

Original Research Article

Transcriptomic profiling reveals similarities between equine IVF and ICSI embryos

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ABSTRACT

In vitro production of equine embryos has been performed using intracytoplasmic sperm injection (ICSI) for the last two decades. Since 2022, a repeatable protocol for conventional *in vitro* fertilization (IVF) provides a successful alternative. However, little is known about the influence of the fertilization method on embryo quality and the transcriptomic profile. In this study, we aimed to examine differentially expressed genes (DEGs) between ICSI and IVF embryos in the horse. Therefore, ten equine sibling blastocysts, produced *in vitro* by either ICSI or IVF from three different mares, were subjected to Full-Length Single-Cell RNA-Sequencing (FLASH-sequencing). As such, 11,518 genes were identified, with no DEGs between ICSI and IVF embryos. Cleavage rates, calculated on collected COCs, of IVF zygotes (55.0 %) were similar to those of ICSI zygotes (51.9 %; $P = 0.74$), but blastocyst rates were higher following ICSI (37.0 % vs 22.5 % calculated on collected COCs and 71.4 % vs 40.9 % calculated on cleaved zygotes; $P = 0.04$ and 0.004 , respectively). The average day of blastocyst development did not differ ($P = 0.55$). In conclusion, gene expression was similar for the two fertilization techniques, supporting the safety of equine IVF for further clinical studies. Overall, the horse provides a valuable model to study long-term effects of assisted reproductive technologies with potential extrapolation to human medicine.

1. Introduction

The use of assisted reproductive technologies (ART) has exponentially increased in the last decades in many species, including the horse [1–4]. Aside from artificial insemination and *in vivo* embryo flushing, *in vitro* production (IVP) of embryos has become immensely popular between breeders. Although conventional *in vitro* fertilization (IVF) had been first performed experimentally in 1990 [5], this technique was not reproducible. Thus, until recently, only ovum pick-up (OPU) followed by

intracytoplasmic sperm injection (ICSI) could be used to produce IVP embryos in the horse. In 2022, Felix et al. unveiled the secrets for successful conventional IVF in the horse, with prolonged incubation of semen in capacitating conditions being the key factor in this process [6]. This protocol has been shown to be repeatable in other labs and was further optimized for IVF with frozen-thawed semen [7,8]. Comparing developmental outcomes between ICSI and IVF showed variable results, with similar cleavage and blastocyst rates demonstrated by Martin-Peleaz et al. [9]. We also found similar blastocyst rates per

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cleaved embryo, while blastocyst rates per collected oocyte were slightly higher after IVF [10]. Another recent publication, however, found the opposite: lower blastocyst rates per cleaved embryos after IVF compared to ICSI [11]. These variabilities could be due to a stallion effect, since both stallion identity and freezing extender affect fertilization rates after IVF [6]. Another explanation could be differences in laboratories, since the IVF technique is fairly new and protocols are constantly being optimized. In human ART, ICSI provides higher fertilization and blastocyst rates than IVF in cases of male infertility [12,13]. A recent study, however, suggested that IVF should be the preferred method of IVP in humans for patients with normal or non severely decreased sperm quality. They reported higher fertilization rates after IVF, no differences in cumulative live birth rates after IVF compared to ICSI, and IVF being more cost-effective and less invasive [14].

Morphokinetic studies have demonstrated that human embryos produced by IVF develop faster and show a larger expansion at the blastocyst stage than ICSI embryos [15]. Also in equine IVF embryos, morphokinetics revealed earlier cleavage and bigger blastocyst expansion compared to embryos derived by ICSI [9]. This could reflect better embryo quality, since morphokinetic selection of embryos in human ART favor fast developing embryos [16]. Indeed, fast cleaving zygotes are correlated with a higher potential for embryo formation, implantation and successful pregnancy [16–20]. Recently, we demonstrated that aneuploidy rates do not differ between equine IVF (1/17 aneuploid) and ICSI (2/18 mosaic) embryos, implying that IVF embryos may have a similar chance of successful pregnancy [10].

To further explore similarities and differences between equine IVF and ICSI embryos, transcriptomics could provide information about activated and differentially expressed genes (DEGs). In the horse, little is known about transcriptomics of IVP embryos. Studies on transcriptomic profiling of equine embryos by RNA-sequencing are mostly limited to *in vivo* embryos [21–23]. Previous studies exploring limited numbers of genes indicate differences between IVP and *in vivo* derived equine embryos and subtle effects of the embryo culture environment [21,24–26]. When Fedorka et al. [27] compared placentas of ICSI-produced pregnancies to *in vivo* produced pregnancies, 1589 DEGs were found. ICSI-pregnancies were associated with pathways relating to hypoxia and ribosome biogenesis [27]. In other species, gene expression between IVF and ICSI embryos is limited to two studies in mice. Between 200 and 1000 DEGs were found in murine blastocysts derived by either ICSI or IVF [28,29]. As such, no publications have investigated the transcriptomic differences between equine IVF and ICSI embryos yet. This

could further provide information about the embryo quality, gene expression and chances of a successful implantation and pregnancy.

Full-Length Single-Cell RNA-Sequencing (FLASH-seq) is a new generation sequencing technique based on Smart-seq 2 [30,31]. The protocol is fast, sensitive and easy-to-use, and can be performed with or without unique molecular identifiers (UMIs) [32]. To assess the transcriptomic profile of equine embryos, FLASH-seq could be used to explore the mRNA diversity and gene expression profiling.

The hypothesis of this study was that RNA-sequencing may reveal subtle differences in transcriptome between equine ICSI and IVF embryos, correlating to their underlying embryo quality.

2. Materials and methods

2.1. Ovum pick-up

A graphical abstract showing the experimental design is presented in Fig. 1. One Warmblood (15 yo, V), one Belgian trotter (16 yo, R) and one Quarter Horse (5 yo, T), were regularly monitored by transrectal ultrasonography (7.5-MHz transducer, transrectal linear probe, MyLabOne, Esaote, Genoa, Italy) to assess the follicular count. When ten or more follicles, with sizes varying between 5 and 25 mm, were counted, the mares were selected for OPU. The OPU-procedure was performed according to our standard protocols, as described by Van den Branden et al. [33]. Just before performing OPU, benzylpenicillin (Penikel®, Kela, Sint Niklaas, Belgium, 20,000 IU/kg, IM) and flunixin meglumine (Wellicox®, Ceva Santé Animale, Naaldwijk, The Netherlands, 1.1 mg/kg, IV) were administered. During the whole procedure, the mares were under sedation of detomidine hydrochloride (Domidine®, Eurovet Animal Health BV, Bladel, The Netherlands, 0.01 mg/kg, IV) and butorphanol tartrate (Dolorex®, MSD Animal Health, Sint-Lambrechts-Woluwe, Belgium, 0.01 mg/kg, IV). To counter intestinal contractions, N-butylscopolammonium bromide (Buscopan®, Boehringer Ingelheim, Brussel, Belgium, 0.3 mg/kg, IV) was administered intravenously. Before inserting the transvaginal probe (Esaote EC123 Intra CavityA; Esaote, Italy), the perineal region was cleaned at least three times using 7.5 % polyvidone iodine antiseptic soap (Iso-Betadine Germicidal Soap 7.5 %; Viatrix Healthcare, Pennsylvania, United States). The ovary was manipulated transrectally and fixed against the vaginal wall. A 12 G x 25 inch double-lumen needle (Minutube, Germany), which was in connection with a prewarmed (38.2 °C) collection bottle, was guided into the follicles. All visible antral follicles

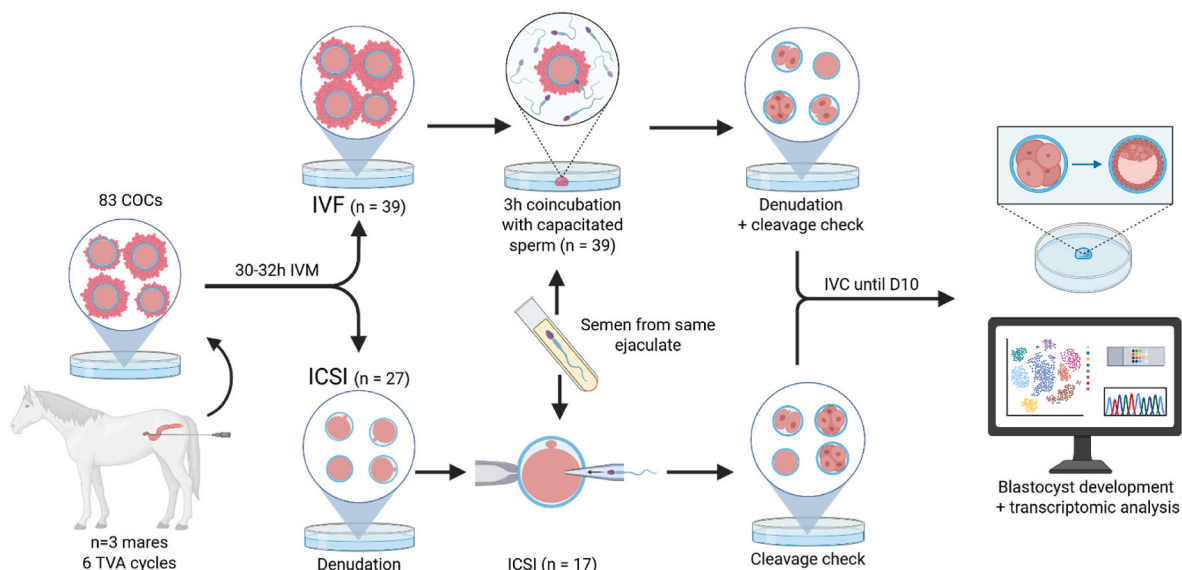


Fig. 1. Graphical abstract depicting the experimental design of this study, created with <https://BioRender.com>.

were punctured, aspirated, and flushed ten times with 1–3 mL of pre-warmed (38.2 °C) flushing medium (Equiplus®, Minitube, Tiefenbach, Germany) while rotating the needle, collecting all follicular content in the prewarmed bottle.

2.2. *In vitro* maturation

The whole content of the collection bottle was poured through a sterile 70 µm oocyte filter (Cell strainer, BD Biosciences, Falcon®, Erembodegem, Belgium) and the cumulus-oocyte complexes (COCs) were searched using a stereomicroscope (Olympus SZX7®, Olympus Corp., Japan) under a laminar air flow. The COCs were collected in Medium 199 with Hank's salts (Gibco, Life Technologies, Merelbeke, Belgium) with 10 % fetal bovine serum (FBS, Gibco). The collected COCs were transferred to maturation medium (Medium 199 with Earl's salts (Gibco) containing 10 % (v/v) FBS (Gibco), 9.4 µg/mL follicle stimulating hormone, 1.88 µg/mL luteinizing hormone (Stimufol®, Reprobiol, Ouffet, Belgium)) and 50 µg/mL gentamycin (Sigma – G1264). *In vitro* maturation was conducted in groups of 8–12 COCs in 500 µL maturation medium under light-weight oil (CooperSurgical, Venlo, The Netherlands) at 38.2 °C in 5 % CO₂ in air for 30–32 h [34].

2.3. Semen preparation

For all fertilization techniques, semen from the same stallion of proven fertility was used.

2.3.1. Fresh semen

Semen was collected on a dummy mount using an artificial vagina (Modified Colorado model, Ghent University, Merelbeke) and processed immediately. The seminal plasma was separated from the semen, after which the gel free portion was extended 1:1 (v:v) with prewarmed (38 °C) extender (INRA96®, IMV Technologies, Maple Grove MN, USA). Using computer assisted sperm analysis (CASA) and a Nucleocounter SP100 (ChemoMetec, Allerød, Denmark), quality parameters were assessed (concentration: 600–780 × 10⁶/mL; total motility: 65–85 %; progressive motility: 30–50 %).

For sperm capacitation, the protocol of Felix et al. (2022) was slightly adjusted for practical reasons. For sperm selection, 200 µL of extended semen was layered under 1 mL of prewarmed G-MOPS (38.2 °C). A swim-up was performed under an angle of 45° at 38.2 °C in air for 20 min. The uppermost 750 µL of the medium was collected and centrifuged at 400×g for 3 min. The sperm pellet was resuspended in 495 µL of Hanks Balanced Salt Solution (HBSS) with calcium and magnesium (Thermo Fisher, Scientific, Massachusetts, USA). The suspension was centrifuged, the supernatant was removed, and the sperm pellet (20–30 µL) was resuspended in equilibrated Fertilization Tyrode's albumin lactate pyruvate (FERT-TALP) supplemented with penicillamine, hypotaurine and epinephrine (FT-PHE) [6] to ~10 million sperm cells/mL. Of this suspension, 5 µL was added to pre-equilibrated 45 µL droplets of FT-PHE (final concentration 1 million/mL) and the droplets were incubated in 5 % CO₂ in air for 22 h.

For ICSI, 200 µL of fresh semen was suspended in 1 mL of preheated (38.2 °C) G-MOPS (Vitrolife, Londerzeel, Belgium) and centrifuged at room temperature (400 g for 3 min at 22 °C). The supernatant was removed, and the pellet was resuspended with 1 mL of G-MOPS and centrifuged a second time. After removal of the supernatant, the sperm suspension was dissolved in 200 µL of G-MOPS. Immediately prior to ICSI, a small amount of the sperm pellet was added to a 5 µL droplet of 7 % polyvinylpyrrolidone (CooperSurgical, Venlo, The Netherlands).

2.3.2. Frozen semen

For frozen semen, the same batch of one ejaculate was used (collection as described above: concentration 770.8 × 10⁶/mL; total motility: 82 %; progressive motility: 46 %). The extended semen was poured into a 50 mL Falcon tube (Thermo Fisher, Scientific,

Massachusetts, USA) and centrifuged at 250×g for 20 min at RT. The supernatant was removed and the pellet was re-suspended in a cryo-protectant (Botucris, BotuPharma, Phoenix, AZ, USA) to a final concentration of approximately 150 × 10⁶/mL. The semen was loaded into 0.5-mL straws at room temperature (RT; 22 °C) and placed horizontally on a rack, after which it underwent a slow freezing process in a computer freezing machine (IceCube 1810; Sy-Lab, Purkersdorf, A). Straws were stored in a container filled with liquid nitrogen (–196 °C) until further use. Post-thaw quality parameters were assessed again using CASA and the Nucleocounter SP100, with a total motility of 81 % and progressive motility of 37 %.

For sperm capacitation, 250 µL of frozen semen (1/2 straw of 500 µL) was thawed for at least 30 s in 4 mL prewarmed (38.2 °C) FT-PHE. Of this suspension, 1 mL was placed into the loading chamber of a Vet-count™ Harvester (MotilityCount ApS, Copenhagen, Denmark) loaded with 800 µL of FT-PHE in the collection chamber. The microfluidics device was incubated in 5 % CO₂ in air for 30 min at 38.2 °C, after which the fluid in the collection chamber, including the semen that migrated through the porous membrane, was collected and centrifuged for 3 min at 400g at RT. The supernatant was removed and the pellet was resuspended in FERT-TALP to ~10 million sperm cells/mL [6]. Of this suspension, 5 µL was added to pre-equilibrated 45 µL droplets of FT-PHE (final concentration 1 million/mL) and the droplets were incubated in 5 % CO₂ in air for 10 h.

For ICSI, a small piece of a straw with frozen semen was thawed in 1 mL of preheated (38.2 °C) G-MOPS and centrifuged (400 g for 3 min, RT). The supernatant was removed, and the pellet was resuspended with 1 mL of G-MOPS and centrifuged a second time. After removal of the supernatant, the sperm suspension was dissolved in 200 µL of G-MOPS. Immediately prior to ICSI, a small amount of the sperm pellet was added to a 5 µL droplet of 7 % polyvinylpyrrolidone.

2.4. Fertilization and culture

After maturation, oocytes from each mare were split in two random groups and subjected to either IVF or ICSI. Both fertilization techniques were executed on the same moment. A total of six OPU-sessions (two replicates/mare) was performed. For mare R, fertilization was performed with fresh semen, for mares T and V frozen-thawed semen was used.

For IVF, COCs were washed in pre-equilibrated FERT-TALP and transferred to the FT-PHE droplets containing 1 million/mL capacitated sperm cells. After 3 h of co-incubation, the COCs were individually transferred to 15 µL droplets of Global® Medium (Coopersurgical, Venlo, The Netherlands) supplemented with 10 % FBS. After 36 h, the zygotes were mechanically denuded from their cumulus cells and checked for cleavage. Cleaved embryos were individually transferred to new droplets of 5 µL of Global® Medium supplemented with 10 % FBS. On the 5th day after fertilization, the embryos were cultured in groups of 5–8 in 20 µL droplets of DMEM/F-12 (Gibco) with 10 % FBS and 50 µg/mL of gentamycin (Sigma – G1264) under oil at 38.2 °C in a humidified atmosphere of 5 % O₂, 6 % CO₂ and 89 % N₂. The blastocyst development was assessed daily from day 7–10 post-fertilization, determined on the first day of visible trophectoderm alignment.

For ICSI, the oocytes were denuded in 0.1 % hyaluronidase (Sigma-Aldrich, Bornem, Belgium) in Medium 199 with Hank's salts with 10 % FBS and those showing an extruded polar body were injected by piezo drill assisted ICSI (PrimeTech, Nakamukaihara Tsuchiura Ibaraki, Japan; speed 3–4, intensity 6–8). The presumptive zygotes were cultured individually in 15 µL droplets of Global® Medium supplemented with 10 % FBS under oil at 38.2 °C in a humidified atmosphere of 5 % O₂, 6 % CO₂ and 89 % N₂. After 36 h, the zygotes were checked for cleavage and individually transferred to new droplets of 5 µL of Global® Medium supplemented with 10 % FBS. Five days post ICSI, the presumptive zygotes were cultured in groups of 5–8 in 20 µL droplets of DMEM/F-12 with 10 % FBS and 50 µg/mL of gentamycin under oil at

38.2 °C in a humidified atmosphere of 5 % O₂, 6 % CO₂ and 89 % N₂. The blastocyst development was assessed daily from day 7–10 post-fertilization. Early blastocysts were collected at the same developmental stage for both IVF and ICSI.

2.5. Statistical analysis

OPU-ICSI session data were manually collected and transferred to a Microsoft Excel (Microsoft Corporation, Redmond, WA) work file. These data were explored, organized, and classified in Microsoft Excel, followed by analysis in IBM SPSS Statistics, Version 29.0.2.0 (Armonk, NY: IBM Corp). Groups were tested for normality using the Shapiro-Wilk test, showing a non-normal distribution for all outcomes. A Mann-Whitney *U* test was used to compare cleavage and blastocyst outcomes between groups. A *P*-value with *P* < 0.05 was considered significant and tending toward significance at *P* ≥ 0.05 and *P* ≤ 0.1.

2.6. FLASH-seq and bioinformatic treatments

Blastocysts were washed in Phosphate Buffered Saline (PBS) and individually transferred to DNA-free tubes containing 2 µL Dulbecco's Phosphate-Buffered Saline (DPBS). The tubes were SNAP-frozen by immersing them in liquid nitrogen and stored at –80 °C until single-embryo analysis at the Department of Developmental and Stem Cell Biology, Institut Pasteur, Université Paris Cité, France.

Library preparation was made according to Festuccia et al. [31], based on the FLASH-seq method [30]. Single-end sequences (50–85 bp, using 50-bp sequencing kits) were generated using Illumina Next-seq 2000 instruments. After trimming Illumina adapter sequences with cutadapt, reads were split in UMI-containing or not with Julia [35]. For UMI, reads were further processed in Julia to remove the invariant adapter sequence, extract the identifier, and the UMI/transcript sequence pairs used to remove duplicates. In this study, the fraction of unique reads ranged between 69 and 90 %.

Conventional and UMI reads were mapped to the transcriptome reference genome (Ens113, EquCab3.0) using STAR (v2.7.10b). Tables of counts were generated with featureCounts (v1.6.0). Analysis of differential expression was made using the DESeq2 package (v1.28.1) with a model taking into account the fertilization method and the mare from which oocytes have been obtained [36]. Genes were considered as differentially expressed (DEG) if adjusted *P*-value < 0.05. Before to proceed to the computation of the Principal Component Analysis (PCA), the count table has been transformed using rlog function of DESeq2 package. Then we used the PCA method proposed in mixOmics package, as it allows to take into account the mare effect (multilevel parameter proposed in PCA function) [37]. Gene Set Enrichment Analysis (GSEA version 4.3.2) was carried out on the normalized expression values of genes to identify enriched biological pathways using gene sets from GO databases (BP, MF and CC), KEGG and hallmark (from MSigDB collections, v2024). The method used for GSEA was gene set permutation. Gene sets were considered as significantly enriched if FDR was below 5 %. Data are available at the Gene Expression Omnibus (GSE2 308657).

3. Results

3.1. Embryo development

Six OPU sessions resulted in 116 aspirated follicles and 83 retrieved oocytes (recovery rate: 71.6 %). Of these 83 COCs, 40 were subjected to IVF and 27 were used for ICSI. The remaining 16 COCs were used for other purposes and not included in the calculations. The COCs used for ICSI had a maturation rate of 63.0 % (17/27). Since IVF was performed on COCs, as cumulus cells are proven necessary for the acrosome reaction [6], maturation rates could not be assessed. Cleavage rate/collected COCs did not differ between ICSI (51.9 %) and IVF (55.0 %; *P* = 0.74). Blastocyst rate/cleaved zygotes were higher for ICSI than IVF cycles

(71.4 % vs 40.9 %, respectively; *P* = 0.004) and blastocyst rate/collected COCs were also higher for ICSI cycles compared to IVF (37.0 % vs 22.5 %, respectively; *P* = 0.04). The descriptive data per mare are shown in Table 1 and Fig. 2. The average day of development showed no differences between ICSI (7.6) and IVF (7.8; *P* = 0.55) (Table 2).

3.2. Transcriptomic profiling

In total, five sibling pairs of embryos were included for transcriptome analysis, using the whole embryo for single-embryo analysis. The selected embryos were one ICSI and one IVF embryo from mare R fertilized with fresh semen, one ICSI and one IVF embryo from mare T fertilized with frozen-thawed semen, and 3 ICSI and 3 IVF embryos from mare V, also fertilized with frozen-thawed semen (Table 1). FLASH-seq libraries showed high-quality sequencing, with the majority of bases having Phred scores ≥ 30, GC content between 46 and 50 %, and 6–27 million reads per sample. The PCA revealed that the first two principal components (axes 1 and 2) explained the largest proportion of the total variance, primarily reflecting inter-mare differences (Fig. 3B). When examining axes 3 and 4 (Fig. 3C), a clearer separation between IVF and ICSI embryos became apparent, albeit to a lesser extent than the mare effect. A total of 11,518 expressed genes were identified across all samples (Supplemental Table 1). No DEGs were detected (adjusted *P*-value > 0.05). When mare R was excluded, so only embryos created with frozen-thawed semen were included, no DEGs were detected either. To identify coordinated gene expression changes, gene expression datasets were analyzed globally using GSEA (Fig. 4A). One positive enrichment of protein secretion pathway was identified in ICSI embryos (Fig. 4B). Several negative enrichments of pathways linked to ribosome were identified in ICSI embryos (representative enrichment plot for is shown in Fig. 4C).

4. Discussion

In vitro production of equine embryos has seen a tremendous increase in the last fifteen years [4]. The recent discovery of a repeatable protocol for conventional IVF in horses may provide an alternative complementing ICSI. To perform this procedure clinically, however, multiple factors need to be assessed and optimized. Aside from the protocol and the stallion effect, IVF should also support consistent embryo production rates and embryo quality, underlying optimal pregnancy and foal health. Few studies assessing embryo quality, indicate high similarities between embryos generated by ICSI or IVF. Morphological quality, based on the grading system by Morris et al. [38], showed no differences between ICSI and IVF embryos [9]. Morphokinetic analysis revealed that IVF embryos showed faster cleavage but similar blastocyst development rates were achieved compared to ICSI [9]. In this study, the average day of blastocyst development was similar for both IVF and ICSI. Further genomic assessment revealed overall high euploidy rates of blastocysts with no differences between the fertilization techniques [10].

To the best of our knowledge, no transcriptomic profiling has been performed to unravel potential differentially expressed genes between ICSI and IVF embryos in the horse. This study demonstrated that none of the 11,518 expressed genes was differentially expressed. GSEA analysis revealed low functional enrichment, which is consistent with the differential expression analysis. The GSEA results may indicate an upregulation of translation and protein biosynthesis in IVF-embryos, possibly linked to the presence of sperm cells attached to the zona pellucida. Yet, they may also reflect distinct biological processes to be differentially regulated between IVF- and ICSI-derived equine embryos. The PCA reveals similar results, with a minor part of the variance related to the fertilization method, but an overall dominance of the mare effect, which may mask subtle transcriptional differences associated with the fertilization technique.

These results could possibly be extrapolated to human IVP, since the

Table 1

Descriptive results from 6 OPU-fertilization sessions in three mares (R, V and T).

Mare	Fertilization	N° COCs	N° MII	MIH rate	N° cleaved	Cleaved/ MII	Cleaved/ COCs	N° blast	Blast/MIH	Blast/ COCs	Blast/ Cleaved	N° blast for RNA-seq
R	ICSI	6	5	83.3 %	5	100 %	83.3 %	2	40.0 %	33.3 %	40.0 %	1
	IVF	13	–	–	6	–	46.2 %	2	–	15.4 %	33.3 %	1
V	ICSI	12	7	58.3 %	4	57.1 %	33.3 %	3	42.9 %	25.0 %	75.0 %	3
	IVF	19	–	–	9	–	47.4 %	5	–	26.3 %	55.6 %	3
T	ICSI	9	5	55.6 %	5	100 %	55.6 %	5	100 %	55.6 %	100 %	1
	IVF	7	–	–	7	–	100 %	2	–	28.6 %	28.6 %	1

COCs = collected immature cumulus-oocyte complexes. MII = matured oocytes shown an extruded polar body. Blast = blastocyst.

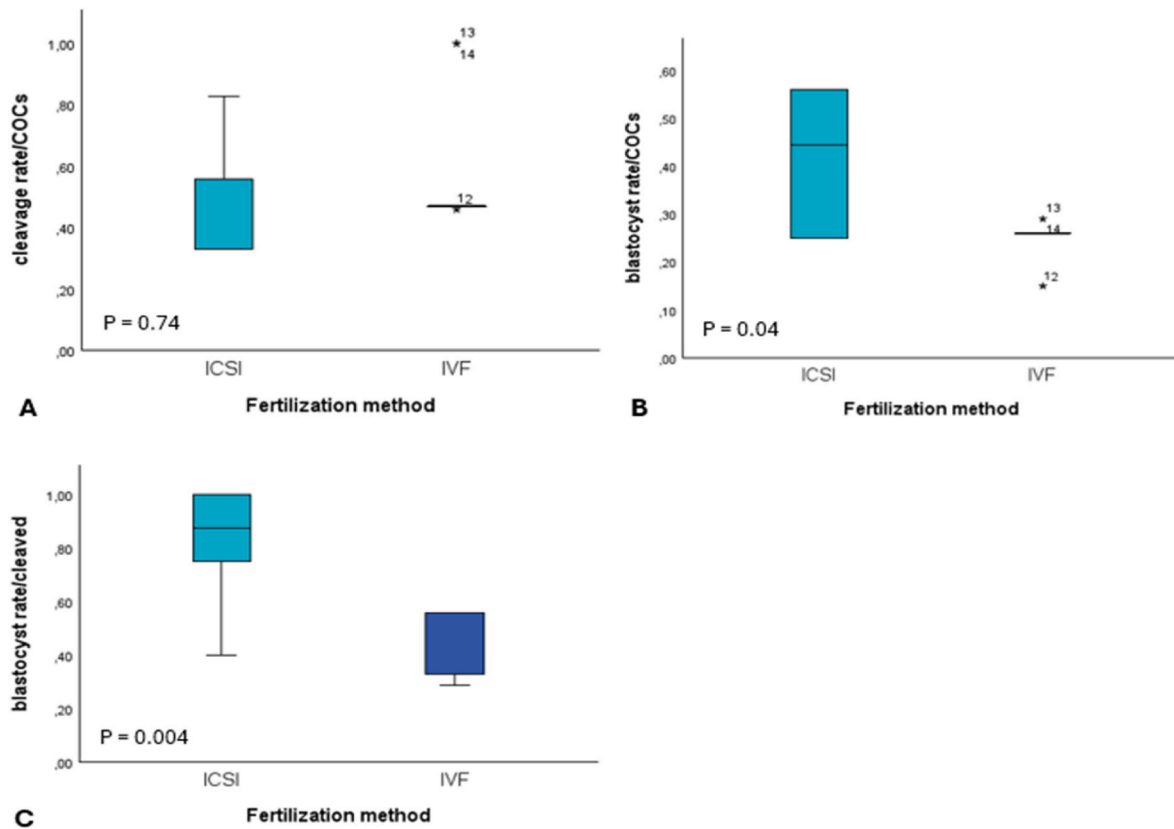


Fig. 2. Boxplots of the developmental rates of 6 OPU-sessions of 3 mares. Cleavage rate/COCs (A) was not different between IVF and ICSI, blastocyst rates calculated on COCs (B) and on cleaved zygotes (C) were significantly different. P-values are indicated on each graph.

Table 2

Day of blastocyst development of embryos produced either by OPI-ICSI or OPU-IVF. No differences were detected between the mean of IVF and ICSI embryos (P = 0.55).

Fertilization	Day 7	Day 8	Day 9	Day 10
ICSI	6	3	0	1
IVF	4	4	0	1

mare can serve as a good model for (aging) women [39,40]. Creating sibling embryos from human oocytes could be ethically questionable and impractical. In humans, IVP is solely used to overcome subfertility, and the preferred technique is selected according to the underlying pathology in couples [14]. Thus, equine models could provide insights in the fundamental molecular differences related between IVF vs ICSI. More research is needed to further unravel genetic and epigenetic variances between both equine IVF and ICSI embryos and the possibility to translate this to human ART.

Of note were the lower blastocyst rates in IVF sessions compared to

ICSI, either calculated on collected COCs (22.5 % vs 37.0 %, respectively; P = 0.04) or on cleaved zygotes (40.9 % vs 71.4 %, respectively; P = 0.004). Cleavage rates calculated on collected COCs did not differ according to the fertilization method (P = 0.74), neither did the day of blastocyst development (P = 0.55). These data confirm the findings of Ramírez-Agámez et al. [11], but contradict other reports [9,10]. However, the study of Broothaers et al. (2025) was performed on slaughterhouse-derived oocytes with substantially lower maturation and ICSI outcomes. Lab and stallion effects can also not be excluded, since IVF-outcomes have disperse outcomes when different studies are compared [6,9,11]. So far, only a few studies compared developmental outcomes of IVF vs ICSI, indicating overall similar capability of blastocyst development for both techniques and the necessity of more research in this topic.

Outcomes with IVF could also be influenced by mare and stallion effects. Since the present study only used one stallion for all fertilizations, the male influence could not be examined. Recent literature, however, suggested evidence for a stallion effect [7,11], but more studies with larger populations are needed. In any case, the mare factor is also important to take in consideration. This study tried to rule out a

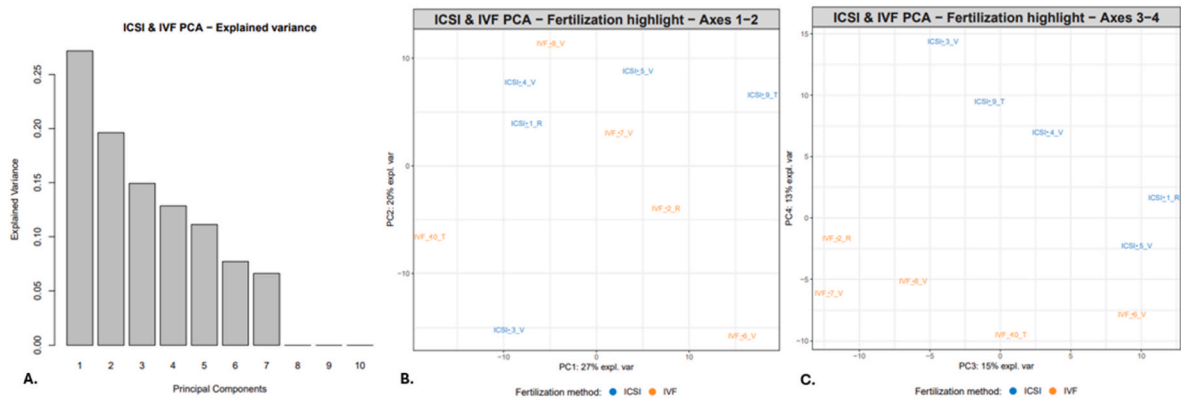


Fig. 3. Principal component analysis (PCA) of the transcriptomic profiles of IVF- and ICSI-derived equine embryos. A.: Scree plot showing the proportion of explained variance for each principal component. B., C.: The variance along axes 1 and 2 was largely driven by inter-mare differences, whereas fertilization method contributed to variance along higher-order components. PCA plot based on axes 3 and 4, illustrating partial separation between IVF (orange) and ICSI (blue) embryos. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

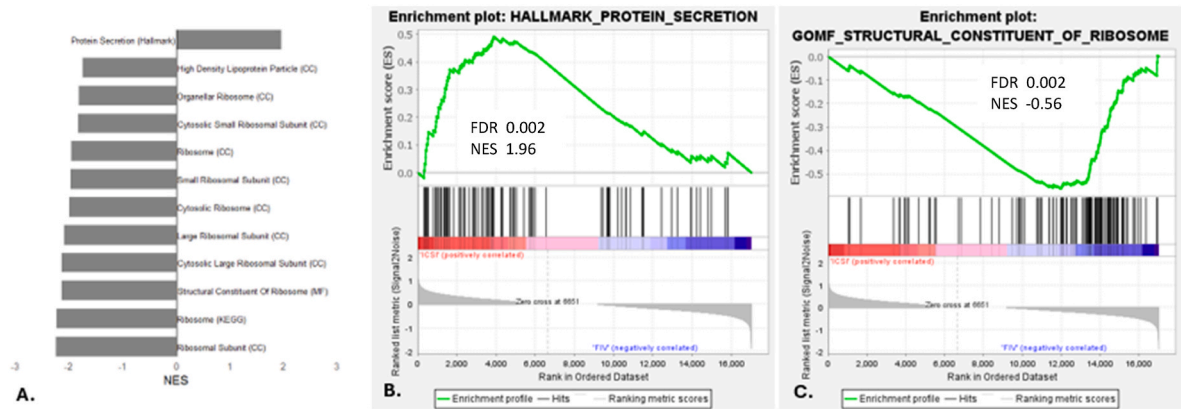


Fig. 4. Gene Set Enrichment Analysis. A.: Results of GSEA GO Cellular Component (CC), GO Molecular Function (MF), KEGG and hallmark showing enriched gene sets (FDR<0.05). A positive Normalized Enrichment Score (NES) value indicates enrichment ICSI embryos, a negative NES indicates enrichment in FIV embryos. B., C.: Enrichment plots for representative pathways.

possible influence of the mare effect by creating sibling blastocysts from three mares. When comparing the PCA, there was a visible mare effect on the transcriptome profile, which was more explicit than the subtle variation observed between fertilization techniques (Fig. 3). This could be attributed to genetic differences between the mares, but to confirm this, larger numbers should be used which was beyond the scope of current study. It has already been demonstrated in multiple species that age is an important factor regarding embryo quality, either *in vivo* or *in vitro* [23,41–43]. Here, the research mares ranged from five to sixteen years old, and they were of different breeds (warmblood, Belgian trotter and Quarter horse), so the multifactorial influence could explain this hierarchical clustering. Another explanation for the distinction between mares could be the use of fresh semen vs frozen semen. The reason for the switch from fertilization with fresh to frozen-thawed semen is that IVF in horses was very new, and only protocols for fresh semen were established. When continuing the study, IVF with frozen-thawed semen became possible and for practical reasons the decision was made to continue with frozen-thawed semen. It was not possible to compare the transcriptomic profile of fresh vs. frozen semen, since it was linked to the individual mare. However, when mare R was excluded, so that only embryos created with frozen-thawed semen were analyzed, there were also no DEGs between the 4 IVF and 4 ICSI embryos. We tried to rule out more variables by using straws of the same stallion. Recent literature showed that developmental outcomes of IVF embryos produced using fresh, cooled or frozen semen did not differ, but gene expression patterns were not included in the analysis [11]. This could be included in future

research, next to other yet unknown factors, such as the influence of IVF on the epigenome, placentation, pregnancy and foal health.

5. Conclusion

Equine ICSI and IVF resulted in similar cleavage rates, but blastocyst rates, either calculated on collected COCs or on cleaved zygotes, were higher for ICSI embryos. When FLASH-seq was performed on the sibling embryos, no DEGs were found in the comparison between ICSI and IVF derived blastocysts. This indicates that conventional IVF results in embryos of similar quality and gene expression as ICSI, substantiating safe clinical production of IVP equine embryos.

CRediT authorship contribution statement

Klaartje Broothaers: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alice Jouneau:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. **Daniel Angel-Velez:** Writing – review & editing, Methodology, Investigation. **Tine De Coster:** Methodology, Investigation. **Nicola Festuccia:** Validation, Formal analysis. **Catherine Archilla:** Formal analysis. **Sophie Calderari:** Writing – review & editing, Visualization, Validation, Formal analysis. **Luc Jouneau:** Formal analysis, Data curation. **Emma Van den Branden:** Writing – review & editing, Investigation. **Sofie Peere:** Writing – review & editing,

Investigation. **Ellen Polfliet**: Writing – review & editing, Investigation. **Manon Dewulf**: Writing – review & editing, Investigation. **Jan Govaere**: Writing – review & editing, Investigation. **Pascale Chavatte-Palmer**: Writing – review & editing, Supervision, Resources, Conceptualization. **Katrien Smits**: Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Ethical approval

The OPU was approved by Ethics Committee of the Faculty of Veterinary Medicine, Ghent University. The study was conducted in accordance with the local legislation and institutional requirements.

Declaration of use of artificial intelligence

During the preparation of this work the author(s) used Chat GPT and DeepL in order to correct spelling mistakes. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Klaartje Broothaers reports financial support was provided by INRAE BREED. Klaartje Broothaers reports financial support was provided by Ghent University. Klaartje Broothaers reports a relationship with Ghent University that includes: employment, funding grants, and non-financial support. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.theriogenology.2025.117749>.

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