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Research Article

Equine in vitro fertilization with frozen–thawed semen is associated with shortened pre-incubation time and modified capacitation-related changes

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Abstract

We recently reported successful equine in vitro fertilization using fresh semen pre-incubated for a prolonged period (22 h) before co-culture with oocytes. In this study, we evaluated the feasibility of equine in vitro fertilization with frozen–thawed sperm and evaluated capacitation-related changes in these sperm over the pre-incubation period. Sperm selected via a commercial sperm separation device yielded significantly higher fertilization than did sperm selected by swim-up or by colloid centrifugation. Using the sperm separation device method, fertilization rates with sperm pre-incubated for 15 min, 3, 6, and 9 h were 7.1, 22.2, 38.5, and 73.3% respectively (9 h vs. 15 min or 3 h, $P < 0.05$). Fertilization rates differed significantly (45.9% vs. 85.5%) between freezing extenders. Blastocysts were produced using frozen–thawed semen from each of three stallions and transfer of nine vitrified-warmed blastocysts to mares yielded seven embryonic vesicles. Anti-protein tyrosine phosphorylation staining of the entire sperm tail increased over pre-incubation, and sperm both with and without staining in the tail bound to the oocyte cumulus after co-incubation. Using the stain $DiSC_3(5)$ and flow cytometric analysis, a population of apparently hyperpolarized sperm was identified at 22 h in fresh sperm that was not seen at any time in frozen–thawed sperm. We conclude that frozen–thawed equine sperm can successfully fertilize oocytes after a shortened pre-incubation time of 9 h, suggesting that the freeze–thawing process induces capacitation-related changes. Our findings on evaluation of pre-incubated sperm indicate that the mechanisms by which frozen–thawed sperm become capable of fertilization may differ from those found in fresh sperm.

Summary Sentence

Frozen–thawed equine sperm selected by a filter-based system can successfully fertilize horse oocytes at high rates after 9 h pre-incubation, but show altered staining for membrane polarization compared to fresh sperm.

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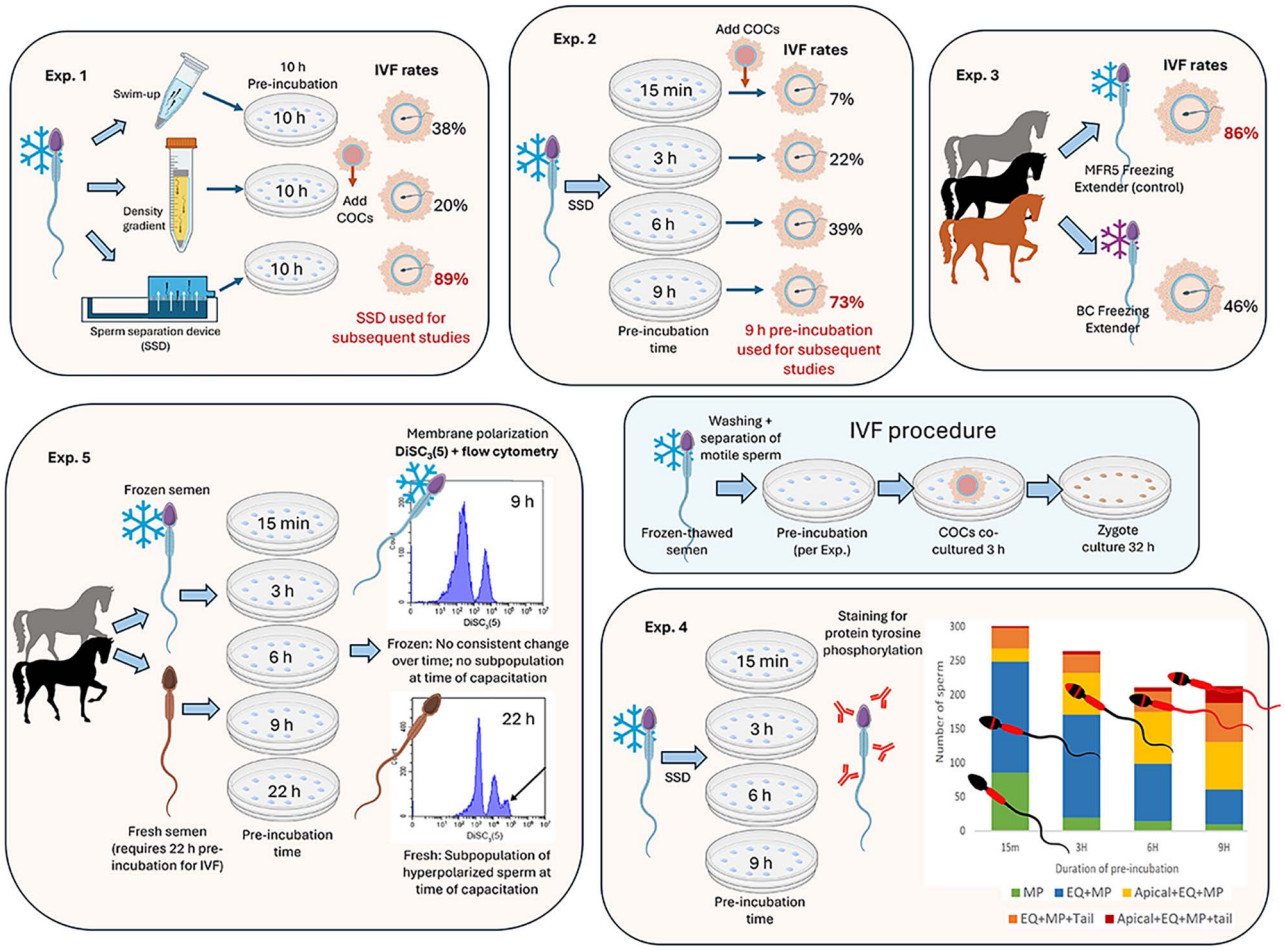
Graphical Abstract



Key words: horse, stallion, sperm, capacitation, IVF, fertilization, membrane, embryo, pregnancy.

Introduction

The first efficient and repeatable method for standard in vitro fertilization (IVF) in the horse was reported in 2022 [1]. This technique used prolonged (20–22 h) pre-incubation of sperm in droplets of a modified Tyrode's Albumin Lactate-Pyruvate (TALP) medium containing penicillamine, hypotaurine, and epinephrine (PHE), to capacitate sperm before the addition of intact cumulus-oocyte complexes (COCs). Sperm and COCs were co-incubated for 3 to 6 h, then COCs were transferred to embryo culture medium for further embryo development. This procedure yielded fertilization rates of over 80%, in vitro blastocyst production rates of 74%, and production of three foals after transfer of three blastocysts. Capacitation of sperm over the pre-incubation period was associated with localization of staining for protein tyrosine phosphorylation (PY) in the sperm apical head, equatorial region and midpiece.

The development of a repeatable protocol for standard equine IVF provides a basis for research in sperm capacitation and fertilization in the horse, and also raises the question of whether standard IVF can be utilized clinically. In vitro embryo production has become an important tool in equine reproductive management. Currently, equine embryos are produced in vitro clinically via intracytoplasmic sperm injection (ICSI), which entails multiple manipulations of the mature oocyte that are potentially detrimental, including holding the oocyte in room atmosphere, denuding it of cumulus, rupturing the oocyte plasma membrane, and injecting an acrosome-intact sperm of the operator's choice, along with associated media, into the oocyte cytoplasm. Standard IVF avoids these steps, thus it is possible that embryo production rates, or pregnancy or foaling rates after transfer to recipient mares, may be higher for embryos produced by IVF than by ICSI. This would make IVF a desirable clinical procedure in equine reproductive management, and the comparison of IVF and ICSI outcomes would provide a meaningful model for human assisted reproduction. However, the IVF studies of Felix et al. [1] utilized only freshly collected semen. For clinical cases, it is important that the procedure works effectively with frozen–thawed semen, as fresh semen is rarely available from the desired sire, and use of frozen semen greatly reduces the labor, variability and cost of sperm management for in vitro embryo production in comparison to that for collection and shipment of fresh semen. The use of

frozen semen would similarly simplify the conduct of research investigating aspects of equine IVF.

Equine frozen–thawed semen has many differences from fresh semen. Sperm are subjected to multiple manipulations during the freezing procedure, which can include centrifugation, exposure to a freezing extender containing a single or combination of cryoprotectants, and the cooling, freezing and thawing processes themselves. Thus, viability, total motility, and progressive motility of frozen–thawed equine sperm typically are decreased 25–40% from their initial values in fresh semen [2, 3]. Notably, frozen–thawed sperm show some changes associated with capacitation [4], a finding termed "cryocapacitation" [5]. In other species, these changes include modifications in intracellular Ca^{2+} and chlortetracycline chloride (CTC) staining patterns [6–10]; actin polymerization [11], and development of patterns of PY associated with capacitated sperm [12]. As a result, in other species cryopreserved sperm can fertilize oocytes in vitro in the absence of factors required for IVF with fresh sperm [8, 13].

In horses, frozen–thawed sperm have been shown to have populations exhibiting increased PY [14, 15] and to have patterns of PY staining [16] similar to that which we identified in fresh sperm capable of fertilizing oocytes in vitro [1]. Frozen–thawed stallion sperm also have been shown to exhibit capacitation-related changes in CTC staining patterns [17, 18], and an increase in plasma membrane fluidity and collapse of membrane phospholipid asymmetry [19–21]. Thus, when utilized for IVF, equine frozen–thawed semen may require different preparation procedures than does fresh semen, or progress along a different capacitation timeline.

The studies reported here were conducted to develop a procedure for performing equine standard IVF with frozen–thawed semen, and to investigate capacitation-related changes in frozen–thawed semen over the pre-incubation (capacitation) period.

Materials and methods

Experimental design

Experiment 1: Effect of selection method on in vitro fertilization rates with frozen–thawed sperm

Preliminary studies (2 replicates each) showed that (A) the use of frozen–thawed semen with the swim-up sperm preparation media and incubation methods developed in the previous study for fresh semen [1] resulted in loss of sperm motility in the droplet by 5 h of pre-incubation; and that (B) separation of frozen–thawed semen using colloid centrifugation rather than swim-up for the first step resulted in some sperm motility at 6 h pre-incubation, but the motility was lost at 22 h.

One trial was performed using a commercial sperm separation device (SSD) for sperm selection (described in General Methods, below). Sperm were still motile at 6 and 10 h of pre-incubation. At 10 h, seven COCs were added to the pre-incubation droplet, co-incubated for 3 h, then transferred to embryo culture medium and cultured for 32 h, which we term *Zygote culture*. At the end of this period we found that 6 of seven oocytes had cleaved, with 3 to 4 normal nuclei each on staining with DAPI, indicating a fertilization rate of 6/7.

Based on this preliminary data, Exp. 1 was conducted to evaluate the effects on fertilization rates with IVF of three different techniques for selection of sperm from frozen–thawed semen: Swim-up (SU), Colloid centrifugation (COL), and use of an SSD. For this experiment, to simplify the procedure from that used previously, [1] we performed laboratory sperm preparation procedures using only two media: FT-PHE, used for all sperm preparation, and TALP-R, used for final resuspension before placement of sperm in the pre-incubation droplets. As fertilization had been achieved in the preliminary SSD study after pre-incubation for 10 h, and in light of previous reports showing evidence of cryocapacitation in equine sperm, as reviewed in the Introduction, we evaluated sperm fertilizing ability after 10 h pre-incubation.

Straws of semen frozen in a commercial extender (MFR5, Animal Reproduction Systems, Chino, CA, USA) from Stallion SM were used. Six replicates were performed. Semen was prepared by SU, COL, and SSD as described under General Methods, below, and prepared sperm were added to pre-incubation droplets. After 10 h sperm pre-incubation, in vitro-matured COCs were added to the sperm droplets. Sperm and COCs were co-incubated for 3 h, then the COCs were transferred to Zygote culture. After the 32-h Zygote culture period, all structures were denuded and stained with DAPI. Cleaved embryos with two or more normal nuclei were considered to be fertilized, as parthenogenesis is rare in equine oocytes, and in our previous study [1], embryo cleavage rates were equivalent to fertilization rates as determined on confocal microscopy. As the meiotic status of the oocytes was unknown at the time they were placed in fertilization droplets (due to presence of the cumulus), after denuding and staining the fertilization rate was calculated as cleaved / (cleaved + metaphase); that is, disregarding degenerating or immature oocytes.

Experiment 2: Pre-incubation period required for IVF with SSD-separated frozen–thawed sperm

The results of Exp. 1 indicated that SSD separation of sperm provided the highest rate of embryo development after IVF, thus SSD separation was used in all subsequent studies. Experiment 2 was conducted to determine the minimum pre-incubation period required for IVF with frozen–thawed sperm prepared using SSD, and to evaluate the ability of oocytes fertilized with frozen–thawed sperm to develop to the blastocyst stage.

Straws of semen from Stallion SM, frozen in MFR5 extender, were prepared using SSD as described in Exp. 1. Three replicates were performed. Sperm were pre-incubated for 15 min (0 h) or for 3, 6, or 9 h before the addition of COCs. Sperm and COCs were co-incubated for 3 h, then COCs were transferred to Zygote culture for 32 h. After this, the cumulus was denuded, uncleaved structures were fixed and stained for evaluation, and cleaved presumptive embryos were transferred to embryo culture medium and cultured up to Day 10 (considering the day of sperm-COC co-incubation as Day 0) to determine blastocyst formation rates. Resulting blastocysts were vitrified as previously described [22].

Time-lapse monitoring equipment (MIRI, Esco Medical) was briefly available and two additional replicates of the 9 h treatment were performed to evaluate zygote development after IVF using this equipment. Six COCs were placed in Zygote culture in the monitoring equipment after co-incubation with sperm and images were recorded at 10 frames per hour for 22 h. Culture conditions within the time-lapse equipment were the same as for standard incubation (6% CO_2, 5% O_2 at 38°C) except that the atmosphere was not humidified.

Experiment 3: Effect of freezing extender on fertilization and blastocyst rates with frozen–thawed semen

Semen was collected from each of three stallions (SM, TH, and LN). Each ejaculate was divided into two equal aliquots and frozen using either MFR5 or Botucrio (BC; BotuPharma, Phoenix, AZ, USA) commercial freezing extender.

For each replicate, straws of semen from both extender treatments from the same stallion were thawed and semen samples were prepared separately by SSD. Sperm were pre-incubated for 9 h, then COCs were added to the droplets, co-incubated for 3 h, then transferred to Zygote culture for 32 h.

Three replicates were performed for each of the three stallions. In two replicates for each stallion, after Zygote culture, all structures were fixed and stained for evaluation of cleavage and nucleus formation. In the third replicate for each stallion, after Zygote culture uncleaved structures were fixed and stained, and cleaved embryos were continued in embryo culture up to Day 10, to verify the ability of frozen–thawed semen from both extender treatments to produce blastocysts after IVF. Resulting blastocysts were vitrified.

To assess the viability after cryopreservation of the blastocysts produced, nine vitrified-warmed blastocysts were transferred to recipient mares (three blastocysts to each of three recipient mares). The recipient mares had ovulated 4 days before transfer. For estimation of embryo day, the blastocysts were considered to be the equivalent of a Day 6 in vivo-produced embryo at the time of transfer. Recipient mares were evaluated via ultrasonography per rectum starting 5 days after transfer and continuing to a minimum of 10 days after transfer (embryo Day 16).

Experiment 4: Evaluation of changes in protein tyrosine phosphorylation in frozen–thawed sperm over the time of pre-incubation

Frozen–thawed semen from all three stallions (SM, LN, and TH; MFR5 extender), was prepared by SSD as described above, and placed in pre-incubation droplets prepared in separate dishes designated to be removed for analysis at four different time points (15 min, 3, 9, and 22 h) during the pre-incubation period. At each time point, sperm were aspirated from pre-incubation droplets and were subjected to immunofluorescence staining to assess PY. One replicate was performed with sperm from each stallion.

Additionally, we investigated the dynamics of PY in sperm during sperm-COC co-incubation. For this, sperm were pre-incubated for 9 h, then COCs were transferred into the droplet. After 1 and 3 h co-incubation, COCs were removed from the droplet and stained intact for PY. Additional COCs were removed from droplets at 1 h and 3 h co-incubation, denuded of cumulus and stained for PY in an attempt to evaluate the PY status of the fertilizing sperm.

Experiment 5: Changes in membrane polarization in fresh and frozen–thawed sperm during the pre-incubation period

Sperm membrane polarization was evaluated using the potentiometric probe, $DiSC_3(5)$. To validate the staining and flow cytometric analysis methods, freshly ejaculated, extended semen from stallion SM was washed and sperm resuspended in GMOPS, then stained with $DiSC_3(5)$ and counterstained with DAPI. Baseline flow cytometric analysis was performed, then staining was assessed after the addition of valinomycin to hyperpolarize the sperm, followed by the addition of increasing concentrations of KCl to valinomycin-treated sperm to depolarize the sperm.

After validating the staining methods, staining with $DiSC_3(5)$ and DAPI was utilized to evaluate changes in sperm membrane polarization in fresh and frozen–thawed sperm over time under IVF pre-incubation conditions. Fresh extended semen and frozen–thawed semen from stallion SM were processed separately using the SSD method described above, and sperm placed into pre-incubation droplets. At 15 min, 3, 6, 9, and 22 h, sperm suspension was aspirated from the pre-incubation droplets and sperm were stained and analyzed via flow cytometry. Two additional replicates assessing frozen–thawed semen at all time points, and two replicates assessing fresh semen at 0 and 22 h were performed with Stallion SM, and two replicates of frozen–thawed semen and one replicate of fresh semen from Stallion TH were assessed at all time points.

Statistical analysis

Because of the low expected values in some cells, pairwise comparisons of cleavage and blastocyst rates between treatments were performed using Fisher's exact test; differences with a $P < 0.05$ were considered significant. For live/dead staining (Exp. 4), differences in proportions of live and dead sperm over time were evaluated by Chi Square with a $P < 0.05$. Differences in category of PY staining within live sperm over time were first evaluated overall by Chi Square, with pairwise comparisons performed using Fisher's Exact Test with Bonferroni's adjustment for multiple comparisons.

General 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 Committee at the University of Pennsylvania (IACUC protocols #806502 and #806952).

Collection and maturation of oocytes

Immature oocytes were collected by transvaginal ultrasound-guided follicle aspiration from Thoroughbred- and Warmblood-type mares, as previously described [23]. The recovered cumulus-oocyte complexes (COCs) were held in commercial embryo holding medium (EmCare, ICPbio Reproduction, WI, USA; or ABT Holding, ABT360, WA, USA) at room temperature overnight then placed in maturation culture as previously described [24]. Briefly, after holding, grossly degenerating COCs were discarded and the remainder of COCs were cultured for 28–32 h in M199 with Earle's salts (Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, ThermoFisher Scientific, MA, USA) and 5 mU/ml FSH (Sioux Biochemicals, IA, USA).

Semen collection and freezing

Three stallions (two Arabian and one Welsh Pony; Stallions SM, TH, and LN, respectively) were utilized, as outlined for each experiment. Semen was frozen in straws using the commercial semen freezing extender MFR5 at 200 million sperm/ml, using a programmable freezer as previously described [25]. For Exp. 3, in which the ejaculate was divided for freezing in each of two extenders, after centrifugation one aliquot was extended with MFR5 and one similarly extended

with BC. Filled straws in the MFR5 treatment were frozen as above; filled straws in the BC treatment were layered on a rack and equilibrated at 5°C for 20 min. The racks were then placed into an insulated box 5 cm above liquid nitrogen for 15 min, after which time straws were plunged into liquid nitrogen.

IVF media preparation

The IVF media used were previously detailed by Felix et al. [1]. Briefly, two modified TALP media [26] were prepared: TALP-R, used for final resuspension of sperm prior to adding to the pre-incubation droplets, and FERT-TALP, used as a base for FT-PHE medium. A solution of PHE [27] as modified previously [1] was prepared and aliquots frozen and used within three weeks of preparation. The PHE solution was added to FERT-TALP to make FT-PHE, with final concentrations of 9 μM hypotaurine, 18 μM penicillamine, and 1.8 μM epinephrine. Then, 45 μl droplets of FT-PHE were prepared under light mineral oil and dishes were equilibrated at 38.2°C in a humidified atmosphere of 5% CO_2 in air overnight before the addition of sperm. Similarly, TALP-R and FT-PHE for use for sperm processing were equilibrated at 5% CO_2 in air overnight before use.

Sperm selection methods

Straws of frozen semen (0.5 ml) were thawed at 38°C for 30 s. Three methods of sperm preparation were used: SU, COL using a commercial colloid preparation (Equipure 90%, Nidacon, Gothenburg, Sweden) and a commercial sperm separation device (SSD; ZyMot (currently available for veterinary use as VetMotl), Gaithersburg, MD, USA).

For swim-up separation, duplicate preparations (200 μl of frozen–thawed semen layered under 1 ml FT-PHE) were incubated at 38.2°C in 5% CO_2 in air for 30 min, with the tube held at 45°. The top 750 μl of media was collected from each tube and each aliquot was increased to 1 ml by adding 250 μl FT-PHE. These were centrifuged at 300 × g for 5 min, then the resulting pellets were combined and resuspended with TALP-R to a volume of 60 μl. This suspension was held at 38.2°C in 5% CO_2 in air for 15 min. During this time, sperm concentration was determined. After the incubation period, a volume of the sperm suspension containing 50 000 sperm was added to the pre-incubation droplet. Additional TALP-R was added, if needed, to make a total added volume of 5 μl, providing a final concentration of 1 million sperm/ml in a 50-μl droplet, as previously described [1].

For colloid separation, duplicate preparations, each of 200 μl of frozen–thawed semen layered on top of 3 ml of 90% Equipure in a 15-ml Falcon tube, were centrifuged at 200 × g for 20 min at room atmosphere and temperature (~22°C). The colloid was removed and the sperm pellets from the tubes were combined and resuspended with 1 ml FT-PHE. This suspension was centrifuged at 300 × g for 5 min, then the resulting pellet was resuspended with TALP-R to a volume of 60 μl. Sperm concentration was determined and sperm added to the pre-incubation droplets as described above.

For use of the sperm separation device (SSD), 400 μl of frozen–thawed semen was diluted with 450 μl FT-PHE and this sperm suspension was injected into the inlet port of the device. Then, 50 μl of FT-PHE was loaded into the outlet port, and the open surface of the filter was covered with 700 μl of FT-PHE. The device was incubated at 38.2°C in 5% CO_2 in air for 30 min. After this, 500 μl of fluid was collected from the outlet port and was diluted with an additional 500 μl of FT-PHE. This was centrifuged at 300 × g for 5 min. The resulting pellet was resuspended with TALP-R and sperm added to the pre-incubation droplets as described above.

Co-incubation of sperm and oocytes

After sperm had been pre-incubated for the designated period as outlined in each experiment, COCs were removed from maturation culture. The period of COC holding before maturation, and onset of maturation culture, were timed so that COCs were always 28–32 h in maturation before addition to fertilization droplets. COCs that appeared grossly degenerated after maturation culture were discarded, and the remainder washed in FERT-TALP without PHE. One to three COCs, in 1 to 2 μl of FERT-TALP medium, were added to each 50-μl FT-PHE droplet containing pre-incubated sperm, and the dishes incubated under 38.2°C in a humidified atmosphere of 5% CO_2 in air for 3 h.

Embryo culture

After the 3-h sperm-oocyte co-incubation period, COCs were removed from the fertilization droplets. This time was designated Time 0 for embryo formation. COCs were cultured for embryo development as described by Felix et al. [1]. Briefly, this consisted of transfer to a commercial human embryo culture medium (Global, LifeGlobal, Guilford, CT, USA) with 10% added FBS for the initial Zygote culture period of 32 h, after which the presumptive embryos were denuded of cumulus via pipetting and evaluated for cleavage. Depending upon the experiment, either all presumptive embryos were fixed and stained with DAPI for evaluation at that time or alternatively, uncleaved structures were fixed and stained while cleaved embryos were continued in embryo culture until Day 5, then transferred to DMEM/F-12 with 10% added FBS for up to Day 10 to assess blastocyst formation. Resulting blastocysts were vitrified as described by Canesin et al. [22].

Staining for protein tyrosine phosphorylation

The pattern of PY was assessed for sperm after 15 min, 3, 6, or 9 h pre-incubation. A minimum of two droplets of sperm in FT-PHE were prepared for each assessment time. The contents of the assigned droplets were aspirated and a volume of 55 μl, containing ~55 000 sperm, was used for assessment. The sperm were fixed, permeabilized, and stained for PY as previously described [1], using an antiphosphotyrosine antibody raised in mouse (anti-phosphotyrosine clone 4G 10, EMD Millipore Sigma) and Alexa Fluor 633 goat anti-mouse IgG (H + L) (ThermoFisher) as the secondary antibody. The preparations were counterstained with Hoechst 33342.

Three replicates were performed, one with sperm from each of the three stallions. On assessment of sperm in the first replicate, a PY staining pattern was noted (PY staining of the entire tail) that was not seen commonly in our previous fresh sperm work [1]. To determine if this PY staining pattern was seen in viable sperm, in the next two replicates sperm were stained for membrane integrity to label non-viable sperm prior to staining for PY. For this, a fixable live/dead staining protocol with anti-agglutination measures was performed as previously described [28], using LIVE/DEAD Fixable Green Dead Cell Stain. After the sperm were fixed by addition of paraformaldehyde as the final step of live/dead staining, they were subjected to PY staining as described above.

Stained sperm were assessed using a fluorescence microscope (Zeiss AxioObserver with 365-nm and 545-nm excitation filters, or Leica DMIRE with 350-nm and 586-nm excitation filters) at 40 X magnification. Fields for evaluation were chosen in brightfield to eliminate bias for specific staining patterns. Images were then acquired for assessment of PY staining at a fixed camera setting to allow semiquantitative analysis of staining patterns.

In the two replicates (Stallions LN and TH) in which sperm were stained for both live/dead and PY, 200 sperm were evaluated visually at each time period for each replicate and the findings combined. The proportion of non-viable sperm was recorded; for viable sperm, PY staining in the apical, equatorial, midpiece and entire tail were scored separately for each sperm.

Immunofluorescence staining of intact COCs and denuded oocytes after co-incubation was performed using the antibodies as for PY staining of sperm, as detailed above. The protocol mirrored that for sperm, however, instead of using coated slides, the COCs were transferred through microdroplets containing the staining solutions.

Staining for membrane polarization with $DiSC_3(5)$.

Validation of $DiSC_3(5)$ staining to detect changes in membrane polarization in equine sperm was performed with fresh semen from Stallion SM. After collection and extension with INRA96, semen was transported to the flow cytometry laboratory (5 min), washed twice and the sperm pellet resuspended in G-MOPS (Vitrolife) to a concentration of 50×10^6 sperm/ml. Stock solutions of $DiSC_3(5)$ (Invitrogen) and DAPI (Invitrogen) were added to the sperm suspension to produce concentrations of 5 nM and 1 μg/ml, respectively, based on the results of preliminary dosage trials. The aliquot was incubated for 10 min at 37°C, then 100 μl of the stained aliquot was placed in each of multiple wells in a 96-well plate and analyzed on a 13-color, 4-laser flow cytometer (CytoFLEX S, Beckman Coulter, Brea, CA) at room temperature (~22°C). The sperm population was gated based on forward scatter and side scatter. For each sample, 10 000 gated events representing sperm were recorded. $DiSC_3(5)$ fluorescence was detected using a 638-nM laser and a 712/25 BP filter, and DAPI fluorescence was detected using a 405-nM laser and a 450/45 BP filter. Unstained and single-stained samples for each fluorochrome were used as controls for autofluorescence and compensation, and to set the positive and negative gates for each stain. No compensation was performed.

After the initial stained sperm suspension was analyzed, the potassium ionophore valinomycin (Sigma) was added to each well to a concentration of 1 μM, to hyperpolarize the sperm, and the sample was analyzed again. To depolarize the sperm, solutions of KCl (Sigma) of increasing concentrations were added, in a fixed volume, to separate wells of valinomycin-treated sperm suspension to achieve an additional 2.5, 5, 10, 20, or 40 mM KCl within the sperm suspensions, and the suspensions were analyzed again. Histograms of $DiSC_3(5)$ staining were generated to visualize the effects of valinomycin and KCl addition on relative changes in membrane polarization.

For the main experiment, fresh extended semen and frozen–thawed semen (MFR5) were both processed using the SSD procedure as described above, and sperm placed into pre-incubation droplets. Separate dishes containing pre-incubation droplets were designated to be removed for analysis at five time points (15 min, 3, 6, 9, and 22 h) during the pre-incubation period. At each time point, 40 μl of the pre-incubation sperm suspension was aspirated from each of five droplets and combined, then 80 μl of this suspension was stained directly with stock solutions of $DiSC_3(5)$ and DAPI (10 μl of each stock solution) to the final concentrations described above and analyzed via flow cytometry.

Results

Experiment 1: Effect of selection method on in vitro fertilization rates with frozen–thawed sperm

The results of Exp. 1 are presented in Table 1. At the end of the 32-h Zygote culture period, a higher fertilization rate per fertilizable oocyte (23/26, 88.5%) was found for sperm selected by SSD than for sperm selected by either SU or COL ($P < 0.001$). The proportion of oocytes found to be degenerating or immature tended to be higher in SU than in SSD ($P = 0.07$). The proportions of cleaved embryos having 2, 3 to 4, or ≥ 5 normal nuclei, respectively, in the different treatments were: SU: 0, 75, and 25%; COL: 20, 40, and 40%; and SSD: 4, 39, and 57%.

Experiment 2: Pre-incubation period required for IVF with SSD-separated frozen–thawed sperm

The fertilization rate, as represented by embryo cleavage, increased significantly over time of sperm pre-incubation, from 7.1% in sperm pre-incubated for 15 min before COC addition, to 73.3% for sperm pre-incubated for 9 h ($P < 0.01$; Table 2). An additional ~5% of oocytes appeared to have been fertilized but were in early stages of activation (anaphase to the first mitotic metaphase) at the time of evaluation (after the 32 h Zygote culture period); these oocytes were included as fertilizable oocytes but were not included in the "fertilized" category. Concomitant with the increase in fertilization rate over time of sperm pre-incubation, the proportion of oocytes found to be still in metaphase decreased significantly, from 60% of all structures at 15 min, to 13.6% at 9 h ($P < 0.05$), indicating that oocytes capable of fertilization were more likely to be fertilized in the longer pre-incubation treatment.

No blastocysts developed on culture of cleaved embryos in the 15 min or 3 h groups. The blastocyst development rates per cleaved embryo cultured in the 6 and 9 h pre-incubation treatments were 3/5, 60% and 6/11, 54.5%. These values were not significantly different.

Of the six time-lapse monitored COCs transferred to the monitoring equipment at the beginning of the zygote culture period, three developed to the blastocyst stage. One of these provided clear visualization of post-fertilization events. In this COC, there appear to be two polar bodies present at the onset of culture (immediately after 3-h co-incubation with sperm), however at 5 h culture the oocyte blebbed rapidly, suggestive of second polar body extrusion. Extrusion of cytoplasmic contents was visualized starting at 14 h after placement in the equipment, and cleavage at 20 h after placement (Supplemental Video 1).

Experiment 3: Effect of freezing extender on fertilization and blastocyst rates with frozen–thawed semen

The results of Exp. 3 are presented in Table 3. The fertilization rate, as represented by embryo cleavage, was significantly

Table 1. Proportions of oocytes in various categories at the end of the 32 h Zygote culture period, after IVF with frozen–thawed sperm selected using different methods

Treatment	n	Degenerating/immature	Metaphase	Fertilized
Swim-up	35	14 (40.0%)*	13 (37.1%)	8/21 (38.1%)[a]
Colloid	31	6 (19.4%)	20 (64.5%)	5/25 (20.0%)[a]
SSD	32	6 (18.8%)	3 (9.4%)	23/26 (88.5%)[b]

Sperm were pre-incubated for 10 h, COCs were co-incubated with sperm for 3 h. After the Zygote culture period, all structures were fixed and stained for determination of chromatin status. Cleaved embryos with ≥2 normal nuclei were considered to represent fertilized oocytes. Fertilization rate is calculated as cleaved / (metaphase + cleaved); no structures were in anaphase, telophase or first mitotic metaphase. SSD, commercial sperm separation device. *The proportion of degenerating/immature oocytes in the Swim-up treatment tended to be higher than that for the SSD treatment ($P = 0.07$). [a,b] within columns, values with different superscripts differ significantly ($P < 0.05$).

Table 2. Proportions of oocytes / embryos in the different stages, after IVF with frozen–thawed sperm pre-incubated for different times before addition of COCs

		Uncleaved – stained Chromatin configuration n (% of total COC)				
Pre-incubation time	n COCs	Immature–Degenerating	Metaphase	Ana-Tel-Mit	Fertilized	Blastocysts (% of cleaved)
15 m	20	6 (30.0%)	12 (60.0%)[a]	1 (5.0%)	1/14 (7.1%)[a]	0
3 h	23	5 (21.7%)	12 (52.2%)[ab]	2 (8.7%)	4/18 (22.2%)[a]	0
6 h	20	7 (35.0%)	8 (40.0%)[a,b,c]	0	5/13 (38.5%)[a,b]	3/5 (60.0%)
9 h	22	7 (31.8%)	3 (13.6%)[c]	1 (4.5%)	11/15 (73.3%)[b]	6/11 (54.5%)

Sperm were selected using the SSD method; COCs were co-incubated for 3 h. Oocytes that were uncleaved at 32 h were fixed and stained for determination of chromatin status; cleaved embryos were continued in culture to determine blastocyst rate. Cleaved embryos were considered to represent fertilized oocytes. Ana, anaphase; Tel, telophase; Mit, first mitotic metaphase. Fertilization rate is calculated as cleaved / (cleaved + metaphase + ana-tel-mit). [a,b,c] within columns, values with different superscripts differ significantly ($P < 0.05$).

Table 3. Proportions of oocytes / embryos in the different stages after IVF with sperm frozen with two different commercial freezing extenders

Freezing extender	n	Immature–Degenerating	Metaphase	Anaphase–telophase	Fertilized	Blastocysts / cleaved embryos cultured (%)
BC	70	9	33[a]	0	28/61 (45.9%)[a]	4/9 (44.4%)
MFR5	71	9	7[b]	2	53/62 (85.5%)[b]	13/19 (68.4%)

Sperm were selected using the SSD method and pre-incubated for 9 h before addition of COCs, which were co-incubated for 3 h. Oocytes that were uncleaved at 32 h were fixed and stained for determination of chromatin status. Cleaved embryos were considered to represent fertilized oocytes. Fertilization rate is calculated as cleaved / (cleaved + metaphase + anaphase + telophase). In two replicates, cleaved embryos were stained to confirm presence of normal nuclei. In one replicate per stallion, cleaved embryos were cultured to determine competence for blastocyst formation. [a,b] within columns, values with different superscripts differ significantly ($P < 0.05$).

higher for oocytes co-incubated with sperm that had been frozen in MFR5 than in BC freezing extender (85.5% vs. 45.9% respectively; $P < 0.001$). Concomitantly, the proportion of oocytes still in metaphase after the Zygote culture period in the MFR5 treatment was significantly lower than that for BC ($P < 0.001$). In the two replicates for each stallion in which all structures were stained after Zygote culture, all cleaved embryos showed the presence of ≥2 normal nuclei. In the replicate in which cleaved embryos were cultured to ascertain potential for blastocyst development, at least one blastocyst was produced from each stallion in each extender. The overall blastocyst rates per cleaved embryo cultured were 13/19 (68.4%) for MFR5 and 4/9 (44.4%) for BC. This difference was not significant ($P > 0.1$).

When evaluated individually, the fertilization rates for sperm from Stallions SM, LN and TH were 19/23 (82.6%), 14/19 (73.7%) and 20/20 (100%), respectively, for extender MFR5 and 4/23 (17.4%), 10/24 (41.7%) and 14/14 (100%) respectively, for extender BC. For both extenders combined, the fertilization rate for Stallion TH, 100%, was significantly higher than that for either of the other two stallions (50.0% and 55.8% for Stallions SM and LN, respectively; $P < 0.0001$).

Three embryo transfers were performed. For each procedure, three vitrified-warmed blastocysts (stallions SM and TH, MFR5 treatment; blastocysts removed and vitrified on Day 7 of culture) were transferred, for a total of nine blastocysts transferred. The number of embryonic vesicles visualized on transrectal ultrasonography in the three recipient mares on Day 13 were 3, 2 and 2, for an overall pregnancy rate of 7/9 (78%; Supplemental Video 2). The embryonic vesicles developed normally to Day 18 to 22, when the pregnancies were terminated by flushing the uterus to recover the conceptuses. In the pregnancy that was maintained until Day 22 before flushing, embryonic heartbeats were seen in both of the two embryos present. Of the seven conceptuses recovered, six had normal-appearing embryos proper; in one conceptus the trophoblast was torn and the embryo proper could not be identified.

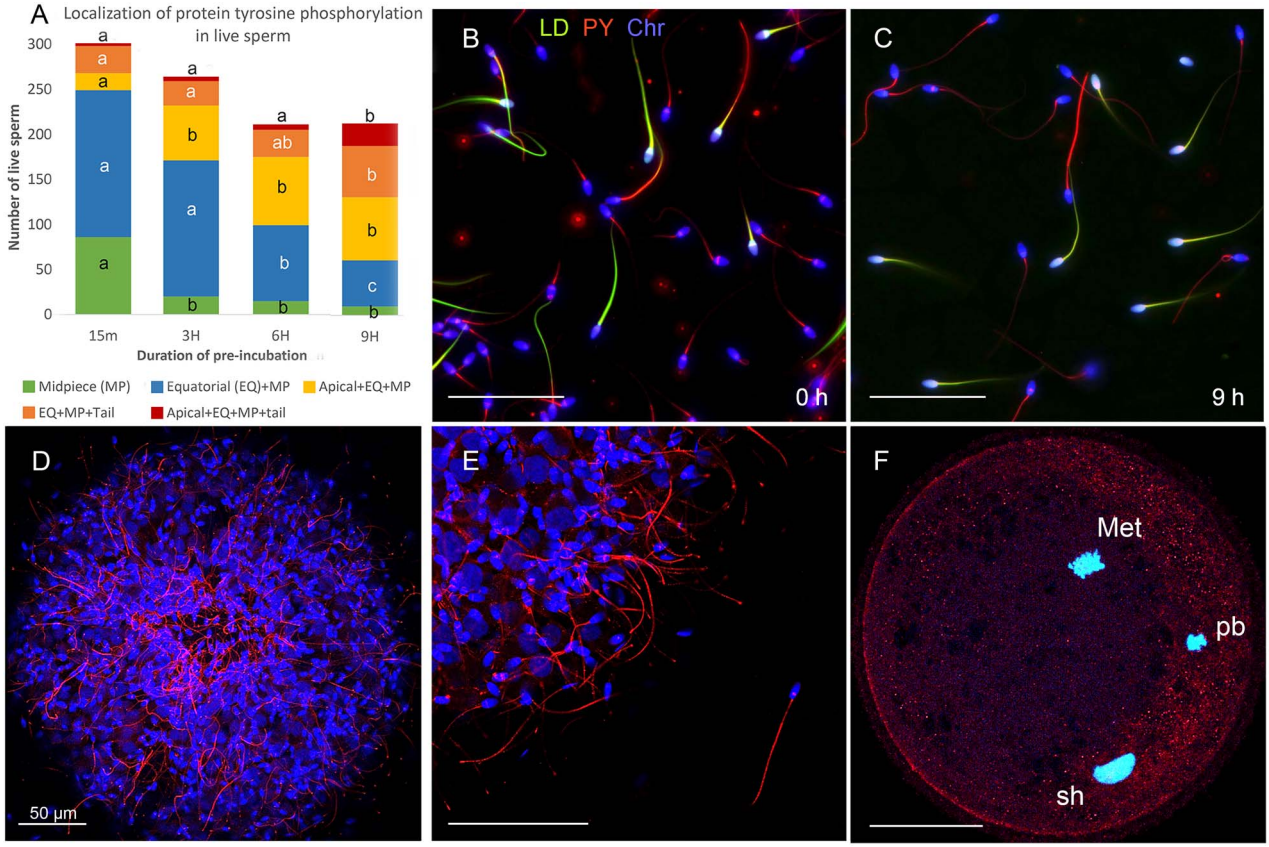


Figure 1. Protein tyrosine phosphorylation (PY) patterns over the period of sperm pre-incubation. (A) proportions of live sperm showing the different staining patterns at different time points. Within staining category, values marked with different letters are significantly different between time points ($P < 0.05$); (B, C) representative photomicrographs of live/dead stained (LD) sperm labeled for PY and chromatin (Chr) at 0 h (15 min) and 9 h pre-incubation; (D E) PY staining patterns of sperm attached to intact COC after 3 h co-incubation; (F) oocyte denuded after co-incubation showing the sperm head (sh), metaphase plate (Met) and polar body (pb). No PY was discernable to indicate the sperm tail. Bar = 50 μm.

Experiment 4: Evaluation of changes in protein tyrosine phosphorylation in frozen–thawed sperm over the time of pre-incubation

Changes in PY staining intensity and pattern over time during the 9 h pre-incubation period are presented in Figure 1. There was a decrease in the proportion of live sperm over the incubation period, from 75.3% at 15 min to 53.3% at 9 h ($P < 0.001$). Within live sperm, there was a significant change in PY staining pattern over the pre-incubation period ($P < 0.001$; Figure 1A-C), with decreasing proportions of sperm having fluorescence of the midpiece only or of the equatorial region and midpiece, and increasing proportions of sperm having fluorescence of the apical region and entire tail.

Evaluation of intact COCs after 1 or 3 h co-incubation with sperm showed that sperm both with and without fluorescence over the entire tail bound to the COC (Figure 1D,E). Evaluation of oocytes denuded before staining demonstrated sperm in the cytoplasm of one of two oocytes evaluated at 1 h co-incubation, and all of three oocytes evaluated at 3 h co-incubation. No distinct PY staining of the sperm tail was visualized in any oocyte (Figure 1F).

Experiment 5: Changes in membrane polarization in fresh and frozen–thawed sperm during the pre-incubation period

For the validation procedure, changes in $DiSC_3(5)$ staining in fresh equine sperm after treatment with valinomycin and then increasing concentrations of KCl are presented in Figure 2. After treatment with valinomycin, sperm that were initially $DiSC_3(5)$-positive, DAPI-negative (membrane-intact sperm exhibiting membrane polarization; quadrant Q5-LR in the flow cytometry scattergrams, Figure 3) showed a heterogenous response, separating into two subpopulations, one showing increased median fluorescence intensity (MFI) and one a slightly decreased MFI. The initial MFI for this quadrant was 4629 ± 136 (mean $\pm$ s.e); and after valinomycin treatment the MFI of the separate peaks were 3264 ± 36 and 7045 ± 133. The initial potassium concentration of the basal G-MOPS was not available from the manufacturer; however, on analysis of the medium (Vitros XT3400 Chemistry System, QuidelOrtho) this was 5.5 mM. Subsequent treatment with increasing concentrations of KCl decreased $DiSC_3(5)$ fluorescence incrementally.

Flow cytometric analysis of $DiSC_3(5)$ and DAPI staining in fresh and frozen–thawed sperm (both processed via SSD) and subjected to different periods of pre-incubation revealed a subpopulation of sperm in Q5-LR staining intensely for $DiSC_3(5)$ (i.e. hyperpolarized sperm) after 22 h pre-incubation in all four fresh-sperm replicates (Figure 3, Suppl. Figure 1). This subpopulation also tended to have lower DAPI staining (was closer to the X-axis). No consistent changes in polarization were observed in frozen–thawed sperm, and an intensely-staining sub-population was not seen at any time point in frozen–thawed sperm from either stallion.

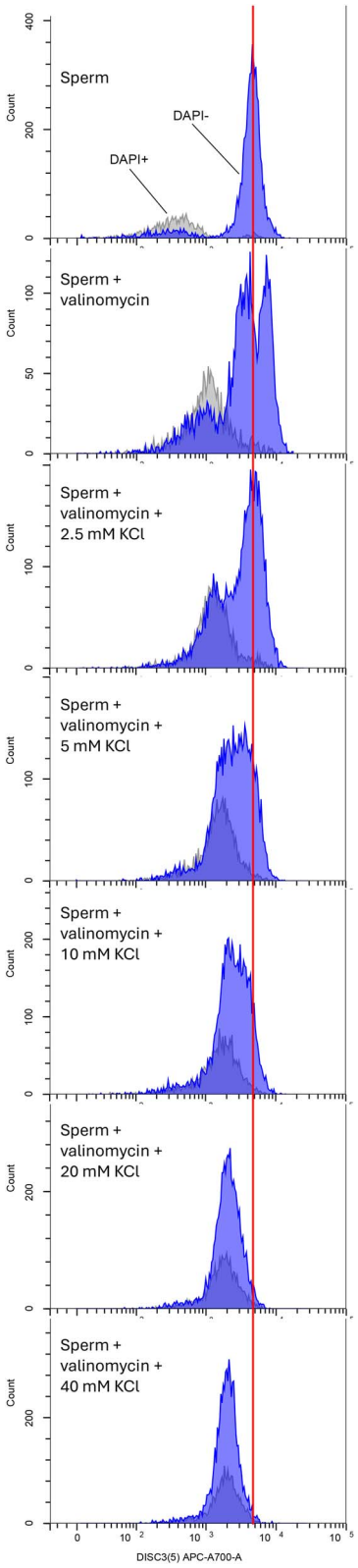


Figure 2. Histograms of DAPI-negative (live) fresh sperm (dark shading) and DAPI-positive (dead) sperm (light shading, resulting in darker shading when overlapping) in basal medium (GMOPS), after addition of 1 μM valinomycin, then after addition of KCl to increase potassium concentration by 2.5, 5, 10, 20 or 40 mM. A line is drawn in the center of the peak of the main population in the initial sample.

Discussion

The results of this study show for the first time that repeatable equine standard in vitro fertilization can be achieved with frozen–thawed semen and that the embryos produced can develop into blastocysts that can establish pregnancy. Notably, appreciable levels of fertilization were achieved with frozen–thawed sperm after only 3 to 6 h of pre-incubation (22% and 39%, respectively), and up to 100% fertilization was achieved with sperm pre-incubated 9 h, whereas in our previous study with fresh sperm, sperm pre-incubated for 6 h were not capable of fertilization [1]. This suggests that the capacitation-related changes previously reported in frozen–thawed equine sperm may speed the onset of functional capacitation (ability to fertilize) in these sperm. Further studies are needed to confirm that the more rapid acquisition of fertilizing capacity is due to the use of frozen–thawed semen, rather than to the sperm selection technique or other aspects modified in this study; for example, utilization of only two media, FT-PHE and TALP-R, for sperm processing.

We found that the method for selection of sperm from frozen–thawed semen had a profound effect on fertilization rates. Selection via a sperm separation device, in which sperm were required to swim through a small-bore filter before selection, was associated with a significantly higher fertilization rate (89%) than were Swim-up or Colloid Centrifugation techniques (20–38% fertilization). In our initial paper [1] and in preliminary trials for the studies in this report we observed an increase in oocyte degeneration when COCs were co-incubated in droplets in which sperm viability appeared low (as shown by few motile sperm observed in the droplet after pre-incubation, at the time of COC placement in droplets). This phenomenon was suggested in the Swim-up treatment in Exp. 1 in the current report, which had a high oocyte degeneration rate. We hypothesize that poor-quality or membrane-damaged sperm may release toxic compounds during prolonged pre-incubation, such as ammonia and hydrogen peroxide generated by the L-amino acid oxidase present in equine sperm [29], that either directly or via formation of toxic compounds such as lipid peroxides [30] adversely affect oocyte viability after COCs are introduced. Thus, it is unclear whether the SSD selection method selects sperm with greater potential for capacitation, or selects against sperm that become compromised during the pre-incubation period and thus release factors into the droplet that adversely affect other sperm or oocytes.

When evaluating the period required for sperm to capacitate, it should be noted that COCs were co-incubated for 3 h, then removed from co-incubation with the cumulus intact (likely containing numerous sperm, Figure 1D, E) to Zygote culture. Thus, fertilization could have occurred at any time in the co-incubation period or potentially during Zygote culture. Possibly related to this, in Exp. 1 we found a tendency for embryos to be more advanced on evaluation after Zygote culture in the SSD treatment than in the SU or COL treatments, suggesting that the SSD-selected sperm may have fertilized more rapidly. The finding in Exp. 4 that one of two oocytes evaluated immediately after 1 h co-incubation were fertilized, as were three of three oocytes evaluated immediately after 3 h co-incubation, demonstrates that frozen–thawed sperm selected by SSD and pre-incubated for 9 h can achieve fertilization rapidly after COCs are introduced.

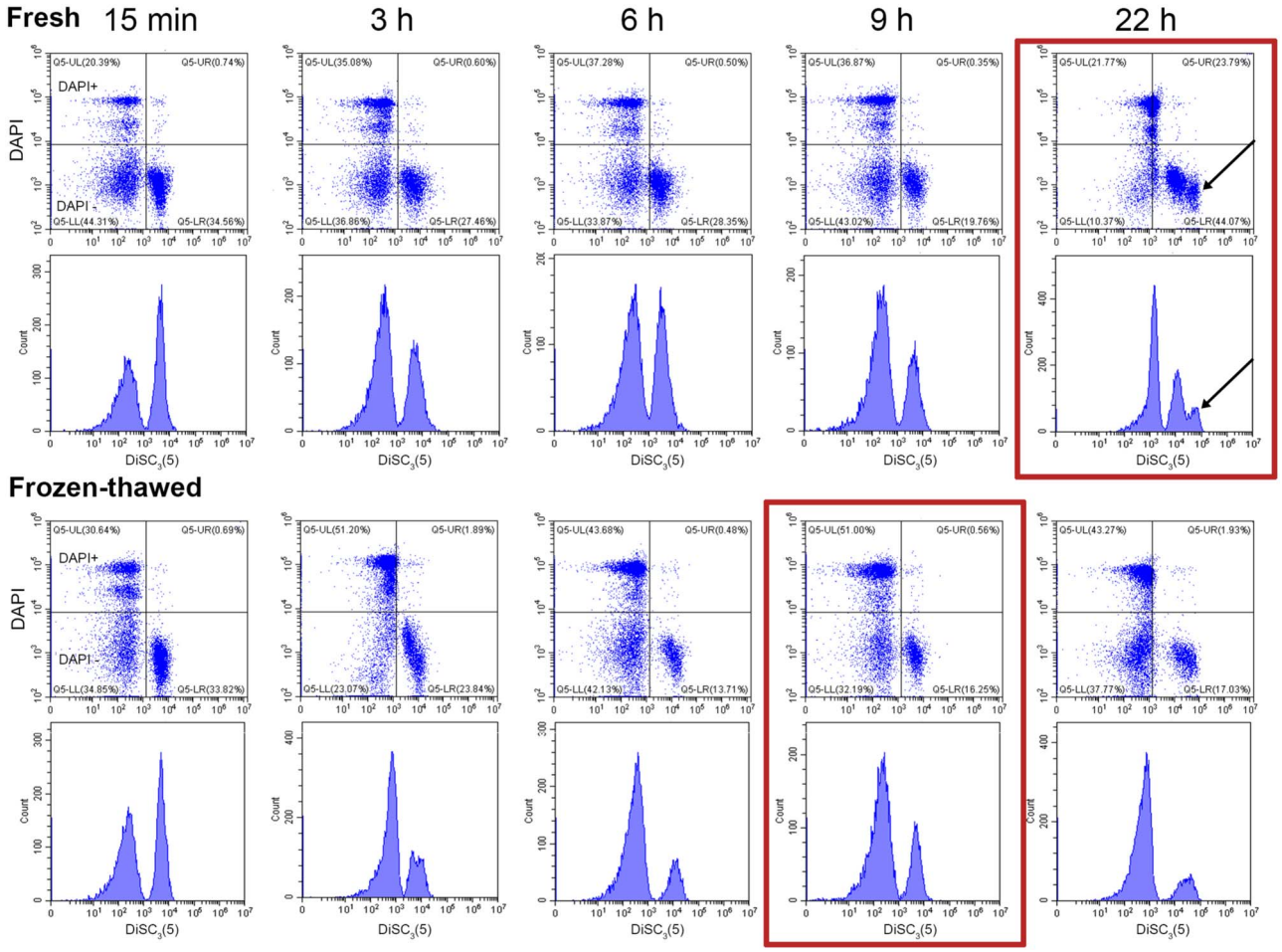


Figure 3. Representative scatterplots and histograms of fresh and frozen–thawed sperm after SSD preparation and pre-incubation in droplets for 0 (15 min), 3, 6, 9, or 22 h. Data obtained at the time the sperm are capable of fertilization are outlined in rectangles. A population of sperm staining intensely for $DiSC_3(5)$ (hyperpolarized sperm; arrow) was seen only in fresh sperm and only after 22 h pre-incubation.

Evaluation of the fertilized COCs via time-lapse imaging showed extrusion of cytoplasmic contents prior to cleavage, as noted previously after equine ICSI [24, 31, 32]. It has been questioned whether this extrusion is associated with compromise of the oolemma during ICSI; however, arguing against this is evidence of cytoplasmic extrusion in still images of in vivo-recovered equine zygotes, and in IVF zygotes from early reports (reviewed by [24]). The clear occurrence of cytoplasmic extrusion in the IVF zygote imaged in our study further indicates that cytoplasmic extrusion in equine oocytes is not an artifact of the ICSI procedure.

For clinical use, blastocysts resulting from IVF must be able to be cryopreserved and produce pregnancies after warming and transfer to recipient mares. Equine embryos produced by ICSI have good rates of pregnancy after either vitrification or freezing [22, 33]; however the role of the ICSI-induced hole in the zona pellucida in supporting post-transfer development of these embryos is unknown. The in vivo-conceived equine embryo does not "hatch" from the zona, rather the zona thins and flakes off the developing embryonic capsule, a glycoprotein envelope which forms between the equine embryo and in inner wall of the zona [34, 35]. In in-vitro produced equine embryos, the capsule does not form and the zona does not thin normally while the embryo is in culture [36, 37], thus these changes must occur after transfer to the uterus. In ICSI embryos, it is possible that after transfer, if the zona does not thin rapidly, the developing blastocyst can escape from the zona through the hole introduced during sperm injection. In embryos produced by IVF, there is no hole in the zona, and additionally the more physiological interaction of the sperm at fertilization may induce a more pronounced cortical granule reaction than seen in ICSI-produced embryos [24], potentially resulting in increased zona hardening. Notably, the embryo vitrification process may then additionally harden the zona, as indicated in a meta-analysis of human data that concluded that assisted hatching (creating a hole in the zona) increased pregnancy rates after transfer of frozen–thawed, but not fresh, embryos to unselected recipients [38]. Because IVF has not previously been repeatably successful in the horse, there is no information available on the viability of equine IVF embryos after vitrification and warming. Our findings that seven of nine transferred vitrified-warmed IVF blastocysts produced pregnancies after transfer was therefore meaningful.

We found a significant difference in fertilizing ability of frozen–thawed sperm associated with the extender used for freezing. It should be noted additionally that a different protocol was used for the BC extender (freezing over liquid nitrogen rather than in a programmable freezer). The two commercial extenders differ in many respects; notably, the BC extender has a higher egg yolk content than does MFR5

and contains dimethylformamide as a cryoprotectant. These components may stabilize the sperm membrane and internal structures and reduce cryocapacitation–related changes. In cattle, some cryocapacitation changes have been noted to be dependent on the type of extender used [39]. In a study we recently completed on use of re-frozen semen for ICSI, we found that frozen–thawed stallion semen that was re-frozen in Botucrio extender had a mean total and progressive motility more than twice those for the same semen re-frozen in MFR5 extender, but yielded a significantly lower cleavage rate after ICSI [25]. In that study using fertilization via ICSI, in contrast to our present findings with IVF, we found no differences in cleavage or blastocyst rates after use of sperm frozen once in either extender; however, Ramirez-Agamez and co-workers [40] reported significantly lower cleavage and blastocyst rates after ICSI with sperm frozen once in extenders with high phospholipid content and containing amides as cryoprotectants. If an extender-dependent increase in membrane stability during freezing and thawing is related to our findings on lowered IVF rates with the BC extender, this is important to note for future studies on the effect of freeze-thawing on capacitation-related changes in equine sperm.

In evaluating status of frozen–thawed semen in our study over the period of pre-incubation, we did not see the same PY acquisition pattern as we saw previously in fresh sperm during the pre-incubation period [1]. In that study, in fresh sperm, there was little anti-PY staining initially, and staining of the apical and equatorial regions, along with the midpiece, increased in intensity over time of pre-incubation. In contrast, in the current study the majority of viable frozen–thawed sperm showed staining in the equatorial region and midpiece when first evaluated after 15 min incubation. Bubenickova and co-workers [16] presented photographs of similar PY staining patterns in non-incubated frozen–thawed equine sperm. We found that the most prominent PY staining pattern increasing during pre-incubation in frozen–thawed sperm was staining of the entire tail. We have detected anti-PY staining of the entire tail previously after exposing equine sperm to conditions that are not associated with fertilizing ability [41, 42], thus we believe that PY staining of the entire tail may not reflect functional capacitation. In support of this, Chung and coworkers [43] showed that PY in capacitated mouse sperm is localized to specific structures in the sperm tail. They noted that non-specific PY can be induced by various treatments and may be abundant, but this PY is not associated with capacitation [43]. To explore the role of PY staining of the entire tail, we attempted to determine the PY staining pattern in frozen–thawed sperm that had successfully fertilized oocytes. Evaluation of co-incubated COCs in Exp. 4 showed that sperm both with and without PY staining of the entire tail were associated with the oocyte cumulus. We were unable to detect PY staining in the fertilizing sperm within the oocyte cytoplasm; this may be due to rapid changes in protein tyrosine phosphorylation status of the tail upon entry into the cytoplasm.

We evaluated membrane hyperpolarization in our sperm treatments as this is considered to be a hallmark of sperm capacitation in other species [44–46]. Little work has been reported on membrane polarization in horse sperm under any conditions. Using spectrophotometry with $DiSC_3(5)$ staining, McPartlin and colleagues [47] demonstrated that exposure to bicarbonate and BSA increased membrane polarization in populations of fresh equine sperm. Ortega Ferrusola and co-workers [48], using flow cytometry with the anionic dye $DiSBAC_2(3)$, reported that freezing and thawing increased depolarization of live sperm. We used the cationic dye $DiSC_3(5)$ and flow cytometric analysis to explore membrane hyperpolarization, following the methods validated by Puga Molina and coauthors in human sperm [49]. These authors found that hyperpolarization, as detected by $DiSC_3(5)$ staining, increased after treatment for capacitation and correlated positively with fertilizing ability in human sperm. Our analysis revealed a subpopulation of hyperpolarized sperm in fresh equine sperm at 22 h pre-incubation, the time that we previously showed these sperm are capable of fertilization [1]. The observation that only a subpopulation of sperm exhibited these changes aligns with findings in mouse sperm, in which only a subpopulation was shown to undergo hyperpolarization during capacitation using flow cytometry analysis with $DiSBAC_2(3)$ [46]. Notably, we could not define a similar hyperpolarized sub-population in frozen–thawed sperm at the time they were maximally fertilization-competent (9 h pre-incubation).

As a cationic probe, $DiSC_3(5)$ may be influenced by mitochondrial membrane potential, and uncouplers of oxidative phosphorylation are often added to reduce contribution from mitochondrial membrane staining. We did not incorporate use of uncouplers, as in mouse sperm use of uncouplers have been shown to have no effect on $DiSC_3(5)$ measurements [50], and we felt they could affect maintenance of sperm function. We did investigate use of $DiSBAC_2(3)$, which as an anionic probe is more specific to plasma membrane polarization. Staining with $DiSBAC_2(3)$ provided similar findings to those for $DiSC_3(5)$ in frozen–thawed sperm in our system; i.e. no evidence of a hyperpolarized subpopulation over the time of pre-incubation (Suppl. Figs. 1 and 2). In fresh sperm, results for $DiSBAC_2(3)$ over time were difficult to interpret and we did not identify the population of hyperpolarized sperm at 22 h that was identified with $DiSC_3(5)$ in the same sperm sample. Because additionally use of $DiSBAC_2(3)$ precludes the use of valinomycin [51], requires a relatively high concentration (we found that 15 μM was needed to define populations in equine sperm) and has a broad emission spectrum that overlaps that of standard viability stains, our initial perception is that $DiSC_3(5)$ is preferable for investigating membrane polarization via flow cytometry in equine sperm. It is possible that the sub-population of intensely-fluorescent sperm identified by $DiSC_3(5)$ at 22 h reflects not only plasma membrane hyperpolarization but also increased mitochondrial membrane potential. While in mice, capacitation is associated with a shift from oxidative phosphorylation to glycolysis [52], equine sperm have been shown to rely almost entirely on oxidative phosphorylation [20, 53], and thus may possibly exhibit increased mitochondrial activity related to capacitation. This is a fertile area for further study.

Our findings on these well-accepted hallmarks of capacitation, protein tyrosine phosphorylation and membrane hyperpolarization, suggest that while frozen–thawed equine sperm prepared as in this report can achieve fertilization followed by normal embryo development, the mechanisms whereby frozen–thawed sperm become capable of fertilization may differ from those found in fresh sperm. The $DiSC_3(5)$ stain identified increases in membrane polarization in subpopulations of fresh sperm prepared for IVF, suggesting capacitation. However, the absence of this subpopulation in frozen–thawed sperm, which also became fertilization-competent,

unfortunately appears to negate $DiSC_3(5)$ staining as a proxy for functional capacitation in work with horse sperm. Development of an accurate test for capacitation status would be of great value to research in capacitation in horse sperm, as evaluating rates of oocyte fertilization is problematic due to the difficulty of obtaining horse oocytes for study. Much further work is warranted on the cellular and molecular changes associated with acquisition of competence for fertilization in both fresh and frozen–thawed equine sperm. The ability to easily acquire abundant fresh and frozen semen from the same individual male, on repeated occasions, may make the horse an excellent model for study of differences in capacitation-related changes in these sperm types.

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Author contributions

MF: Conceptualization, methodology and investigation.
TD: Investigation.
EW: Methodology, conceptualization, investigation, data interpretation.
RL: Methodology, investigation, data interpretation.
CO: Methodology, conceptualization, and investigation.
RS: Methodology and investigation.
KH: Conceptualization, formal analysis, methodology, project administration, supervision, data interpretation, visualization, and writing.

Conflict of Interest: The authors declare that they have no competing interests.

Data availability

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

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