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IVF with frozen-thawed sperm after prolonged capacitation yields comparable results to ICSI in horses: A morphokinetics study

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ABSTRACT

Intracytoplasmic sperm injection (ICSI) is the current clinical practice for the *in vitro* production of equine embryos. The use of conventional fertilization methods such as *in vitro* fertilization (IVF), has historically been associated with poor success in horses. However, recent improvements have led to better outcomes with IVF, though only when using fresh semen, which limits its use in clinical practice. IVF remains in its infancy in equine reproduction, and several unknowns remain about the technique. One significant gap in knowledge concerns the morphokinetics of IVF embryos and how they differ from their ICSI counterparts. To address this, we performed IVF using frozen-thawed sperm from five different stallions following sperm selection and a prolonged capacitation period of 10 h, on a total of 109 oocytes. We then analyzed the cleavage rate (cleaved/initial oocytes), blastocyst rate (blastocyst/initial zygotes), and blastocyst development (blastocyst/cleaved zygotes) of the IVF cycles, and compared them with those of the clinical ICSI cycles during the same period. We also evaluated time-lapse images of the developed embryos to assess developmental time points such as time to morula compaction and blastocyst expansion, as well as morula and blastocyst sizes. Overall, developmental rates were not different between IVF and ICSI cycles (blastocyst rate 41.1 % IVF and 41.8 % ICSI, $p > 0.05$). However, development proceeded faster in IVF cycles (blastocyst expansion IVF 155.5 ± 18.5 h; ICSI 167.2 ± 19.6 h; $p < 0.05$) and IVF embryos were also larger (blastocyst area IVF 22608 ± 2857 μm^2; ICSI 20806 ± 1505 μm^2; $p < 0.05$). The faster development and larger size might suggest a more advanced developmental stage. The implications of these findings need to be further evaluated to assess their association with pregnancy potential. The successful developmental rates achieved in IVF cycles demonstrate the potential of this technique for clinical application, although the amount of frozen-thawed semen required is significantly higher in IVF than in ICSI, which is an important consideration for mare and stallion owners. Nonetheless, the use of frozen-thawed semen in equine IVF, coupled with comparable blastocyst rate, presents promising potential for broader clinical adoption of the IVF technique.

1. Introduction

In vitro production of embryos (IVP) in horses has become a routine procedure performed worldwide. Equine IVP enables the production of offspring from mares and stallions previously unable to reproduce using more traditional techniques. With advancements in the procedure and improved outcomes, IVP is no longer limited to subfertile animals. Due to its high success rate, it is now possible to produce more embryos compared to traditional methods, such as breeding and embryo collection [1]. IVP is gaining significant traction in the equine industry, offering new opportunities for genetic advancement and reproductive success. Intracytoplasmic sperm injection (ICSI) is the most commonly used fertilization technique for IVP, largely due to the historical failure of conventional *in vitro* fertilization (IVF) methods in horses [2–4]. However, recent advances in IVF using fresh stallion sperm [5] offer a new perspective on equine assisted reproductive technologies. The inability to capacitate stallion sperm under laboratory conditions was the main reason proposed for previous IVF failures [4], along with

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proposed deficiencies in oocyte maturation and embryo culture conditions. While these issues hindered progress in the past, advancements in IVP have largely overcome the latter challenges.

Male gamete cells are derived from spermatogonial stem cells in the seminiferous tubules in a sequential process that continues throughout the male's reproductive life [6]. At the end of spermatogenesis and spermiogenesis, sperm cells are transported through the epididymis, where they undergo final maturation changes [7]. Once they reach the tail of the epididymis, they become capable of fertilization through natural breeding. Nonetheless, sperm must still undergo capacitation in the female reproductive tract before being capable of fertilizing the oocytes. Capacitation is mainly characterized by the removal of lipids from the sperm membrane along with the acquisition of hyperactivated motility [8]. While the process of sperm capacitation in horses remains poorly understood, prolonged culture in capacitation media before the addition of cumulus-oocyte complexes (COC) has shown success in overcoming this barrier [5,9]. To date, successful embryo production and live foals have been achieved using high-quality fresh sperm [5,10,11], whereas reports of successful IVF from frozen sperm include only cleavage stage zygotes to 16-cells [9,12].

The use of frozen-thawed sperm is a standard practice in commercial IVP, enabling the use of stallions that are geographically distant from the oocyte source. In addition to this, it allows the use of sperm from stallions engaged in sports careers, from which sperm collection is undesired during competition periods. Frozen-thawed sperm also allows the use of sperm from deceased stallions [13]. Cryopreserved sperm also uses minimal amounts of sperm, as it is routine to cut a small piece of the straw per ICSI session [14]. Hence, it is paramount to develop an equally efficient way of producing embryos via IVF using frozen-thawed sperm if the technique is to be used commercially.

Another yet-to-be-determined aspect of IVF is embryo morphokinetics. Embryo morphokinetics refers to the detailed tracking and analysis of embryo development over time using time-lapse imaging technology [15–18]. Non-invasive time-lapse microscopy, which allows for continuous in-incubator monitoring, has emerged as a valuable tool for observing embryo development without disrupting the culture environment [19–21]. This approach allows researchers and clinicians to observe key developmental milestones, such as the timing of cleavage, morula formation, and blastocyst expansion, in near real-time. Traditional monitoring methods, which may compromise embryos by requiring removal from the incubator for periodic microscopic assessment, are limited in their ability to capture the exact timing of developmental events.

One of the primary advantages of embryo morphokinetics is its ability to provide a non-invasive method for assessing embryo quality, as developmental patterns and timing can be correlated with the potential for embryo formation, implantation and successful pregnancy. In human and non-human primates, this method has demonstrated a very high sensitivity in predicting blastocyst formation [22–24]. By monitoring these events without disturbing the embryo, clinicians can make informed decisions on embryo selection, reducing the need for invasive biopsy procedures. For instance, it is known that the timing to reach the two-cell stage and blastocyst formation are associated with embryo viability and the likelihood of successful development [16–18]. However, it remains unclear whether equine embryos produced by IVF follow similar precisely-timed mitotic patterns as those produced by ICSI. Since IVF is likely a more natural method of *in vitro* embryo production compared to ICSI, understanding these developmental dynamics could provide insights not only for optimizing IVF conditions but also for improving the outcomes of ICSI by refining its culture methods and enhancing blastocyst success rates in equine reproductive practices. For these reasons, our goals were 1) to develop an efficient protocol for equine IVF with frozen-thawed sperm and 2) to compare early embryonic development characteristics in blastocysts produced *in vitro* by IVF and ICSI.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

EquiPlus OPU Recovery Medium (Minitube, Verona, WI) was used for equine oocyte aspiration and searching.

2.2. Media preparation

Maturation medium consisted of 36.0 % Global medium (LifeGlobal, Guilford, CT), 54.0 % DMEM/F-12 (Thermo Fisher Scientific, Waltham, MA), 25 μg/mL gentamicin (Thermo Fisher Scientific, Waltham, MA), 0.1 mM sodium pyruvate, 6.0 % fetal bovine serum (FBS), 10 μL/mL insulin-transferrin-selenium solution (Thermo Fisher Scientific, Waltham, MA), 10 % dominant stimulated follicle follicular fluid, 8.8 mU ovine FSH (National Hormone And Peptide Program) and 1.1 mU porcine somatotropin (Harbor-UCLA Research and Education Institute). The culture medium consisted of 54 % DMEM/F-12, 40 % Global® (Cooper Surgical Inc Ballerop, Denmark), 6 % FBS, 10 μl/mL 1 insulin-transferrin-selenium solution and 0.1 mM sodium pyruvate.

For sperm capacitation, all media was prepared as previously described [5]. Fert-Talp (FT) was composed of 114 mM NaCl, 3.2 mM KCl, 25 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.05 mM $MgCl_2$, 10 mM HEPES, 1.5 mM $CaCl_2$, 4.6 mM D-glucose, 10 mM sodium lactate, 0.5 mM sodium pyruvate and 6 mg/mL BSA in UltraPure distilled water. PHE solution was prepared in UltraPure distilled water as previously described [5] and consisted of 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. The solution was stored in Eppendorf tubes with 200 μl aliquots wrapped in aluminum foil at −20 °C.

2.3. Stallion pre-selection

The suitability of all stallions (n = 5) for IVF was tested before use. Stallions were deemed suitable when sperm progressive motility was higher than 20.0 % after 10 h of capacitation in droplets as described in section 2.6.

2.4. Oocyte collection and transportation

The general experimental design is presented in Fig. 1. For IVF, oocytes were obtained by transvaginal aspiration (TVA) as previously described [14,25] by two clinics with ample experience in the technique (Veterinary Teaching Hospital, University of California, Davis, CA and Genetech, Purcell, OK). The oocytes were donated to the Veterinary Assisted Reproduction (VetART) Laboratory of the University of California, Davis, for research purposes. Oocytes were transported to the laboratory after collection and maintained in a temperature-controlled container (MicroQ, Micro Q Technologies, AZ) at 22 °C. All oocytes were held in equine commercial holding media (EquiHold, Minitube USA Inc. Verona, WI) for 24 h before transfer to maturation media.

2.5. Oocyte maturation

After holding, our routine equine IVP protocols were followed as previously described [14,15,25]. Briefly, COCs were transferred to the maturation medium in 25 μL droplets under light mineral oil (Irvine Scientific, Santa Ana, CA) overlay. Oocytes were matured for 31 h at 38.2 °C in a humidified atmosphere of 5.8 % CO_2, 5.0 % O_2, and 89.2 % N_2.

2.6. Sperm capacitation

Frozen sperm from five different stallions was used for IVF

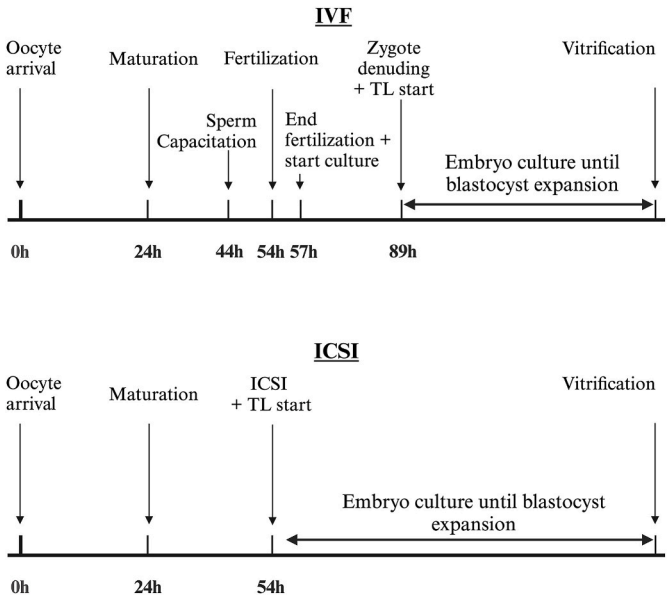

Fig. 1. Experimental design. Timeline of IVF and ICSI cycles from the arrival of oocytes to the clinic until the vitrification after blastocyst expansion. Time is presented in hours. For morphokinetic timepoints time 0 was the end of fertilization in IVF cycles and time of sperm injection in ICSI cycles.

fertilization. Fertilization media was prepared as previously described [5,9]. Medium used were FT and Fert-Talp-PHE (FT-PHE). Briefly, aliquots of PHE solution were thawed at room temperature protected from the light 15 min before media preparation. To create FT-PHE, 120 μL of PHE were added to each 3 mL of FT. Both media were pre-equilibrated at 38.2 °C, 5.0 % CO_2 and atmospheric O_2 in a humidified incubator for a minimum of 2 h. Sperm capacitation and fertilization were performed as previously described [5,9] with minor modifications. Briefly, fertilization plates were prepared with 45 μL droplets of FT-PHE with an embryo grade mineral oil overlay and equilibrated overnight at 38.2 °C, 5 % CO_2 and atmospheric O_2 in a humidified incubator. Frozen straws were thawed in a water bath at 38 °C for 60 s and its contents were expelled in a prewarmed 15 mL conical tube. A total of 600 μL of FT were gently mixed with the thawed sperm. Sperm total motility (TM) was evaluated using computer assisted sperm analysis system (CASA, Hamilton Thorne, Beverly, MA) and only straws with a TM over 40 % were used. Sperm selection was performed with VetMotl™ (Vetmol Multi 850 μL VetMotl, Inc. Gaithersburg, MD) as previously described [26]. Briefly, 850 μL of diluted sperm were loaded into the microfluidic chamber inlet and 750 μL of FT were added to the upper chamber. Devices were incubated for 30 min at 38.2 °C, 5 % CO_2, and atmospheric O_2 in a humidified incubator, after which 500 μL of the upper chamber were collected for further processing. A total of 500 μL of FT-PHE were added to the selected sperm and centrifuged at 300 G for 5 min. The supernatant was removed, and the pellet was diluted in 100 μL of FT-PHE. Sperm concentration was measured and subsequently added to the fertilization droplets at a final concentration of 1 million/mL. Fertilization plates were incubated at 38.2 °C, 5 % CO_2 and atmospheric O_2 in a humidified incubator for 10 h until the addition of matured COCs.

2.7. Fertilization

2.7.1. IVF

After sperm capacitation was performed for 10 h, sperm total motility was analyzed by subjective visual analysis in the fertilization droplets by one experienced operator, ensuring all droplets maintained total motility above 20 %. The post maturation COCs were rinsed in equilibrated FT-PHE and added to the fertilization droplets with capacitated sperm prepared previously. One COC was added per droplet and co-cultured with capacitated sperm for 3 h at 38.2 °C, 5 % CO_2 and atmospheric O_2 in a humidified incubator.

2.7.2. ICSI

For ICSI fertilization, data from our commercial program during the same period as the IVF experiments was collected. Briefly, after maturation, COCs were stripped of cumulus cells by repeated pipetting in 2 % hyaluronidase in G-MOPS (Vitrolife, Gothenburg, Sweden) supplemented with 10 % FBS. The maturation status of the oocytes was assessed by the presence of a polar body within the perivitelline space [14]. Only matured oocytes were injected with frozen-thawed sperm from stallions selected by the respective clients, using conventional needles (COOK Medical, Bloomington, IN). The swim-up technique [27] was used as the sperm selection method.

2.8. In vitro embryo culture

Presumptive zygotes fertilized by IVF were washed in culture media (composition previously described [25]) and placed in 35 μL droplets with an oil overlay and cultured at 38.2 °C under 5.8 % CO_2, 5 % O_2 and 89.2 % N_2 for 32 h. After this, presumptive zygotes were denuded of cumulus cells by repeated pipetting and placed in culture media into the MIRI®TL time-lapse imaging incubator (Esco Technologies, St Louis, MO) and cultured at 38.2 °C under 5.8 % CO_2, 5 % O_2 and 89.2 % N_2. The culture medium was changed after four days of time-lapse (TL) incubation start. The culture was concluded when embryos reached the blastocyst stage as determined by embryo pulsing and the presence of a clear trophectoderm layer [15]. For a subset of 13 oocytes fertilized with two different stallions, denuding was performed at 24 h instead of 32 h to determine the optimal timing of cumulus cell removal. All produced blastocyst were vitrified.

Presumptive zygotes fertilized by ICSI were placed in culture media and incubated in the MIRI®TL time-lapse imaging incubator with the same conditions as previously stated [14,25]. The culture medium was changed four days after ICSI. The embryos were cultured until they reached the blastocyst stage and were subsequently vitrified.

2.9. Morphokinetics

Time-lapse images of all embryos (n = 23) produced by IVF and a subset of 22 randomly selected embryos produced by ICSI were retrospectively analyzed by a trained operator. Images from one of the IVF embryo were blurred, so 22 TL videos were available for final analysis. Time points to morula compaction and blastocyst expansion were recorded. In addition to this, measurements of the diameter and calculated area of the compacted morula and expanded blastocyst were also recorded. Measurements are explained in Fig. 2. Areas were calculated from the two-dimensional measurements and the formula of ellipse area ($A = \pi ab$; where A is the area, and a and b are the two axes of the ellipse, calculated by dividing each diameter in 2). For the compacted morula, measurements of the compacted blastomeres and of the inner zona pellucida were used to determine the area occupied by active blastomeres by subtracting the area of the morula from the area of the inner zona. Both measurements were taken during and after the first pulsing event to determine the changes in the volume.

For expanded blastocysts, measurements were taken at the beginning of blastocyst pulsation and the detection of thinning of the ZP [15]. The diameter and calculated area of the blastocyst, inner and outer zona pellucida, were used to determine the area occupied by active blastomeres within the zona pellucida as well as change in thickness of the zona pellucida caused by the blastocyst expansion.

Maximal area of the outer edge of zona pellucida at blastocyst pulsing was used as embryonic size. Differences between morula, blastocyst and zona pellucida area during contraction and expansion were used to determine pulsing vigor.

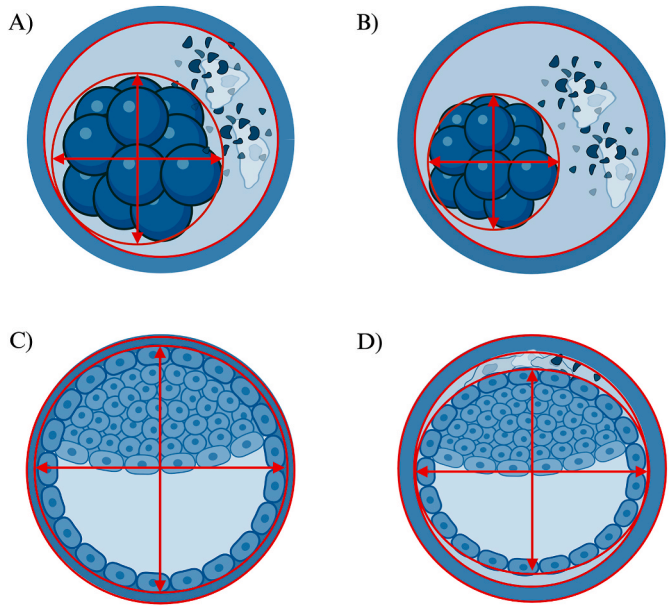

Fig. 2. Measurement of embryonic morphology after IVF and ICSI A) Compacted morula before pulsing. Red circles represent area of morula and inner zona pellucida. Red arrows represent the diameter of the morula. B) compacted morula during pulsing. Red circles represent the area of morula and inner zona pellucida. Red arrows represent the diameter of the morula. C) Expanded blastocyst during pulsing with zona pellucida thinning. Red circles represent the area of the blastocyst, inner and outer zona pellucida. Red arrows represent the diameter of the blastocyst. D) Expanded blastocyst after pulsing. Red circles represent the area of blastocyst, inner and outer zona pellucida. Red arrows represent the diameter of the blastocyst. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.10. Arrested zygote evaluation

Time-lapse recordings from all cycles were evaluated to determine at what developmental stage cleaved zygotes were arrested. The arrest stage was separated into three categories: early arrest in arrested embryos up to eight-cells or before embryonic genome activation [28]; middle arrest for embryos arrested from 16-cells to morula stage; or late arrest when arrested after morula compaction.

2.11. Statistical analysis

Statistical analysis was performed using JMP® 18 (SAS Institute, Cary, NC). Development rates were calculated from IVF and ICSI cycles and presented in percentages. Maturation Rate (MR) is the number of matured oocytes per initial number of oocytes and was only determined in ICSI cycles since oocytes cannot be denuded before fertilization in IVF. Cleavage Rate (CR) is defined as the number of zygotes cleaved/injected oocyte, Cleavage per Total Oocyte Rate (CTO) is the number of cleaved zygotes/initial oocyte number. Cleavage rate was only possible to calculate in ICSI cycles due to the presence of cumulus cells in IVF group during the first 32 h. Blastocyst Rate (BR) was calculated as the number of blastocysts/injected oocytes (for ICSI), whereas Blastocyst per Total Oocytes Rate (BTO) is the number of blastocysts/initial oocyte number. Blastocyst Development Rate (BD) was calculated as the number of blastocyst/cleaved zygotes and was calculated for both fertilization techniques. A Chi-square comparison test with a significance threshold of $p < 0.05$ was performed to compare the development rates of IVF and ICSI cycles.

For time points and volumes obtained by time-lapse analysis, data was analyzed for normal distribution with a Shapiro-Wilk test. Timepoints were normally distributed and were compared between IVF and ICSI cycles with a *t*-test. All the volumes were not normally distributed and thus were compared between IVF and ICSI cycles with a Wilcoxon test. Data is presented in mean ± SD for ease of interpretation. The significance level was set at $p < 0.05$.

3. Results

3.1. Development rates

A total of 109 oocytes were used for the IVF cycles, involving five different stallions. A total of 11 cycles were conducted, with 6–14 oocytes per cycle, and only one stallion used per cycle. Blastocyst rates (blastocyst/initial number of oocytes) per stallion/per cycle are presented in Table 1. On average, we obtained 2.09 embryos per cycle. During the same period, a total of 416 oocytes were retrieved for ICSI using sperm from 16 different stallions over 32 cycles, yielding an average of 2.38 embryos per cycle.

The developmental rates for both IVF and ICSI methods are presented in Table 2. A comparison of these rates revealed no statistical differences between the developmental outcomes of IVF and ICSI cycles ($p > 0.05$).

3.2. Morphokinetics

The time to first blastocyst expansion pulse was significantly shorter in embryos produced by IVF (155.5 ± 18.5 h) than for ICSI embryos (167.2 ± 19.6 h; $p = 0.0287$). We detected a similar trend for the time to morula compaction, though it was not statistically significant (134 ± 17 h for IVF and 143.5 ± 15h for ICSI; $p = 0.0573$).

The area of the morula and the inner zona were larger in the IVF embryos at both the contraction and expansion stages. Moreover, the area inside the inner and outer zona pellucida were larger in the IVF embryos than in the ICSI embryos at the expansion stage of the blastocyst. The measured areas are presented in Table 3. The change in zona thickness at the time of blastocyst expansion was also significantly different, with IVF embryos having a larger difference in zona thickness (903.1 ± 713.6 μm^2) than the ICSI embryos (474.2 ± 363 μm^2; $p = 0.0242$). The rest of the measured parameters were not different between IVF and ICSI embryos ($p > 0.05$).

3.3. Embryonic arrest

The percentage of early embryonic arrest in ICSI cycles was 50.0 % (54/108) whereas in IVF cycles early embryonic arrest was 31.3 % (10/32); $p = 0.059$. Embryos arrested at mid-stage were 40.7 % (44/108) for ICSI and 65.6 % (21/32) for IVF method, $p = 0.01$; and late-arrested embryos were 9.3 % and 3.1 % for ICSI and IVF, respectively ($p = 0.25$).

Table 1
Overall blastocyst rate (blastocyst/total oocytes) per stallion and cycle. Blastocyst rate expressed in percentage of total numbers.

STALLION	NUMBER OF CYCLES	BTO CYCLE 1	BTO CYCLE 2	BTO CYCLE 3	BTO CYCLE 4
A	4	30.0 % (3/10)	25.0 % (2/8)	33.3 % (2/6)	21.4 % (3/14)
B	1	0.0 % (0/7)	–	–	–
C	3	0.0 % (0/12)	57.1 % (4/7)	53.8 % (7/13)	
D	2	25.0 % (1/4)	0.0 % (0/14)	–	–
E	1	7.1 % (1/14)	–	–	–

Table 2
Comparison of overall developmental rates from IVF and ICSI cycles. Maturation rate: matured oocytes/total oocytes; Cleavage/initial: cleaved/total oocytes; Cleavage rate: cleaved/injected oocytes; Blastocyst/initial: blastocyst/total oocytes; Blastocyst rate: blastocyst/injected oocytes; Blastocyst development: blastocyst/cleaved oocytes.

Fertilization	Maturation Rate	Cleavage/Initial	Cleavage Rate	Blastocyst/Initial	Blastocyst Rate	Blastocyst Development
IVF	N/A	51.4 % (56/109)	N/A	21.1 % (23/109)	N/A	41.1 % (23/56)
ICSI	64.2 % (267/416)	43.8 % (182/416)	68.2 % (182/267)	18.3 % (76/416)	28.5 % (76/267)	41.8 % (76/182)

Table 3
Measurements of the area of morula (M), morula inner zona (MIZ), blastocyst (B), blastocyst inner zona (BIZ) and blastocyst outer zona (BOZ) embryos created by IVF and ICSI at contraction (Con) and expansion (Ex) during the first morula compaction pulse and zona-thinning pulse at blastocyst expansion. Measurements are expressed in mean ± SD μm^2. Different superscript letters signify statistical difference within columns.

	M		MIZ		B		BIZ		BOZ	
	Con	Ex	Con	Ex	Con	Ex	Con	Ex	Con	Ex
IVF	8099 ± 1,464[a]	9436 ± 1,629[a]	12,939 ± 1,593[a]	13,324 ± 1,472[a]	10,831 ± 21,28[a]	13,798 ± 2,088[a]	13,527 ± 1,839[a]	14,744 ± 1,900[a]	21,817 ± 2,876[a]	22,608 ± 28,57[a]
ICSI	7152 ± 1,623[b]	7934 ± 1,816[b]	11,844 ± 1,080[b]	12,056 ± 1,049[b]	9701 ± 3,019[a]	11,958 ± 2,383[b]	12,522 ± 12,16[a]	13,388 ± 1,101[a]	20,173 ± 1,959[b]	20,806 ± 1,505[b]

4. Discussion

Fertilization is the first step in embryonic development involving a series of complex events requiring a well-orchestrated machinery within the oocyte, which contains all the necessary organelles to transform two haploid gametes into an embryo. Hence, comparing embryonic development across different fertilization methods can provide valuable insights into their respective impact on embryo development. Here, we observed no difference in the development rates of ICSI and IVF cycles while using frozen-thawed semen for both cases, demonstrating the efficacy of the technique. It is important to note that maturation rates in IVF cycles are challenging to assess, as oocytes are not denuded before fertilization, and presence of polar body cannot be assessed until 32 h post-fertilization when cumulus cells are removed. This limitation prevents the evaluation of fertilization rates, as we cannot determine how many of the matured oocytes are fertilized. Nonetheless, we compared cleavage rates based on the initial oocyte numbers of both techniques, and no significant differences were observed. This could suggest similar fertilization capabilities between the methods, although there remains the possibility that ICSI might induce oocyte activation without successful fertilization [29], a point we will explore further in the discussion.

Interestingly, IVF embryos developed at a faster rate, which could indicate superior embryonic developmental capabilities. Several studies have demonstrated that faster developmental speed in equine ICSI-produced embryos are associated with a higher pregnancy potential [30–32], which could suggest a higher competency of IVF-produced embryos. Caution is warranted in the interpretation of these findings, as no data on pregnancy establishment, early pregnancy loss, or foaling rates is available for IVF-produced embryos in this study. Additionally, long-term studies on the effects of the fertilization technique on the offspring are lacking. Nevertheless, these results mark a promising start in this novel approach.

Notably, IVF embryos were larger at the time of morula compaction and blastocyst expansion, showing a greater change in the zona pellucida thickness while pulsing. The fact that blastocyst size at the contraction stage did not differ, but all parameters were larger during the expansion stage in the IVF group, could suggest that the pulsing event is more vigorous in this group. Pulsing occurs in equine embryos during blastocyst formation in both *in vivo* and *in vitro* embryos [15], and it has been postulated that this phenomenon is due the pumping of fluid into the inner cavity to form the blastocoele and to osmolar changes in the blastocoele fluid [33]. Stronger blastocyst expansion has also been associated with a higher implantation rate in human embryos [34], although the significance for equine embryos remains unknown. Overall, observed differences in size and pulsing vigor between IVF and ICSI embryos, in conjunction with their faster development, could suggest a more advanced development stage in IVF embryos. However, further studies are required to compare the developmental stages at a molecular level and the timing of cell lineage differentiation between IVF and ICSI embryos.

Early embryonic development relies on maternal RNAs and proteins accumulated in the ooplasm of the oocyte during oocyte growth and maturation in the form of mitochondrial-associated granules [35] and subcortical-maternal complexes [36], while the embryonic genome remains relatively silent [37]. The maternal-embryonic transition in horses has been described to occur at the four-to eight-cell stage, with a minor wave of transcription occurring at the four-cell stage and final embryonic gene expression being established at the eight-cell stage [28,38]. For this reason, in horses, as well as in other species, this is a critical stage during *in vitro* culture, and a high percentage of embryos are arrested due to the incapability to undergo the oocyte-to embryo transition [39]. Our study shows a distinct difference in arrest timing between fertilization methods, with ICSI embryos showing similar arrest rates before and after embryonic genome activation, whereas IVF embryos primarily arrest at later stages. This could be attributed to differences in oocyte activation or genome activation. In humans, the leading cause of fertilization failure in ICSI cycles is incomplete oocyte activation, while in IVF cycles, it is sperm penetration failure [40,41]. Oocyte activation is modulated by sperm factors such as PLCZ1, which, upon contact with the ooplasm, produce characteristic calcium oscillations that initiate the cascade of events that resume meiosis and allow for fertilization to occur [8,42,43], even in ICSI cycles [8]. According to our data, cleavage rates are similar, suggesting that total fertilization failure rates might not differ between ICSI and IVF cycles in horses. If both techniques achieve similar fertilization rates, the downstream effects of the fertilization method might have an influence on the ability of the newly formed zygote to initiate genomic activation, which might suggest a technique or male effect in the zygotic-embryonic transition. One striking difference between IVF and ICSI is the method of sperm selection, as the sperm penetrating the oocyte is subjectively chosen by the operator in the case of ICSI, whereas in IVF cycles, there is a natural selection. Sperm DNA fragmentation is known to impact early embryonic development, embryo quality, and increase embryonic arrest rates [44,45], and other sperm-derived factors, such as mRNAs and centrioles, are also essential for proper embryonic development [46,47]. The effect of the fertilization method could also result from mechanical damage to the oocyte during ICSI. This technique often involves the aspiration of some of the ooplasm before injecting the sperm cell, which could disrupt the subcortical maternal protein complexes that are critical for zygotic

genome activation [36]. Disruption caused by the injection and ooplasm aspiration may impair the zygote's ability to transition from maternal to embryonic gene expression, thus hindering development at the genome activation stage. Further studies in horses are needed to investigate the presence and location of protein aggregates and to determine whether ICSI affects these critical factors. The influence of fertilization method on oocyte organelle damage, oocyte activation, and embryonic genome activation need further investigation to determine if they are the cause of different arrest patterns in ICSI versus IVF cycles.

Regarding the stallion effect, it is essential to note the high variability in success rates not only between different stallions but also within the same stallion across multiple trials (Table 2). This significant variation underscores the importance of initial sperm quality. Previous studies on IVF using fresh semen with prolonged capacitation reported similar findings, where acceptable fertilization rates were achieved only with high-quality sperm processed immediately after collection [5]. That study also emphasized the need for frequent collection and precise semen handling techniques to optimize results. Our findings similarly indicate a requirement for high-quality sperm, as evidenced by variability between different straws/cycles of the same stallion, as well as stallion-to-stallion variability. These factors are critical, as they present challenges to the generalized application of this technique, supporting a stallion-specific approach.

It is also important to note that the IVF protocol was established within a fully functional commercial ICSI laboratory, suggesting that the technique may be successfully applied in similar settings. However, the developmental rates within a newly established laboratory remain to be determined. The commercial viability of the technique will also depend on sperm availability and stallion efficiency.

5. Conclusion

Overall, we report the first successful production of IVF-derived blastocyst in horses using frozen semen following prolonged sperm capacitation. We found a similar success rate between IVF and ICSI using frozen-thawed sperm, with IVF embryos exhibiting faster progression to the blastocyst stage. Further studies are required to assess the pregnancy potential of these embryos before IVF can be fully established as a commercial tool. Nonetheless, IVF in horses is a promising technique that offers valuable opportunities to study fertilization in an *in vitro* setting.

CRediT authorship contribution statement

Soledad Martin-Pelaez: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Alejandro de la Fuente:** Writing – review & editing, Data curation. **Kazuki Takahashi:** Writing – review & editing, Data curation. **Itzel Tirado Perez:** Writing – review & editing, Investigation. **Jazmin Orozco:** Writing – review & editing, Investigation. **Carolina T.C. Okada:** Writing – review & editing, Resources. **Carlos Ramires Neto:** Writing – review & editing, Resources. **Stuart Meyers:** Writing – review & editing, Conceptualization. **Pouya Dini:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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