboratory for processing (~5 min transport), whereas in successful replicates, the semen was taken to the laboratory immediately after collection. In the two replicates performed with Stallion SM in which sperm were transported immediately and had good motility at the end of pre-incubation, the blastocyst production rates per estimated MII oocyte were 4/32 (12.5%) and 2/6 (33.3%).

The mare that received the transferred Day 7 blastocyst from the main experiment (TVA oocytes, 0GL treatment, and Stallion SM) was found to be pregnant on transrectal ultrasonography of the uterus 5 days after the transfer (embryo day 12) with an embryonic vesicle 3 mm in diameter visualized. This pregnancy progressed normally and a healthy 40-kg colt was born on December 28, 2021 at 334 days gestation (Figure 1B). Gross and histological examination of the placenta showed no abnormalities. The colt continued to develop normally and was 9 months old at the time of writing. Hair bulbs from the colt, stallion, recipient mare, and mares used as oocyte donors were sent for testing for

Table 1. Chromatin configuration of oocytes co-incubated with sperm that had or had not been treated with A23187 (1 μM for 1 min) prior to pre-incubation for 24 h then co-incubation with COCs for 6 h

Replicate	Treatment	*n*	Deg	Met	Fertilized			Total fertilized	Fertilization rate
					2N	3–4N	5–6N		
1	A23187	22	13	8	1	0	0	1	1/9 (11%)
1	No-A23187	23	5	11	2	4	1	7	7/18 (39%)
2	No-A23187	12	2	5	3	2	0	5	5/10 (50%)
3	No-A23187	7	0	4	1	1	1	3	3/7 (43%)

After co-incubation, oocytes were cultured for 36 h then evaluated on DAPI staining. Deg, degenerating; Met, oocyte chromatin in metaphase (with or without visible polar body chromatin); 2N, etc., embryos with given number of normal nuclei as assessed on DAPI staining; these were considered to be fertilized. Fertilization rate is calculated as fertilized/(metaphase + fertilized).

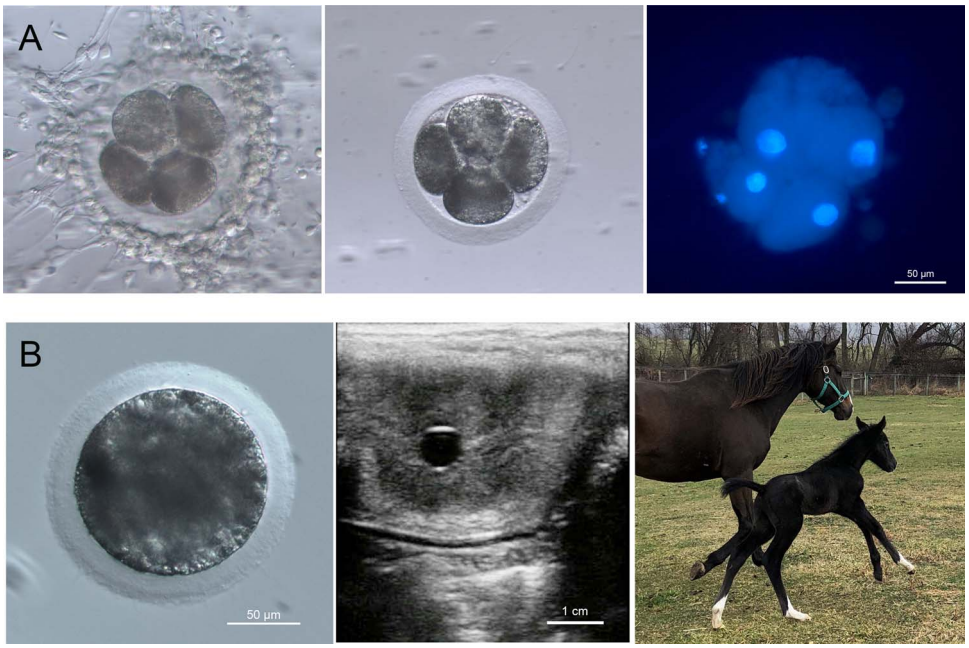

Figure 1. (A) Four-cell embryo resulting from IVF with sperm incubated for 24 h before exposure to COCs. The COCs were co-incubated with sperm for 6 h then removed and cultured for 36 h. The embryo is shown before cumulus removal, after cumulus removal, and after staining with DAPI, demonstrating four normal nuclei and two polar bodies. (B) Day 7 blastocyst resulting from the 0GL treatment in Experiment 1, which was transferred to a recipient mare, with ultrasonographic image of the uterus of the recipient mare on embryo day 13, showing a normal-appearing 7-mm diameter embryonic vesicle and the resulting foal two days after birth.

genetic markers to an outside laboratory (Veterinary Genetics Laboratory at the University of California at Davis). Parentage testing by comparison of microsatellite allele type for the 15 equine identification markers noted above confirmed that the recipient mare was excluded as the colt's dam and that the stallion (SM) and one of the mares, KT, from which oocytes were recovered for that replicate of IVF, qualified as the sire and dam of the colt.

In the seventh replicate, performed with 0GL only and not included in the data analysis for this experiment, of the seven oocytes co-incubated with sperm, after the zygote culture period two were degenerating, one was in metaphase and four had cleaved and were cultured further. One seven-day blastocyst was produced. This blastocyst was transferred to a recipient mare which was found to be pregnant on ultrasonography per rectum at embryo day 12. The pregnancy developed normally and a healthy estimated 50-kg colt was born at 351 days gestation. Gross and histological examination of the placenta showed no abnormalities. The colt continued to develop normally and was 7 months old at the time of writing. Microsatellite typing confirmed that the recipient mare was excluded as the colt's dam and that the stallion (SM) and one of the mares, DW, from which oocytes were recovered for that replicate of IVF, qualified as the sire and dam of the colt.

Preliminary study 3: use of extended semen for IVF

In the first replicate using semen that had been extended immediately after collection, all oocytes were fixed and were stained after the zygote culture period. Of the five oocytes evaluated, three were degenerating and two had cleaved with three to four nuclei. In the second replicate, embryos were cultured to determine the blastocyst production. In this replicate, after the zygote culture period, of 15 oocytes, 2 were degenerating, 2 were in metaphase and 11 had cleaved. The cleaved embryos were cultured further and seven developed to the blastocyst stage, all on Day 7 of culture. Based on these results, all further trials and experiments were conducted using semen extended immediately after collection.

One blastocyst from the second replicate was transferred to a recipient mare; this blastocyst had resulted from the fertilization of an oocyte recovered postmortem. The recipient mare

Table 2. Embryo development after co-incubation of oocytes with sperm that had been pre-incubated for 22 h with or without PHE before COC addition

Treatment	*n*	Deg	Met	Fertilized					Total fertilized	Fertilization rate
				PN	2N	3–4N	5–6N	9–10N		
PHE	18	2	2	1	1	5	3	2	12	12/16 (75%)[a]
No-PHE	18	11	7	0	0	0	0	0	0	0/7[b]

Oocytes were co-incubated with sperm for 6 h then subjected to zygote culture for 36 h before fixation and staining. Results on staining are presented. Deg, degenerating; Met, oocyte chromatin in metaphase (with or without visible polar body chromatin) with no sperm chromatin present; PN, pronucleus formation with oocyte and sperm chromatin present; 2N, etc., embryos with given number of normal nuclei as assessed on DAPI staining. Oocytes with PN and cleaved embryos with normal nuclei were considered to be fertilized. Fertilization rate is calculated as fertilized/(metaphase + fertilized). a, b: Fertilization rate differed significantly ($P < 0.001$).

Table 3. Fertilization rate 1–9 h after exposure to sperm that had been pre-incubated for 22 h before COC addition

Study	Treatment	Deg not stained	Lost during staining	Stained				
				Deg on stain	Metaphase-no sperm	Fertilized	Met + Fert	% Fertilized
1	3H	4	3	3	2	14	16	87.5
	6H	3	3	0	2	11	13	84.6
	9H	5	4	3	1	12	13	92.3
2	1H	2	1	0	3	11	14	78.6
	2H	2	1	2	1	11	12	91.7
	3H	1	1	5	0	12	12	100.0

Oocytes were evaluated after 3 or 6 h co-incubation with sperm, or 6 h coincubation followed by 3 h zygote culture (9H, Study 1), or after 1, 2 or 3 h co-incubation with sperm (Study 2). Oocyte-cumulus complexes with obvious signs of degeneration after co-incubation were discarded before staining; the remainder were denuded and stained with DAPI. Sperm were labeled with MitoTracker before pre-incubation and oocytes with sperm tails and associated chromatin visible in the cytoplasm were considered fertilized. Met + Fert, total of oocytes in metaphase plus those fertilized. Fertilization rate is calculated as fertilized/(metaphase + fertilized). There were no significant difference in fertilization rate among treatments ($P > 0.05$).

was found to be pregnant by transrectal ultrasonography on embryo day 12. The pregnancy developed normally and a healthy 61 kg filly was born at 355 days of gestation. The filly developed normally and was 6 months old at the time of writing. Microsatellite typing confirmed that the recipient mare was excluded as the filly's dam and that the stallion (SM) and one of the mares from which oocytes were recovered postmortem for that replicate of IVF, qualified as the sire and dam of the colt.

A photograph of the three foals born after transfer of IVF-produced in vitro-cultured blastocysts is presented in Supplemental Figure S1.

Experiment 2. Effect of removal of PHE on fertilization rates

Three replicates were performed. Of 44 COCs placed into maturation culture, 8 (18.2%) were classified as degenerating after maturation, based on cumulus and cytoplasmic morphology, and were not placed into co-incubation. The remaining 36 COCs were co-incubated with sperm (18 oocytes in each treatment; Table 2). In the PHE (control) treatment, the fertilization rate, as determined by staining of all oocytes and embryos after the zygote culture period, was 12/16 (75%). In the no-PHE treatment, the fertilization rate was 0/7; this was significantly different from the PHE group ($P < 0.001$). In the no-PHE treatment, 11 of the 18 oocytes (61%) were degenerated after the zygote culture period, whereas in the PHE group, the degeneration rate was 2/18 (11%); this higher degeneration in the no-PHE treatment was seen in every replicate (3/7, 3/3, and 5/8 were degenerated for no-PHE vs. 0/9, 0/3, and 2/6 for PHE). It is possible that incubation of oocytes in the FERT droplet for 6 h with dead or dying sperm (sperm that had been incubated overnight without PHE) caused oocyte damage or death, and during the subsequent 36-h zygote culture period, the oocyte chromatin degenerated.

Experiment 3. Time of sperm penetration of oocytes after initiation of co-incubation

Study 1. Evaluation of oocytes at 3, 6, or 9 h after onset of co-incubation

Four replicates were performed. Of 84 COCs placed into maturation culture, 8 (9.5%) were classified as degenerating after maturation and were not placed into co-incubation. One dish of six oocytes was lost during processing. The results for the remaining 70 oocytes are presented in Table 3. The proportion of oocytes fertilized (sperm chromatin and tail present within the oocyte cytoplasm; oocyte at MII or further stage of meiosis) at 3 h was 14/16 (87.5%). There was no significant difference in the rate of fertilization between oocytes evaluated at 3, 6, or 9 h after onset of co-incubation ($P > 0.5$).

Study 2. Evaluation of oocytes at 1, 2, or 3 h after onset of co-incubation

Three replicates were performed. Of 59 COCs placed into maturation culture, 6 (10.2%) were classified as degenerating after maturation and were not placed into co-incubation. Results for the remaining 53 oocytes are presented in Table 3. The proportion of oocytes fertilized (sperm chromatin and tail present within the oocyte cytoplasm) at 1 h was 11/14 (78.6%). There were no significant differences in the rate of fertilization between oocytes evaluated at 1, 2, or 3 h after onset of co-incubation ($P > 0.2$).

Evaluation of early events after fertilization

To assess the kinetics of sperm chromatin remodeling and the progress of oocyte meiosis at different times after fertilization, results of Study 1 and Study 2 were combined. Fertilized oocytes were classified as being in metaphase, anaphase, or the PN stage and sperm chromatin as condensed, decondensing, or PN (larger, more circular, and less dense than

Table 4. Progress of oocyte meiosis and sperm decondensation over time after onset of sperm–oocyte co-incubation

Hour	*n* oocytes	Oocytes metaphase		Total oocytes metaphase	Oocytes anaphase-telophase		Total oocytes anaphase-telophase	Oocytes decond/PN
		Sperm cond	Sperm decond		Sperm cond	Sperm decond/PN		Sperm decond/PN
1	11	4 (36.4%)	6 (54.5%)	10 (90.9%)	1 (9.1%)	0.0	1 (9.1%)	0.0
2	11	2 (18.2%)	4 (36.4%)	6 (54.5%)	1 (9.1%)	4 (36.4%)	5 (45.5%)	0.0
3	26	3 (11.5%)	5 (19.2%)	8 (30.8%)	0.0	18 (69.2%)	18 (69.2%)	0.0
6	11	0.0	0.0	0.0	1 (9.1%)	8 (72.7%)	9 (81.8%)	2 (18.2%)
9	12	1 (8.3%)	1 (8.3%)	2 (16.7%)	0.0	7 (58.3%)	7 (58.3%)	3 (25.0%)

Sperm were pre-incubated for 22 h before COCs were introduced. Oocytes were evaluated at the given hours after onset of co-incubation. Cond, condensed chromatin; Decond, decondensed chromatin.

"decondensing"). The results are presented in Table 4 and Figure 2. At 1 h, 1/11 (9.1%) of oocytes had progressed to anaphase and 6/11 (54.5%) of oocytes showed sperm that had started chromatin decondensation. An increasing proportion of oocytes progressed into anaphase/telophase over hours 1–3 (9.1, 45.5, and 69.2%, respectively); the first oocyte PN formation was seen at 6 h.

Of the 71 total fertilized oocytes (oocytes with MII or further oocyte chromatin) evaluated, 10 (14.3%) appeared to have been penetrated by more than one sperm; the proportion of oocytes that showed polyspermy at the different time points were 1/11 (9.1%), 4/10 (40%), 4/26 (15.4%), 0/11, and 1/12 (8.3%) at 1, 2, 3, 6, and 9 h. For the 10 total fertilized oocytes showing polyspermy, 7 oocytes had one additional sperm, 2 oocytes had two additional sperm, and 1 oocyte had three additional sperm.

In both studies, it was noted that oocytes with immature or abnormal chromatin (these oocytes were not counted in the "fertilized" category) were often penetrated by multiple sperm. Of 13 oocytes evaluated that had immature or degenerating chromatin, 12 (92%) had been penetrated by sperm, and all 12 had multiple sperm (from 2 to 23 sperm; mean 9.9, median 7.5) visible within the cytoplasm (Figure 2B).

On confocal microscopy of intact COCs (removed after 3 h co-incubation), all three COCs imaged had been fertilized. Externally, sperm could be seen embedded in the cumulus (Figure 3A and B). Evaluation of PNA staining indicated that almost all sperm attached to the cumulus were acrosome-reacted; few were seen to have an intact acrosome (Figure 3B and E). Internally, the sperm and oocyte chromatin and sperm tail could be visualized in the oocyte cytoplasm (Figure 3C and D). Apparent acrosomal ghosts could be seen at the periphery of the zona pellucida.

Three replicates of IVF (sperm pre-incubated for 22 h and COCs co-incubated for 3 h) were performed to produce blastocysts for the genetic analysis for polyploidy. Overall, 26 COCs were co-incubated with sperm; after the zygote culture period, 17 were cleaved and were cultured further. Staining of the uncleaved oocytes showed seven oocytes to be immature or degenerating, one in MII, and one fertilized but arrested. From the 17 cleaved embryos, 14 blastocysts were produced (3 Day 6, Figure 4; 11 Day 7 and 2 Day 8) for an overall blastocyst rate of 14/19 (73.7%) per estimated MII oocyte exposed to sperm.

Evaluation of the allele number for the examined microsatellites was performed on 10 of the blastocysts. All embryos were found to be diploid and the stallion (SM) used for IVF qualified as the sire. Of the 10 embryos, 5 were female and 5 were male.

Experiment 4. Effect of duration of sperm pre-incubation (15 min, 6 h, or 22 h) on fertilizing ability of sperm

Three replicates were performed. Of 103 COCs placed into maturation culture, 9 (8.7%) were classified as degenerating after maturation and were not placed into co-incubation. Three COCs were used for another study. Results for the remaining 91 oocytes are presented in Table 5. When sperm were pre-incubated for 15 min or 6 h before exposure to COCs, no fertilization was seen in the oocytes after 1 h of co-incubation. For sperm that were pre-incubated for 22 h before exposure to COCs, 8/17 oocytes were found to be fertilized after 1 h co-incubation. We also assessed the proportion of immature or degenerating oocytes that were penetrated by sperm, as a potential indicator of sperm capacitation; in the 22-h pre-incubation treatment, 5 of 7 of degenerating/immature oocytes had been penetrated by sperm, whereas none of 24 degenerating/immature oocytes had been penetrated in the 0 or 6 h pre-incubation treatments.

Flow cytometric evaluation of membrane integrity and acrosome status of sperm in pre-incubation droplets showed that the proportion of membrane-damaged (non-viable) sperm tended to increase between 15 min and 22 h (22.8 ± 0.9 and $44.7 \pm 6.6\%$, respectively: $P = 0.07$). There was no increase in the proportion of viable acrosome-reacted sperm over the period of pre-incubation (1.6 ± 1.4 and $0.2 \pm 0.1\%$ for 15 min and 22 h, respectively; $P > 0.3$).

Evaluation of PY patterns of sperm removed from the pre-incubation droplets after 15 min, 6 h, or 22 h showed that anti-phosphotyrosine staining started to become apparent in the midpiece, in an equatorial band, and at the apical region of the head at 6 h and this staining was more pronounced in the same regions at 22 h (Figure 5A). No appreciable PY staining was seen in cooled extended sperm at these time points.

Antiphosphotyrosine staining of sperm incubated in MW medium in air (which was initially pH 7.25 and achieved a pH of 8.12 at 6 h and 8.85 at 22 h) showed an intense PY staining of the principal piece and endpiece, and less intense staining of the midpiece, at 6 h incubation (Figure 5B). This staining pattern intensified further after 22 h incubation. No appreciable PY staining was seen in the head of sperm incubated in this high pH medium. Sperm in the IVF treatment showed staining of the midpiece, equatorial band, and apical region at 22 h as for the previous study.

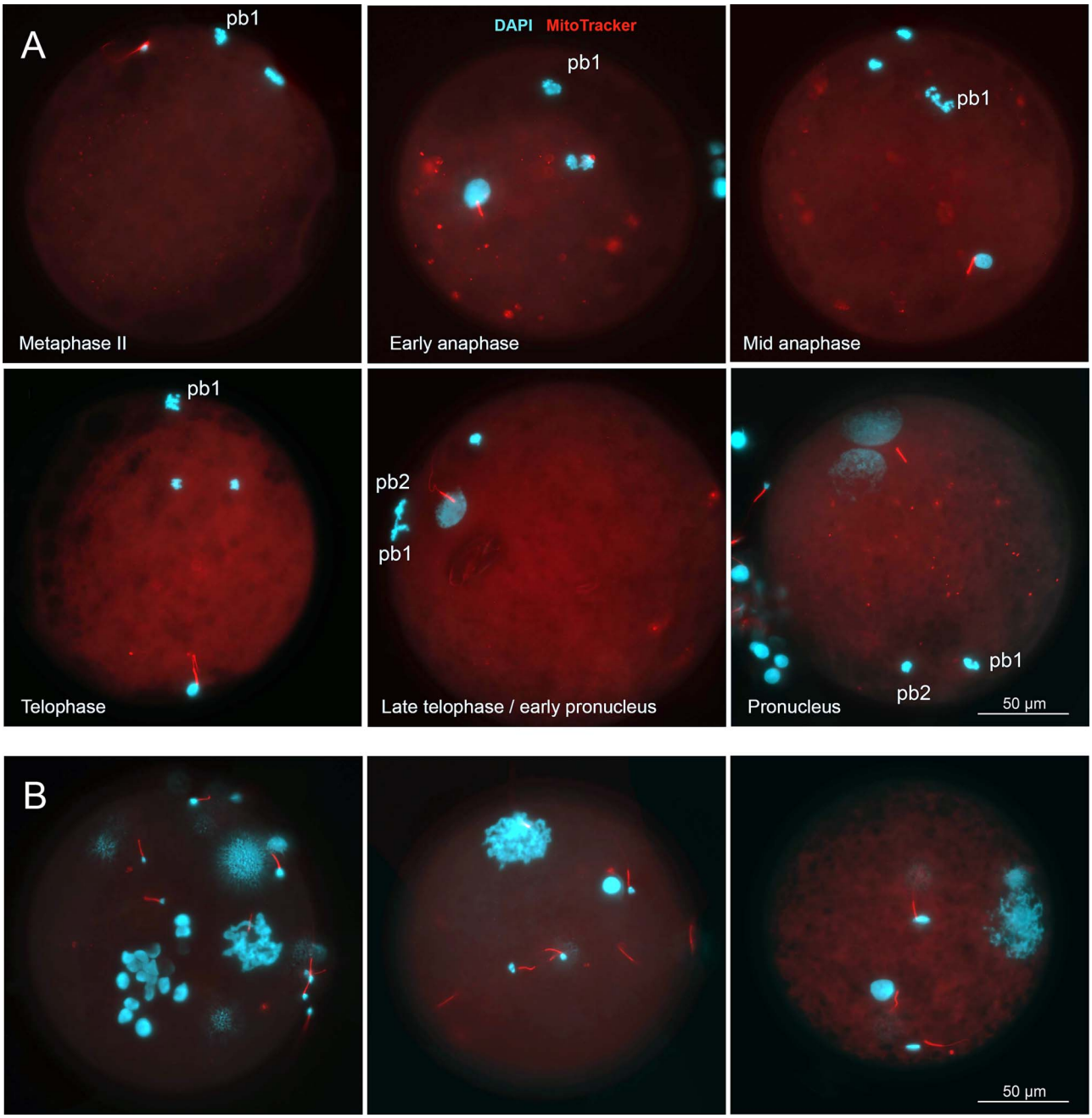

Figure 2. Chromatin configurations after equine IVF. Sperm mitochondria were stained with MitoTracker Red CMXRos before exposure to oocytes. (A) Progressive phases of oocyte and sperm chromatin reorganization after fertilization. The phase of oocyte chromatin is noted on each panel; sperm chromatin is indicated by proximity to stained tail. pb1, first polar body; pb2, second polar body. Time after onset of co-incubation was 3 h (first four panels), 6 h (late telophase), and 9 h (PN). (B) Oocytes with immature chromatin configurations showing polyspermy after co-incubation with sperm that had been pre-incubated for 22 h before the addition of COCs. Sperm mitochondria were stained with MitoTracker Red CMXRos before exposure to oocytes. Oocytes were imaged 3 h or 9 h (last panel) after the onset of sperm–COC coincubation.

Table 5. Fertilization rates of oocytes co-incubated for 1 h with sperm that had been pre-incubated for different times before COC addition

Time sperm pre-incubation	Deg not stained	Lost during staining	Stained			
			Degenerating/ immature no sperm	Degenerating/ immature + sperm	Metaphase-no sperm	Fertilized
15 min	6	1	10	0	16	0[b]
6 h	6	1	14	0	12	0[b]
22 h	1	0	2	5	9	8 (47.1%)[a]

Met, metaphase. Fertilization rate is calculated as fertilized/(metaphase + fertilized). Values with different superscripts differ significantly ($P < 0.01$).

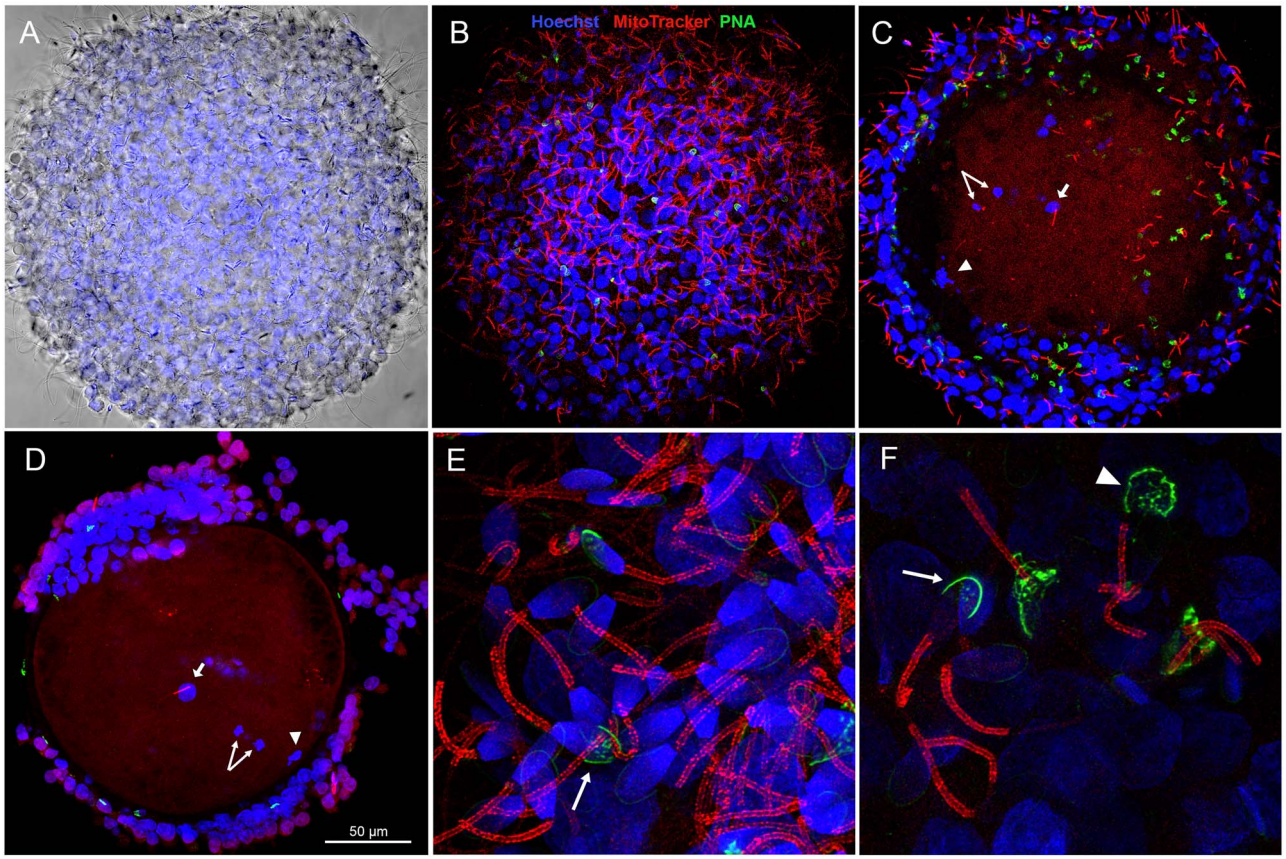

Figure 3. Confocal microscopic images of intact COCs 3 h after exposure to sperm that had been pre-incubated for 22 h before COC addition. COCs were labelled with Hoechst 33342 (chromatin); MitoTracker Red CMXRos (sperm tail mitochondria); and PNA-Alexa Fluor 488 (acrosome). (A and B) Images taken at the surface of a COC; few of the attached sperm have intact acrosomes. (C and D) Images through cytoplasm of two COCs showing the sperm chromatin and tail (short arrow), oocyte chromatin in anaphase (long arrows), and first polar body (arrowhead). Acrosomal ghosts can be seen at the periphery of the zona pellucida. (E) Enlarged view of sperm heads in the cumulus showing that most sperm have lost the acrosome (lack of PNA staining over apical region); an intact acrosome is shown by the arrow; (F) enlarged view of sperm heads in cumulus showing PNA staining of apparent intact (arrow) and reacting (triangle) acrosomes.

Discussion

We present here for the first time a repeatable and effective method for equine standard IVF. The method was successful (yielded >50% fertilization) with sperm from both stallions that we utilized for this study. Additionally, we used only in vitro-matured oocytes and achieved fertilization, blastocyst development, and foal production from the oocytes recovered both via TVA and postmortem. This is the first report of production of blastocysts in vitro after standard IVF in the horse and of foals resulting from transfer of these blastocysts. To the best of our knowledge, these are the only foals that have been produced from standard IVF other than the two foals reported by the laboratory of Eric Palmer in 1991 and 1992 after use of in vivo-matured oocytes, A23187-treated sperm, and surgical transfer of cleaved embryos to the oviduct [8, 9].

The reproducible fertilization of equine oocytes in vitro is de facto evidence that the developed methods result in the in vitro capacitation of equine sperm. This allowed us to perform the first investigations of functional sperm capacitation in this species. We found that sperm pre-incubated for 22 h appeared to be fully capacitated in that they penetrated 47–79% of oocytes within 1 h of exposure. The sperm chromatin was already decondensed within the oocyte cytoplasm in 55% of fertilized oocytes after 1 h of co-incubation. By contrast, sperm pre-incubated for 15 min or 6 h before co-incubation with COCs did not fertilize within 1 h, indicating that in this system, >6 h pre-incubation is needed to induce functional capacitation.

Flow-cytometric analysis of sperm after 22 h in pre-incubation droplets showed that there was essentially no increase in viable acrosome-reacted sperm, indicating that capacitation in this system was not associated with spontaneous acrosome reaction. However, confocal microscopy using the PNA lectin showed that almost all sperm in the cumulus after 3 h co-incubation were acrosome-reacted (Figure 3E and F). This suggests that exposure to the cumulus induced the acrosome reaction in the capacitated sperm.

In other species, capacitation has been associated with PY of a specific set of sperm proteins [36]. We found that a distinct pattern of PY developed over time in pre-incubation culture. This pattern of PY staining in the midpiece, apical, and equatorial regions is in striking contrast to patterns that have been reported previously (e.g., the strong principal piece/faint midpiece staining reported after incubation in alkaline or calcium-deficient media [34, 37, 38], staining of an equatorial band with or without staining of the flagellum [39]) and therefore suggests that these other patterns of tyrosine PY may not be indicative of true capacitation. As horse oocytes are difficult to obtain, it is possible that the specific staining pattern seen in our study may represent a useful measure to

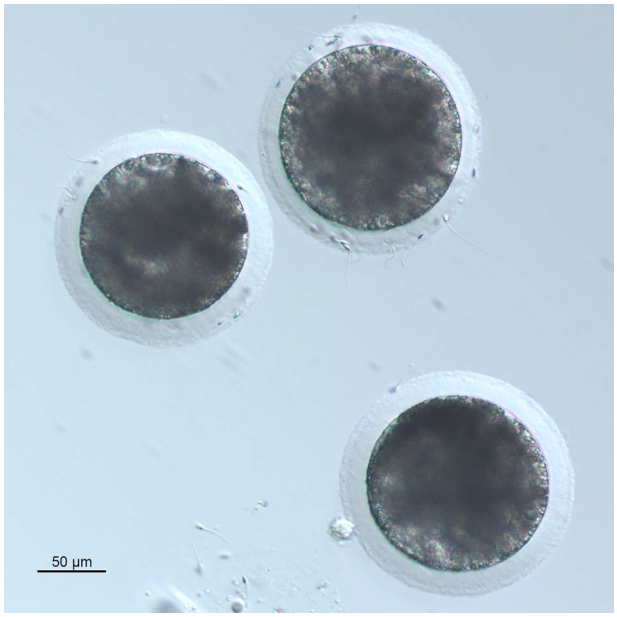

Figure 4. Three Day-6 blastocysts resulting from IVF with the final developed system. Note the supernumerary sperm in the zonae pellucidae. Sperm were pre-incubated for 22 h before addition of COCs. Sperm and COCs were co-incubated for 3 h before COCs were removed to zygote culture.

evaluate sperm capacitation in this species. Interestingly, a PY-staining pattern similar to that which we observed appeared to be visible in images taken during flow cytometry in frozen–thawed equine sperm [40], suggesting that capacitation-like changes may occur during cryopreservation ("cryocapacitation"). Our in vitro system now provides us with the tools needed to study PY as it relates to capacitation in equine sperm.

The long period of pre-incubation needed to achieve capacitation may define one of the barriers to standard IVF in the horse to date; i.e., a long period may be required for capacitation, but it is difficult to maintain motility of equine sperm in culture [23]. We utilized PHE, the components of which stimulate sperm motility and increase the incidence of the acrosome reaction in a variety of species [41]. We found that sperm incubated for 22 h without PHE did not fertilize oocytes, but this may be due simply to loss of viability or motility rather than a direct effect of PHE on sperm capacitation. Further work is needed in this area.

Alm and Torner used PHE in combination with heparin with frozen–thawed sperm and evaluated the timing of sperm penetration after equine IVF; they reported up to 46% penetration and up to 29% sperm decondensation/meiotic progression [10]. Their findings on kinetics of sperm penetration appeared similar to ours, e.g., the first sperm penetration was reported at 2 h, even though there was essentially no sperm pre-incubation. Possibly the use of heparin, and of frozen–thawed sperm, may have accelerated the sperm capacitation process. Unfortunately, the findings reported by this group [10, 13] have not been repeated by other laboratories; our laboratory has attempted to replicate their procedures but with poor success [7, 16]. The establishment of the current system now provides a baseline protocol with which variations, such as use of frozen–thawed semen, and capacitating factors, such as heparin, can be evaluated.

One of the challenges in IVF research is verifying fertilization status, especially in species such as the horse that have an opaque oocyte cytoplasm. The ability to stain sperm mitochondria during swim-up and achieve fertilization was a remarkable asset in accurately assessing the fertilization status. Using this method, we noted that immature and degenerating oocytes were typically penetrated by numerous sperm. This occurred only in the 22-h sperm pre-incubation treatment and so appeared to require sperm capacitation. The polyspermy in immature or degenerating oocytes indicates that these oocytes lacked mature cortical granules and thus failed to undergo zona hardening after sperm fused with the oocyte, allowing additional sperm to penetrate the zona. Limited polyspermy was also seen in a proportion of fertilized MII oocytes, and the incidence of this counterintuitively appeared to decrease over time from the onset of co-incubation (e.g., from 40% at 2 h to 8.3% at 9 h). The vivid nature of this reduction in polyspermy over time, which was reflected in normal ploidy of blastocysts produced in this system, led us to consider whether there was an active mechanism for elimination of excess sperm in viable MII horse oocytes. This is an area that warrants further investigation.

We found that leaving the oocyte cumulus intact was associated with fertilization, as previously reported by Alm and Torner [13]. As the inner layers of the cumulus of equine oocytes after in vitro maturation appear dense (Figure 3A), the ability of sperm to penetrate the equine cumulus in vitro has been a recurring question. Our findings on confocal microscopy that large numbers of acrosome-reacted sperm were found throughout the cumulus (Figure 3B and E) indicate that sperm underwent the acrosome reaction in response to the cumulus and traversed the dense cumulus without problem.

In Experiment 1, we focused on the potential for blastocyst formation after IVF in this system. Concern regarding the effect of the FT-PHE on the oocyte and previous reports suggesting that short incubation times yielded better-quality embryos after human IVF [42] led us to our original fairly short co-culture time of 6 h; in many IVF protocols attempted previously in the horse, the oocytes have been co-incubated with sperm for prolonged periods (18–24 h) [8, 11, 13, 18, 19]. The finding that fertilization rates were essentially 100% by 3 h after exposure of COCs to sperm in our system allowed us to reduce the co-incubation time further to 3 h. This timing was used in the final replicates focused on embryo culture and resulted in a high blastocyst production rate (74% per estimated MII oocyte).

Further work is needed to compare equine blastocyst development side by side with that achieved after ICSI. Compared with ICSI, the IVF procedure has the advantages not only of fertilization with self-selected, capacitated, acrosome-reacted sperm, associated with the more physiological fusion of the sperm with the oocyte and induction of the subsequent activation cascade, but also that oocytes are not handled in room atmosphere, exposed to temperature and medium changes, or physically insulted by the denuding and injection processes. Notably, the ICSI oocyte is denuded before injection and thus is devoid of cumulus during early embryo development, whereas in the developed IVF system, the oocyte retained its cumulus for the first 36 h of embryo development. Even when not directly in communication with the oocyte, the cumulus may serve a critical role in modulating the medium environment, including secretion of paracrine stimulating factors [43].

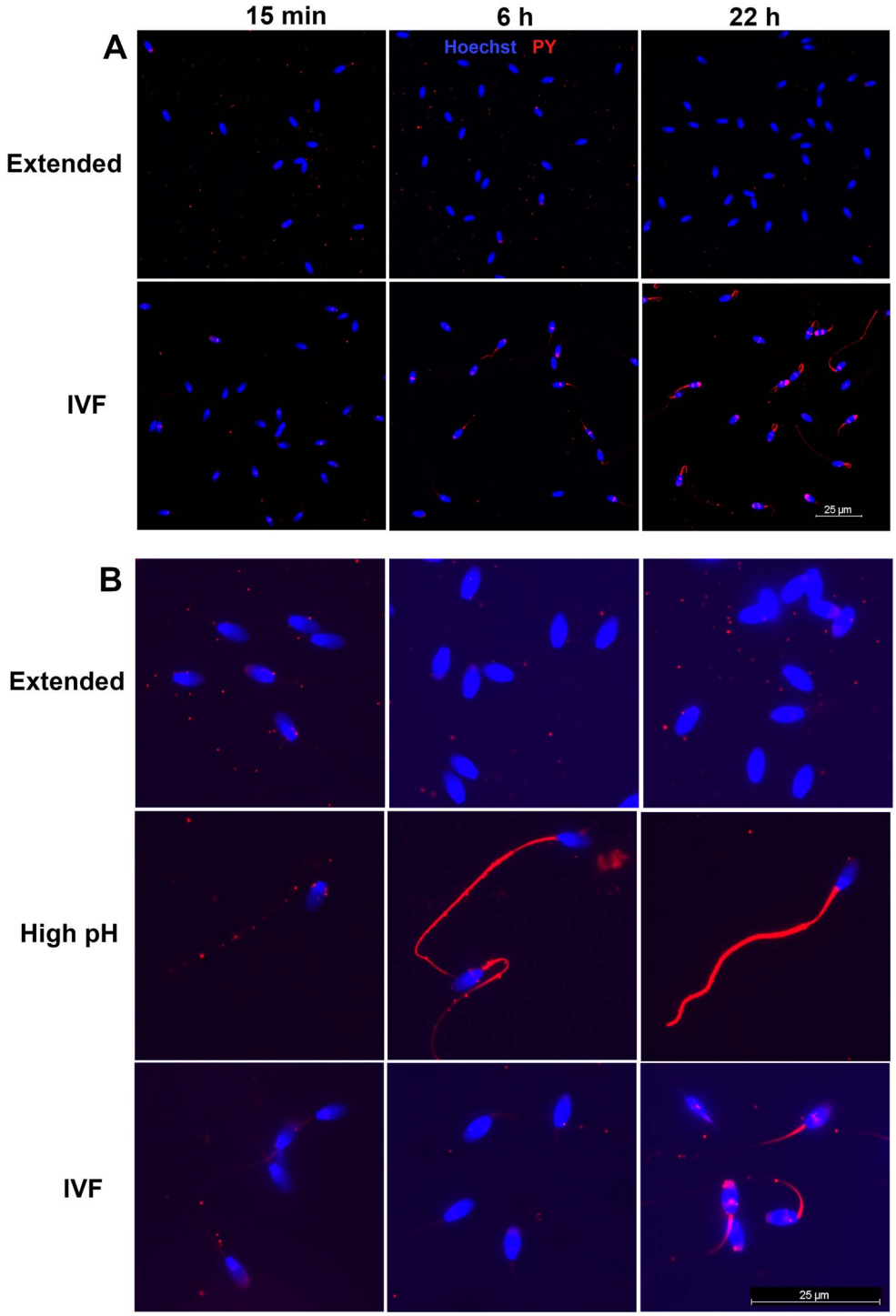

Figure 5. (A) Protein tyrosine phosphorylation patterns in sperm that had been extended in INRA96 and kept in a cooled-storage device for 15 min, 6 h, or 22 h before staining with anti-phosphotyrosine antibodies (extended) or that were processed as described for the developed IVF protocol and pre-incubated in droplets of FT-PHE for the same periods of time (IVF). Photomicrographs were taken with confocal microscopy. (B) PY patterns in sperm that had been extended in INRA96 and kept in a cooled-storage device for 15 min, 6 h, or 22 h before staining with anti-phosphotyrosine antibodies (extended), sperm that were washed and exposed to Modified Whitten's medium incubated in air to achieve a high pH for the same periods of time (High pH), or that were processed as described for the developed IVF protocol and pre-incubated for the same periods of time (IVF). Photomicrographs were taken with standard wide-field fluorescence microscopy.

In our studies, to allow the scheduling of maturation culture so that oocytes were at 30 h maturation when utilized, we held immature COCs overnight at room temperature, a long-established protocol in our lab [44], or for one or two nights at 15°C, which we have shown supports normal maturation and blastocyst rates after ICSI [45]. While we did not directly compare these holding techniques, in studies in which two holding techniques were used in the same oocyte/sperm replicates, final maturation rates per COC put into holding were equivalent (e.g., 16/36, 44%, for one

night at 15°C and 17/36; 47%, for two nights at 15°C) and blastocyst rates per co-incubated COC were equivalent (e.g., 4/9, 44%, for postmortem COCs held one night at RT; and 3/6, 50%, for TVA COCs held two nights at 15°C).

For clinical use, the IVF system would need to be functional with cooled-transported and frozen–thawed semen, as the desired stallion is unlikely to be available locally for the use of fresh semen; much further work is needed in this area. One aspect we noted anecdotally was that optimum stallion management (collection frequency) and semen handling were needed for best results. This may be related to our finding that the incubation of oocytes in droplets in which sperm were dying (i.e., in the no-PHE treatment) was associated not only with lack of fertilization but also with oocyte degeneration, suggesting that the maintenance of sperm membrane integrity during the entire procedure may be vital to its success.

Research utilizing IVF has been crucial to our understanding of the physiological processes of sperm capacitation, oocyte developmental competence, and fertilization in other species. We now have a tool to enable these studies to begin in the horse.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Acknowledgments

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Conflict of Interest

The authors have declared that no conflict of interest exists.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Authors' contributions

Conceptualization was by MF and KH; methodology was by MF, RT, and KH; investigation was by MF, RT, and TD; funding acquisition, project administration, and writing were the responsibility of KH.

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