Research in Veterinary Science 171 (2024) 105222

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Characterization of preovulatory follicular fluid secretome and its effects on equine oocytes during *in vitro* maturation

Marcos Luis-Calero [a], Federica Marinaro [b], Pablo Fernández-Hernández [a], José M. Ortiz-Rodríguez [a], Javier G. Casado [c], Eva Pericuesta [b], Alfonso Gutiérrez-Adán [b], Esperanza González [d], Mikel Azkargorta [e], Ricardo Conde [f], Maider Bizkarguenaga [f], Nieves Embade [f], Félix Elortza [e], Juan M. Falcón-Pérez [d], Óscar Millet [f], Lauro González-Fernández [g,1], Beatriz Macías-García [a,1,*]

[a] *Departamento de Medicina Animal, Grupo de Investigación Medicina Interna Veterinaria (MINVET), Instituto de Investigación INBIO G+C, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain*
[b] *Departamento de Reproducción Animal, INIA-CSIC, Madrid, Spain*
[c] *Unidad de inmunología, Departamento de Fisiología, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain*
[d] *Exosomes Laboratory, CIC bioGUNE-BRTA, Derio, Spain*
[e] *Proteomics Platform, CIC bioGUNE-BRTA, Derio, Spain*
[f] *Precision Medicine and Metabolism Laboratory, CIC bioGUNE, Basque Research and Technology Alliance (BRTA), Bizkaia, Spain*
[g] *Departamento de Bioquímica y Biología Molecular y Genética, Grupo de Investigación Señalización Intracelular y Tecnología de la Reproducción (SINTREP), Instituto de Investigación INBIO G+C, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain*

ARTICLE INFO

Keywords:
Horse
Oocyte
In vitro maturation
Secretome
Meiotic competence
Gamete quality

ABSTRACT

In vitro maturation (IVM) of oocytes is clinically used in horses to produce blastocysts but current conditions used for horses are suboptimal. We analyzed the composition of equine preovulatory follicular fluid (FF) secretome and tested its effects on meiotic competence and gene expression in oocytes subjected to IVM. Preovulatory FF was obtained, concentrated using ultrafiltration with *cut-off* of 10 kDa, and stored at −80 °C. The metabolic and proteomic composition was analyzed, and its ultrastructural composition was assessed by cryo-transmission microscopy. Oocytes obtained *post-mortem* or by ovum pick up (OPU) were subjected to IVM in the absence (control) or presence of 20 or 40 μg/ml (S20 or S40) of secretome. Oocytes were then analyzed for chromatin configuration or snap frozen for gene expression analysis. Proteomic analysis detected 255 proteins in the *Equus caballus* database, mostly related to the complement cascade and cholesterol metabolism. Metabolomic analysis yielded 14 metabolites and cryo-transmission electron microscopy analysis revealed the presence of extracellular vesicles (EVs). No significant differences were detected in maturation rates among treatments. However, the expression of *GDF9* and *BMP15* significantly increased in OPU-derived oocytes compared to *post-mortem* oocytes (fold increase ± SEM: 9.4 ± 0.1 *vs.* 1 ± 0.5 for *BMP15* and 9.9 ± 0.3 *vs.* 1 ± 0.5 for *GDF9*, respectively; $p < 0.05$). Secretome addition increased the expression of *TNFAIP6* in S40 regardless of the oocyte source. Further research is necessary to fully understand whether secretome addition influences the developmental competence of equine oocytes.

1. Introduction

Assisted reproductive techniques (ARTs) are in high demand for producing equine blastocysts *in vitro*. Intracytoplasmic sperm injection (ICSI) is the preferred technique for horses, but the results are improvable, with only 9.85–39.2% of initially injected oocytes reaching the blastocyst stage, depending on the mare's breed (Lazzari et al., 2020; Brom-de-Luna et al., 2021). *In vitro* maturation (IVM) of equine immature oocytes obtained clinically *in vivo* by ovum pick up (OPU) or *post-mortem*, is a crucial step in equine embryo production (Hinrichs, 2018).

* Corresponding author at: Facultad de Veterinaria, Avda. de la Universidad s/n, 10004 Cáceres, Spain.
E-mail addresses: lgonfer@unex.es (L. González-Fernández), bemaciasg@unex.es (B. Macías-García).
[1] These authors contributed equally as senior investigators to this study.

https://doi.org/10.1016/j.rvsc.2024.105222
Received 24 November 2023; Received in revised form 3 January 2024; Accepted 8 March 2024
Available online 11 March 2024
0034-5288/© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Different conditions and additives, such as follicle-stimulating hormone, epidermal growth factor (Sánchez-Calabuig et al., 2021), luteinizing hormone, or sodium pyruvate (Lewis et al., 2020), have been used to improve IVM, but success rates are limited, ranging between 50% and 60%, regardless of the oocyte source (OPU or *post-mortem*) (Hinrichs, 2018; Claes and Stout, 2022). This is partially related to the fact that oocytes are matured under non-physiological conditions, as the media used for maturation differs greatly from the follicular environment (Fernández-Hernández et al., 2020).

Previous studies have shown that follicular fluid (FF) contains nine metabolites not present in the commonly used media for equine oocyte IVM (TCM-199 and DMEM/F-12), and the concentrations of core metabolites like lactate or glucose vary vividly among FF and these media (Fernández-Hernández et al., 2020). FF is a complex and variable milieu that provides for the oocyte's needs during the follicular wave, promoting its progression to metaphase II to resume meiosis. This biological fluid contains proteins, lipids, extracellular vesicles (EVs), metabolites, hormones, enzymes, DNA, and mRNA, among other components, and its cargo varies with the changing phases of the estrous cycle (Spacek and Carnevale, 2018). Although oocyte maturation in 100% equine FF is known to be the best physiological condition for successful maturation, this approach has not significantly improved oocyte meiotic or developmental competence in horses (Caillaud et al., 2008; Dell'Aquila et al., 1997; Douet et al., 2017; Hinrichs et al., 2002). The FF is a transudate from the thecal cell layer vasculature that also contains the "secretome" (Deftu et al., 2020). The term "secretome" was first used in the context of proteins secreted by *Bacillus subtilis* (Tjalsma et al., 2000), and is composed of all the proteins secreted by the cells and includes cytokines, growth factors, hormones, enzymes, glycoproteins, coagulation factors, and EVs. EVs are a type of membrane-encapsulated particles, including exosomes and microvesicles, that carry regulatory molecules, such as RNA species (microRNAs, long non-coding RNAs, mRNAs), lipids, metabolites or DNA fragments, among other factors, from the donor to recipient cells, facilitating cell-to-cell communication (Mishra and Banerjee, 2023; Harmati et al., 2021). These bioactive compounds play an essential role in oocyte maturation, determining the oocyte's meiotic and developmental fate (Cakmak et al., 2016).

In equine oocytes, the addition of EVs retrieved by ultracentrifugation from FF obtained from small follicles (< 20 mm) to the IVM medium has been shown to increase oocyte maturation rate in compact cumulus oocyte complexes (COCs) (Gabryś et al., 2022). However, EVs only represent a fraction of the secretome and are challenging to obtain, thereby leading to the disadvantage of leaving behind important bioactive molecules such as cytokines that can have an impact not only on nuclear maturation but also on cytoplasmic maturation of the oocyte (Cakmak et al., 2016). Therefore, the objective of our study was to characterize the homologous secretome retrieved from preovulatory FF and investigate the effect of its addition on IVM of equine oocytes obtained *in vivo* or *post-mortem*.

2. Materials and methods

Unless stated otherwise, all chemicals were purchased from Merck-Sigma Aldrich Quimica (Barcelona, Spain). Heparin (H3393); FBS (12103C); TCM 199 Earle's salts (M4530); TCM Hank's salts (M0393); Recombinant FSH from porcine pituitary (F2293); Hyaluronidase from bovine testis (H3506); Hoechst 33258 (14530); Poly; Polivinyl alcohol (363138).

2.1. Follicular fluid collection

Follicular fluid (FF) was retrieved from six research mares by flank aspiration after stimulation of a preovulatory follicle (> 35 mm and uterine oedema 2–3 in a 1–3 scale) with 3000 IU of hCG intravenous (Veterin Corion, DFV, Spain). After 32 h if uterine oedema had dropped, the follicle was smooth on palpation and some discomfort was observed in the mare upon follicle palpation, the mare was assumed to had responded to hCG and the FF was aspirated by the flank as previously reported (Hinrichs et al., 1998); if the follicle did not show signs of clinical response to hCG the follicular fluid was not aspirated. Briefly, the flank ipsilateral to the preovulatory follicle was shaved and scrubbed as for surgery. After, the flank was infiltrated with one ml of local anesthetic (Xylazine at 1%), a scalpel blade was used to open the skin and muscle, and an equine trochar was placed through the skin and muscles of the flank into the peritoneal cavity. The ovary was held in place against the end of the trochar cannula manually per rectum using one hand, and the 12 g needle was introduced through the trochar into the preovulatory follicle using the other hand. The content of the follicle was then aspirated using a 50 ml syringe attached to a plastic tube. The aspirated FF was centrifuged at 4 °C at 700*g* for 10 min, the supernatant was placed in a clean tube and centrifuged again at 2000*g* for 10 min at 4 °C to discard cell debris. Clean FF was then divided into aliquots (5 ml) and frozen at −80 °C until use.

2.2. Secretome obtention

FF from six different mares was used (30 ml in total). Five ml of FF of each individual mare were thawed at room temperature (RT; 22–25 °C) until thawing was visually observed, diluted 1:3 in sterile phosphate-bufferedsaline (PBS) and filtered through a 0.22 μm syringe filter (Frisenette, Knebel, Denmark). The obtained fluid (15 ml each) was placed onto 10 K Amicon® Ultra-15 Centrifugal Filter Units (Merck-Millipore) and centrifuged at 4000*g* for 1 h at 4 °C as previously reported (Ormazabal et al., 2022). Secretome (1000 μl approximately) was retrieved from the top funnel of the Amicom filter and placed into a sterile Eppendorf tube using a sterile pipette. After thorough resuspension of the obtained secretome by gentle pipetting, protein concentration of fresh secretome was measured using a Bio-Rad DC protein assay following the manufacturer's instructions. Secretome of each mare was then aliquoted and frozen at −80 °C until use for secretome analysis. For oocyte IVM experiments, secretome from 2 mares (Alba and Nuna) was used based on their age (4 years) and their clinical response to the hCG (notable oedema decrease by ultrasound and vivid follicle softening by rectal palpation). After thawing, the aliquots were discarded, and a new aliquot was used for each experiment.

2.3. Oocyte collection

Oocytes were collected *in vivo* by ovum pick up (OPU) or *post-mortem* from a commercial slaughterhouse (Incarsa, Burgos, Spain).

2.3.1. OPU procedure

OPU was performed on a research mare herd hosted at the University of Extremadura (seven mares in total aged 3–14 years) housed in group paddocks with free-choice hay and water. All experimental procedures were performed according to Institutional, National and European regulations and were approved by the Institutional Animal Care and Use Committee at the University of Extremadura and Junta de Extremadura (Ref. MAM/JSR). Aspiration of all follicles over 5 mm was performed once every 14 days in a given mare through OPU as described previously (Choi et al., 2016) disregarding the cycle stage. OPU sessions were scheduled so that two mares underwent OPU on a given day. In general, aspiration was not performed unless more than eight follicles over 5 mm diameter were present. Briefly, mares were tranquilized and held in stocks. The operator positioned a transvaginal ultrasound probe with a needle guide and held the ovary per rectum. The diameter of each follicle was recorded before the follicle was punctured by passing a 12-gauge double-lumen needle (Minitub Ibérica, Tarragona, Spain) through the needle guide through the vaginal wall into the follicle. When possible, each follicle was flushed six to eight times with Equiflush medium (Minitub Ibérica, Tarragona, Spain) added with 4 IU/ml heparin and the follicle was curetted by repeated rotation of the needle. The

aspirated fluid was filtered through a nylon cell strainer with 70 μm pores (Falcon, Durham, NC, USA) and the collected cellular material was rinsed into a Petri dish. Oocytes were searched under a dissection microscope and located oocytes were washed twice in M199 Hanks' salts medium (Gibco, Madrid, Spain) added with 10% fetal bovine serum or FBS (*v*/v; Thermo Fisher, Madrid, Spain). Once all the oocytes were retrieved, they were immediately subjected to *in vitro* maturation. The oocytes were obtained in 19 different OPU sessions.

2.3.2. Post-mortem collection

Ovaries were collected at a slaughterhouse (Incarsa, Burgos, Spain) and transport was performed within 5 h from slaughter at 15 °C. Immature cumulus–oocyte complexes (COCs) were obtained by follicular scraping or using a vacuum pump (Cook, Limerick Ireland) in Equiflush medium (Minitub Ibérica, Tarragona, Spain) added with 4 IU/ml heparin. The oocytes were collected and maintained in a holding medium [HM: TCM-199 supplemented with Earle's salts (40%; *v*/v) + TCM-199 supplemented with Hank's salts (40%; v/v) + FBS (20%; v/v) and 25 μg/ml gentamicin (Gibco, Madrid, Spain)] for a maximum of 18 h at RT in the dark. The next morning, the oocytes were subjected to IVM. The oocytes were obtained on 5 different days.

2.4. In vitro maturation

COCs were cultured in M-199 with Earle's salts and 10% FBS (*v*/v; Gibco, Madrid Spain), 5 μU/ml FSH and 25 μg/ml of gentamicin, being this medium the control group. Secretome was added at 20 μg/ml (S20) or 40 μg/ml (S40) as previously reported (Marinaro et al., 2019) and COCs were cultured in groups (maximum of 25 COCs) in 500 μl wells when retrieved *post-mortem* or in droplets at a rate of 20 μl/oocyte if retrieved by OPU. The IVM droplets were covered with mineral oil (NidOil, Nidacon, Sweden), allowed to equilibrate for a minimum of 3 h and IVM was conducted for 26–28 h at 38.5 °C under an atmosphere of 5% CO_2 in air with maximum humidity.

2.4.1. Evaluation of oocyte degeneration and maturation status

Oocyte maturation was evaluated after 26–28 h. All IVM experiments were performed in the laboratory located at the Veterinary Clinical Hospital of the University of Extremadura (Cáceres, Spain) from October of 2021 to November of 2022. For the OPU assays, the oocytes were retrieved in 19 different sessions while in the *post-mortem* oocytes, 5 different sessions were included. First, cumulus cells were removed from the oocytes by meticulous pipetting using stripper capillary tubes of decreasing diameter (175–125 μm; Cooper Surgical, Barcelona, Spain) with PBS supplemented with 0.01% PVA (PBS + PVA) until no granulosa cells were visualized on the zona pellucida. Prior pipetting, hyaluronidase was used at 0.1% (*w/v*) for 1 min. Denuded oocytes were then fixed in 4% formaldehyde in PBS + PVA for 24 h at 4 °C. Then, oocytes were thoroughly washed in PBS + PVA and stained with 2.5 μg/ml Hoechst 33342 (Thermo Fisher, Madrid) in PBS + PVA at 37 °C for 10 min in the dark. Oocytes were then mounted on slides using glycerol and a coverslip, sealed with nail polish and allowed to air dry. Oocytes were classified based on DNA integrity and conformation as: germinal vesicle (GV), metaphase I (MI) or MII using a Nikon Eclipse 50i fluorescence microscope equipped with an ultraviolet lamp and a 60× objective following previously validated criteria (González-Fernández et al., 2018). Oocytes were considered as degenerated when no DNA was present or if unidentifiable chromatin configurations were observed.

2.5. Nuclear magnetic resonance (NMR) secretome sample preparation and measurements

2.5.1. Secretome sample preparation

On the day of analysis, samples were thawed for 30 min slowly in ice. A total of 10 μl of sample was diluted in 189 μl of 0.2 M potassium phosphate buffer in deuterium oxide (D_2O) with a pH of 7.4 ± 0.5 and 1.11 μl of TSP-d6 (3-(Trimethylsilyl)propanoic acid), to reach a final volume of 200 μl. Samples were vortexed for 10 s and added into a 3 mm NMR tube.

2.5.2. Secretome NMR measurements

Samples were measured at 300 K on a 600 MHz IVDr (Bruker BioSpin, Germany), with a thermostatized automatic sample changer (SampleJet) and a double resonance broadband probeheadprobe head (BBI) with a z gradient coil and BOSS-III shim system.

Before sample acquisition, the spectrometer was calibrated with two different samples: methanol and sucrose to check the temperature (300*K*) and optimal shimming respectively. Three main spectra were acquired for all the samples. Standard one-dimension ^{1}H NOESY spectrum (*noesygppr1d*) with water presaturation was acquired with 4 dummy (DS) and 32 accumulated (NS) scans. A 1D ^{1}H Carr-Purcell-Meiboom-Gill (CPMG) experiment (*cpmgpr1d*) was acquired with 4 DS and 256 NS, and a two-dimensional J-resolved experiment (*jresgpprqf*) was acquired to help on the signal multiplicity identification. Spectra were acquired and processed using the TopSpin 3.6.2 software (Bruker Biospin GmbH). Free induction decays were multiplied by an exponential function equivalent to 0.3 Hz line broadening before applying Fourier transform. All transformed spectra were corrected for phase and baseline distortions and referenced to the TSP singlet at 0 ppm.

The concentrations of the identified metabolites are expressed in micromolar units. The calculation of metabolites' concentration was done relatively to integrate the signal of each metabolite and the number of protons associated with that signal. Metabolite concentration was then extrapolated using TSP singlet at 0 ppm. The metabolites found are presented in Table 1 and were determined following previously validated methods (Fernández-Hernández et al., 2020).

2.6. Hybrid trapped ion mobility spectrometry – Quadrupole time of flight mass spectrometer assays (timsTOF assay)

Secretome samples were incubated in a buffer containing 7 M urea 2 M Thiourea 4% CHAPS and 5 mM DTT and protein was digested following the filter-aided FASP protocol described by Wisniewski et al., with minor modifications (Wiśniewski et al., 2009). Trypsin was added to a trypsin:protein ratio of 1:50 and the mixture was incubated overnight at 37 °C, dried out in a RVC2 25 speedvac concentrator (Christ) and resuspended in 0.1% formic acid (FA). Peptides were further desalted and resuspended in 0.1% FA using C18 stage tips (Millipore).

Samples were analyzed in a hybrid trapped ion mobility spectrometry – quadrupole time of flight mass spectrometer (timsTOF Pro with PASEF, Bruker Daltonics) coupled online to an Evosep ONE liquid chromatograph (Evosep). Two hundred ng were directly loaded onto the

Table 1
Chemical shift assignment, multiplicity, and number of contributing protons for the identified metabolites.

Metabolites	Peak for integration (ppm)	Multiplicity	Number of protons
Lactic acid	4.1	q	1
Glucose	5.23	d	1
Tyrosine	6.9	m	2
Glycerol	3.64	m	4
Lysine	3	t	2
Succinic acid	2.389	s	4
Pyruvic acid	2.363	s	3
Glutamic acid	2.335	m	2
Acetic acid	1.906	s	4
Alanine	1.47	d	3
Threonine	1.316	d	3
3-hydroxyisovaleric acid	1.237	d	3
Valine	1.03	D	3
Isoleucine	1.004	D	3
Leucine	0.96	t	6

Evosep ONE and resolved using the 30 samples-per-day protocol. Protein identification and quantification were carried out using PEAKS software (Bioinformatics solutions). Searches were carried out against a database consisting of horse entries (Uniprot) with precursor and fragment tolerances of 20 ppm and 0.05 Da.

2.6.1. Proteomic analysis

Gene names for the Uniprot accession numbers of the identified proteins were obtained from UniProt release 2023_03 (https://www.uniprot.org/) (The UniProt Consortium, 2023) and the DAVID Bioinformatics "Gene ID Conversion Tool" (https://david.ncifcrf.gov/conversion.jsp) (Sherman et al., 2022). Enrichment and pathway analyses were performed on unique gene symbols to avoid redundancy. The enrichment analysis was performed with the "Functional Annotation Tool" provided by DAVID Bioinformatics (https://david.ncifcrf.gov/tools.jsp), using Benjamini-Hochberg False Discovery Rate (FDR) for multiple test correction. The overrepresentation pathway analysis was carried out with the Analysis Tools of Reactome (https://reactome.org/PathwayBrowser/#TOOL=AT) (Gillespie et al., 2022), exploring the gene names associated with the identified equine proteins in the *Homo sapiens* database. Finally, to identify protein-protein interaction networks, multiple equine proteins belonging to pathways of interest were analyzed with STRING (https://string-db.org/) (Szklarczyk et al., 2015).

2.7. Gene expression analysis

Pools of COCs were used for RNA extraction. In *post-mortem* samples, 25 oocytes per experimental group (control, S20 and S40; 75 oocytes in total) retrieved on 3 different days were used. In the OPU group, 20 COCs for control, 26 for S20 and 23 for S40 retrieved in 5 different sessions were used (69 COCs in total). COCs were pooled and three replicates per experimental group were used for RNA extraction. Poly (A) RNA was extracted using the Dynabeads® mRNA DIRECT™ Micro Kit (Ambion®, Thermo Fisher Scientific Inc., Norway) following the manufacturer's instructions. Immediately after RNA extraction, reverse transcription (RT) reaction was carried out using a Moloney murine leukaemia virus (MMLV) Reverse Transcriptase 1st-Strand cDNA Synthesis Kit following the manufacturer's instructions (Epicentre Technologies Corp., USA) in a total volume of 40 μl to prime the RT reaction and produce cDNA. Tubes were heated to 70 °C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 50 units of reverse transcriptase. Next, the tubes were incubated at 25 °C for 10 min to promote the annealing of random primers, followed by 60 min at 37 °C to allow the RT of RNA and finally an incubation for 5 min at 85 °C to denature the enzyme.

All mRNA transcripts were quantified using quantitative polymerase chain reaction (qPCR) and were run in the following conditions: 95 °C for 3 min, 35 cycles of 94 °C for 15 s, 56 °C for 30 s and 72 °C for 15 s, followed by a final extension step for 10 s and carried out in duplicate for all genes of interest in the Rotorgene 6000 Real-Time Cycler TM (Corbett Research, Sydney, Australia) by adding a 2-ml aliquot of each sample (~60 μg/μl) to the PCR mix (GoTaq qPCR Master Mix, Promega Corporation, Madison, WI, USA) containing the specific primers selected to amplify the selected genes. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are given in Supplementary Table 1. The comparative cycle threshold (CT) method was used to quantify expression levels. All primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries when possible. Primers were previously validated for adequate primer efficiency and the specificity of their PCR products was confirmed by electrophoresis on a 2% agarose gel. The PCR conditions were tested to achieve efficiencies close to 1. Each RT-qPCR reaction was performed in a final volume of 20 μl, containing 0.25 mM of forward and reverse primers, 10 μl of GoTaq RT-qPCR Master Mix (Promega) and 2 μl of each cDNA sample using a Rotorgene 6000 Real-Time Cycler (Corbett Research, Sydney, Australia) and SYBR Green as double-stranded DNA-specific fluorescent dye. All mRNA transcripts were quantified in duplicate. The comparative quantification cycle (Cq) method was used to quantify expression levels (Sánchez-Calabuig et al., 2021).

In this study, we examined the quantitative expression of candidate genes associated with oocyte quality (*BMP15*, *TNFAIP6*, and *GDF9*), cytokine and growth factor expression (*VEGFA*, *IGF1*, and *TNF*), and developmental competence (*HSP90AA1*) in *post-mortem* and OPU-retrieved oocytes that were matured with or without added secretome. To identify the most suitable housekeeping gene for normalizing mRNA levels, we first evaluated the expression of three housekeeping genes (*H2AFZ*, *RN18S*, and *GAPDH*) and found that *H2AFZ* was the most stable under our experimental conditions. We then used *H2AFZ* for the normalization of mRNA transcripts since its expression was directly proportional to the amount of mRNA present in non-normalized reverse transcription reactions.

2.8. Cryo-transmission electron microscopy

Freshly glow-discharged 200-mesh grids (R2/1; QUANTIFOIL) were placed inside the chamber of an EM GP2 Automatic Plunge Freezing (Leica Company), which was maintained at 8 °C temperature and relative humidity close to saturation (90%). Four microliters of the sample were dropped onto the grid for 30 s. After incubation, most of the liquid on the grid was removed by blotting (blot time was 2 s, the number of blots was set to 1 and no extra movement was applied) with absorbent standard filter paper (Ø 55 mm, Grade 595, Hahnemühle). After the blotting step, the grid was abruptly plunged into a liquid ethane bath automatically set to −184 °C. Once the specimen was frozen, the vitrified grid was removed from the plunger and stored under liquid nitrogen inside a cryo-grid storage box. Cryo-TEM analysis of the samples was performed on a JEM-2200FS/CR (JEOL Europe, Croissy-sur-Seine, France) transmission electron microscope. This microscope is equipped with a field emission gun (FEG) operated at 200 kV and an in-column Ω energy filter.

During imaging, no-tilted zero-loss two-dimensional (2D) images were recorded under low-dose conditions, utilizing the 'Minimum Dose System (MDS)' of Jeol software with a total dose on the order of 30–40 electrons/$Å^2$ per exposure, at defocus values ranging from 1.5 to 4.0 μm. The in-column Omega energy filter of the microscope helped us to record images with improved signal-to-noise ratio (SNR) by zero-loss filtering, using an energy selecting slit width of 20 eV centered at the zero-loss peak of the energy spectra. Digital images were recorded in linear mode on a 3840 × 3712 (5 μm pixels) Gatan K2 Summit direct detection camera (Gatan Inc.) using DigitalMicrograph™ (Gatan Inc.) software at nominal magnifications of 2000× and 20,000× with a pixel size of 1.8 nm and 0.2 nm respectively.

2.9. Statistical analysis

All statistical tests were performed using SigmaStat (Jandel Scientific, San Rafael, CA, USA) and Sigma Plot 12.0 for windows (Systat Software, Chicago, IL, USA). To test normality, a Saphiro-Wilk test was used and to test the equality of variances, a Levene's test was performed. Data for oocyte's chromatin configuration showed normal distribution and homogeneous variances, thus, they were compared using a one-way analysis of variance ANOVA followed by Dunn's *post hoc* test. Data for relative mRNA abundance that followed a normal distribution and had homogeneous variances were compared using one-way analysis of variance ANOVA followed by Fisher LSD method *post hoc* test. If data followed a normal distribution but failed the equal variance test or presented a non-gaussian distribution, they were compared using the non-parametric Kruskal Wallis test. Values were considered significantly different when the p-value was <0.05.

3. Results

3.1. Secretome metabolite identification

We used NMR spectroscopy to quantitatively interrogate the metabolism of the secretome, from a cohort of six individual secretomes, obtained from 6 different mares. First, we assigned the chemical shift for each metabolite by using a randomly selected secretome sample. We then compared the spectra of the follicular fluid secretome to the metabolites identified in our previous studies in horses (Fernández-Hernández et al., 2020; González-Fernández et al., 2020). Table 2 shows the concentrations of the 14 detected metabolites in μM. Data are presented as mean ± Standard Error of the Mean (SEM) and, importantly, only the 14 metabolites that were detected in all the samples are shown.

3.2. Secretome proteomic analysis

Protein identification and quantification were carried out using PEAKS software (Bioinformatics solutions). After spectra analysis, a total of 343 proteins was found in the pool of secretome from six mares though UHPLC-TOF/MS. Only the identified proteins with at least two spectral counts annotated to the organism name *Equus caballus* (organism identifier: 9796) were considered for this study (n = 255) and are listed in Supplementary Table 2.

The secretome proteins were associated to official gene symbols and 125 unique genes were used for the enrichment and pathway analyses. For the enrichment analysis, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used as annotation databases, revealing enriched terms as *extracellular space* (GO:0005615, 61 proteins), *innate immune response* (GO:0045087, 13 proteins), and *serine-type endopeptidase inhibitor activity* (GO:0004867, 21 proteins) (Supplementary Table 3 and Fig. 1A). To complete the enrichment analysis, the 125 unique gene names corresponding to the initial 255 equine proteins were analyzed with Reactome for a high-level pathway overview (Fig. 1B). According to the KEGG and GO analysis, many proteins were involved in lipid, phospholipid and lipoprotein metabolism (ecb04979, GO:0010873, GO:0042157), activity (GO:0051006, GO:0060228), exchange (GO:0033344, GO:0043691), clearance (GO:0034382) and binding (GO:0005543, GO:0034380, GO:0034375, GO:0031210, GO:0070653, GO:0010903). This was corroborated by the Reactome analysis, where the category *Plasma lipoprotein assembly, remodeling, and clearance* (R-HSA-174824) were overrepresented (data not shown). Similarly, the enrichment of the KEGG category *Complement and coagulation cascades* (ecb04610) was confirmed by the overrepresentation of the Reactome pathways *Innate Immune System* (R-HSA-168249), *Hemostasis* (R-HSA-109582), *Platelet activation, signaling and aggregation* (R-HSA-76002), and *Complement cascade* (R-HSA-166658). Lastly, to understand the interaction between the proteins included in these events, STRING analyses were performed, revealing high-confident interactions between many follicular fluid proteins (Fig. 1C and D).

Table 2
List of the metabolites detected in the secretome of equine preovulatory FF.

Metabolite	Concentration (μM)
Acetate	21.0 ± 2.2
Alanine	22.0 ± 2.5
Glucose	115.0 ± 13.8
Glycerol	4228.0 ± 512.0
Glutamic acid	30.0 ± 5.3
Isoleucine	10.0 ± 1.3
Lactate	136.0 ± 5.6
Leucine	15.3 ± 1.3
Succinate	5.5 ± 1.0
Pyruvate	2.8 ± 0.4
Threonine	2.0 ± 0.4
Valine	12.7 ± 1.8
Tyrosine	34.7 ± 3.4
3-hydroxyisovaleric acid	4.9 ± 1.0

3.3. Transmission electron microscopy

In our samples, CryoTEM experiments extracellular vesicles presenting a clear plasmalemma could be observed. Other non-identified particles could also be visualized (Fig. 2).

3.4. Chromatin configuration

Oocyte maturation was evaluated after 26–28 h but no significant differences were found within in MII, MI, GV or DEG despite secretome addition ($p > 0.05$; Table 3A and B) when oocytes were retrieved by OPU or *post-mortem*. However, when OPU *vs. post-mortem* oocytes sources were compared statistically significant differences were observed in control for germinal vesicle stage (27.8 *vs.* 8.7% OPU *vs. post-mortem* respectively; $p < 0.05$) and degenerated oocytes (16.7 *vs.* 33.3% OPU *vs. post-mortem* respectively; $p < 0.05$). In the S40 treatment, more degenerated oocytes were observed in the *post-mortem* group than in the OPU group (35.7 *vs.* 17.8% respectively; $p < 0.05$). Hence, secretome addition, at least at the concentrations used in the present work, does not affect the nuclear maturation of equine oocytes obtained by OPU or *post-mortem* and does not induce a deleterious effect as it could be suspected if higher degeneration rates were observed in S20 or S40 groups compared to control. Interestingly, a tendency for increased metaphase II (MII) and lower metaphase I (MI) was observed in *post-mortem* and ovum pick-up (OPU) groups when secretome was added at 40 μg/ml to IVM medium (Table 3).

3.5. Relative mRNA expression of genes related to oocyte quality, embryo competence, growth factors and cytokines

Although maturation rates did not differ in the presence or absence of secretome, significant differences were observed in genes related to oocyte quality, such as *BMP15*, *GDF9*, and *TNFAIP6*. We found statistically significant differences in *BMP15* and *GDF9* expression between OPU and slaughterhouse groups (Fold increase ± SEM: 9.4 ± 0.1 *vs.* 1 ± 0.5 for *BMP15* and 9.9 ± 0.3 *vs.* 1 ± 0.5 for *GDF9*, respectively; $p < 0.05$). However, secretome addition did not have any effect within either group (OPU or slaughterhouse) despite secretome addition at 20 or 40 μg/ml in *BMP15* or *GDF9* expression (Fig. 3).

In contrast, we observed an enhancement in *TNFAIP6* expression in OPU-retrieved oocytes with secretome addition compared to the control group (Fold increase ± SEM: 1.0 ± 0.3 for control, 8.3 ± 0.2 for S20, and 16.8 ± 0.3 for S40; $p < 0.05$). This increase was also seen in the slaughterhouse group for control and S40, and between S20 and S40, but not between control and S20 (Fold increase ± SEM: 1.7 ± 0.5 for control, 2.0 ± 0.3 for S20, and 5.2 ± 0.1 for S40; $p < 0.05$). *HSP90AA1*, a gene previously shown to be related with embryo quality in horses (Smits et al., 2011), did not vary despite secretome addition at any of the dosages tested or the group (OPU *vs.* slaughterhouse) (Fig. 3).

Regarding the relative mRNA expression of cytokines, insulin-like growth factor 1 (*IGF1*) did not show significant differences among groups (OPU *vs.* slaughterhouse) or in the presence or absence of secretome at the different dosages tested ($p > 0.05$). The mRNA expression of vascular endothelial growth factor (*VEGFA*) significantly differed among groups (OPU *vs.* slaughterhouse; $p < 0.05$) but not within the same group despite secretome addition ($p > 0.05$; Fig. 4). Finally, the mRNA relative expression of tumor necrosis factor alpha (*TNF*) increased in the OPU group when secretome was added at 20 and 40 μg/ml compared to the control (Fold increase: 1.0 ± 0.1 for control, and 2.7 ± 0.1 for S20 and 5.2 ± 0.1 for S40; $p < 0.05$). This increase was also observed in the slaughterhouse group when comparing the control group to the group treated with 40 μg/ml of secretome (Fold increase: 2.3 ± 0.1 for control and 5.0 ± 0.6 for S40; $p < 0.05$) (Fig. 4).

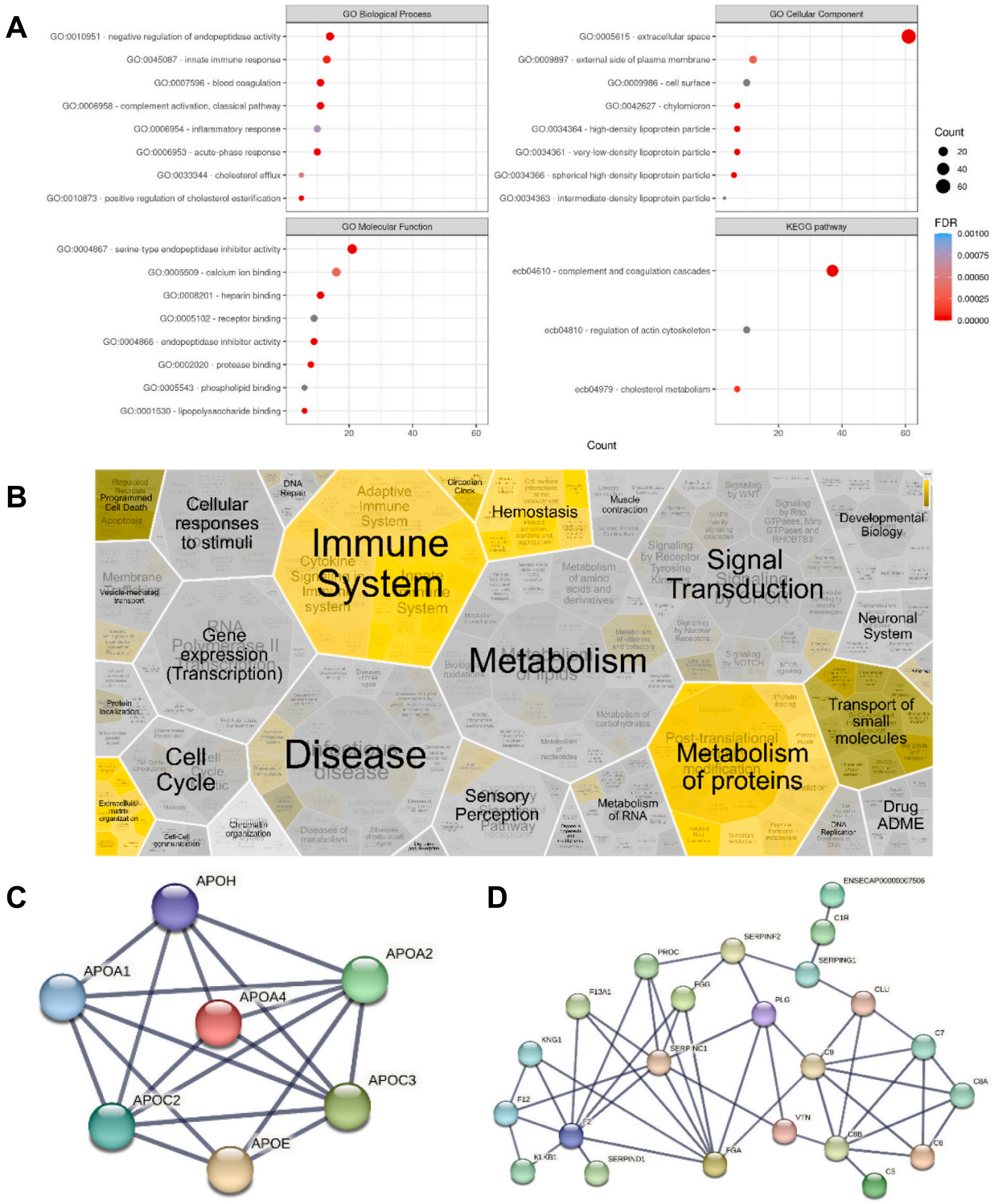

Fig. 1. Proteomic analysis of the secretome obtained from preovulatory equine follicular fluid. Panel (A) displays significant Gene Ontology (GO) and KEGG annotations of the identified proteins from equine follicular fluid secretome, obtained through the Functional Annotation Tool provided by DAVID Bioinformatics. The most representative terms with FDR < 0.01 are shown, and "Count" represents the number of proteins within the corresponding category annotation. Panel (B) shows a Voronoi pathway visualization (Reacfoam) for all proteins ($n = 255$, see Supplementary Table 1) associated with gene symbols ($n = 125$), where a Reactome overrepresentation pathway analysis was performed by exploring the corresponding gene names in the *Homo sapiens* database. Significantly enriched pathways are shown in bright yellow ($p \approx 0$) or grayish yellow ($0.05 < p < 0$). Panels (C) and (D) display protein-protein interaction networks among the proteins found in equine follicular fluid secretome belonging to the KEGG categories *Cholesterol metabolism* (ecb04979) and *Complement and coagulation cascades* (ecb04610), respectively. Lines between nodes indicate edges (representing protein-protein associations) with the highest confidence (0.900). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

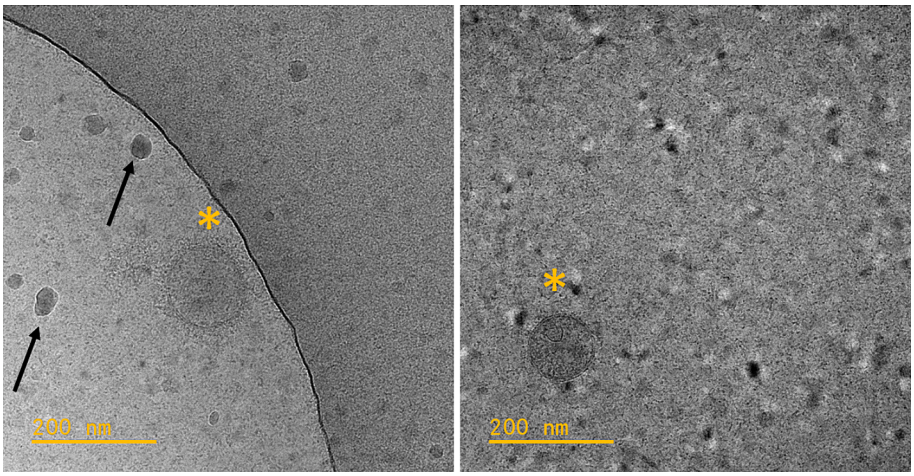

Fig. 2. CryoTEM of the secretome obtained from equine preovulatory follicular fluid. Extracellular vesicles (*) and other non-identified particles (arrowhead) were observed in the different samples analyzed.

Table 3
Chromatin configuration after IVM in oocytes retrieved by OPU or *post-mortem*.

A. OPU	Maturation stage			
Treatment group	MII	MI	GV	DEG
CONTROL (n = 126)	47 (37.3)	23 (18.2)	35 (27.8)[a]	21 (16.7)[a]
S20 (n = 109)	45 (41.3)	19 (17.4)	19 (17.4)	26 (23.8)
S40 (n = 107)	46 (43.0)	18 (16.8)	24 (22.4)	19 (17.8)[a]
B. POST-MORTEM	Maturation stage			
Treatment group	MII	MI	GV	DEG
CONTROL (n = 69)	27 (39.1)	13 (18.8)	6 (8.7)[b]	23 (33.3)[b]
S20 (n = 75)	28 (37.3)	10 (13.3)	10 (13.3)	27 (36.0)
S40 (n = 70)	33 (47.1)	7 (10)	5 (7.1)	25 (35.7)[b]

Values show the total number of oocytes in each group, with percentages in parentheses. Total number of oocytes used per treatment is reported in the first column. Within columns, values bearing different letters in the OPU assays differ significantly with the homologous treatment *post-mortem* ($p < 0.05$). MII, metaphase II; MI, metaphase I; GV, germinal vesicle; DEG, degenerated.

4. Discussion

In this study, we demonstrate for the first time the effects of adding secretome retrieved from equine preovulatory FF during *in vitro* maturation on equine oocytes. Previous studies have shown that the composition of current oocyte IVM media differs widely from that of preovulatory FF in equids (Fernández-Hernández et al., 2020; Miro et al., 2022). This difference may partly explain the low efficiency of *in vitro* embryo production in horses, where high maturation (> 60%) and cleavage rates (> 70%) are achieved but development to the blastocyst stage is still low, varying between 11% and 40% (Lazzari et al., 2020; Brom-de-Luna et al., 2021; Choi et al., 2016; Galli et al., 2016). The initial oocyte quality and the attainment of nuclear and cytoplasmic maturation are known to be determinant for the developmental competence of the female gamete, and variations in media used for oocyte IVM can influence embryo development and blastocyst cell number (Watson, 2007). In horses, attempts have been made to enhance the meiotic and developmental competence of equine oocytes by using native FF in the maturation media, but these attempts have not resulted in significant improvements in maturation rates or developmental competence (Spacek and Carnevale, 2018; Douet et al., 2017). These results can be explained by the fact that the follicular environment is a rapidly changing milieu that is constantly renewed, eliminating by-products. Therefore, when native reproductive fluids are added, even at low concentrations, during *in vitro* culture (over 5% *v*/v), these molecules can cause oocyte and embryo damage, as previously demonstrated in bovine embryos when oviductal fluid was added during culture (Lopera-Vasquez et al., 2017).

In this sense, secretome is known to influence cell function in both healthy and diseased cells. Therefore, a thorough investigation of the FF secretome in horses has the potential to provide novel insights into the mechanisms regulating oocyte meiotic and developmental competence, and could also help to identify new biomarkers of disease, as has been described in humans (Bonetti et al., 2019). Regarding the metabolic composition of the equine secretome, previous studies have shown that it differs significantly from preovulatory FF (PFF) (Fernández-Hernández et al., 2020; Gérard et al., 2015). Acetyl carnitine, acetate, alanine, aspartate, carnitine, choline, citrate, creatine, creatine phosphate, fumarate, glycine, histamine, and histidine are all detected in PFF, but are not present in the secretome. Furthermore, our group's previous results have shown that the detected metabolites are present at lower concentrations in the secretome compared to PFF (in μM *vs.* mM, respectively) (Fernández-Hernández et al., 2020). The lower concentration of metabolites in the secretome may be explained by the fact that metabolites have low molecular weight, and the molecular weight *cut-off* of 10 K used to obtain the secretome may influence their enrichment.

It is well known that oocyte growth and developmental competence are supported by the varying composition of FF secretome, which regulates cumulus cell function and expansion, granulosa cell proliferation, and influences the metabolism of somatic cells surrounding the oocyte (Cakmak et al., 2016). Hence, a better understanding of the proteomic and transcriptomic profile of the secretome of the FF might be the key to improving oocyte developmental competence in horses. For this reason, mass spectrometry was performed to explore the proteome of equine secretome from preovulatory FF. First, the proteomic analysis revealed that 65 proteins were listed under the term "*Extracellular space*" (GO:0005615), showing the massive presence of secreted proteins in our samples (Fig. 1). Second, most of the identified proteins were related to the complement-coagulation cascades, coinciding with previous works in humans (Shen et al., 2017), buffalo (Fu et al., 2016), yak (Pei et al., 2022), pig (Paes et al., 2020), and cow (Uzbekova et al., 2020; Afedi et al., 2021). On the other hand, it has already been suggested that inflammation has a key role in folliculogenesis and ovulation (Boots and Jungheim, 2015) and that complement proteins are directly involved in follicle and oocyte development (Jarkovska et al., 2010). Additional enriched categories were related to lipid metabolism, binding and efflux. Notably, our pathway analysis underscored the close interrelation and mutual interactions among apolipoproteins. It is known that lipoproteins are important for oocyte meiosis and follicular cholesterol homeostasis, but also that hypercholesterolemia causes oocyte cholesterol

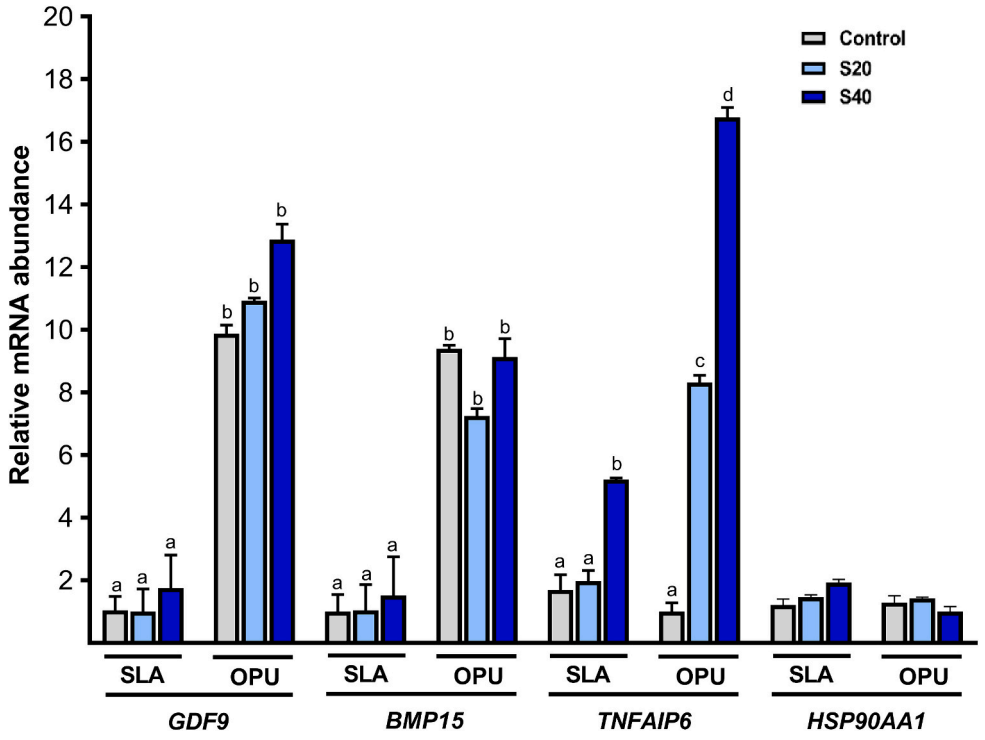

Fig. 3. Relative mRNA abundance of genes related to oocyte quality, meiotic competence, and embryo quality in equine cumulus-oocyte complexes (COCs) retrieved either *in vivo* (OPU) or *post-mortem* (SLA) and matured in the absence or presence of secretome from follicular fluid at 20 μg/ml (S20) or 40 μg/ml (S40). The bars represent the fold change, and the expression of the target genes was normalized against the housekeeping gene *H2AFZ*. The genes shown in the figure are TNF alpha induced protein 6 (*TNFAIP6*), growth differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*) and heat shock protein 90 alpha family class A member 1 (*HSP90AA1*). The data are the mean ± SEM of three replicates. Bars representing a single gene marked with different letters indicate a significant difference ($p < 0.05$).

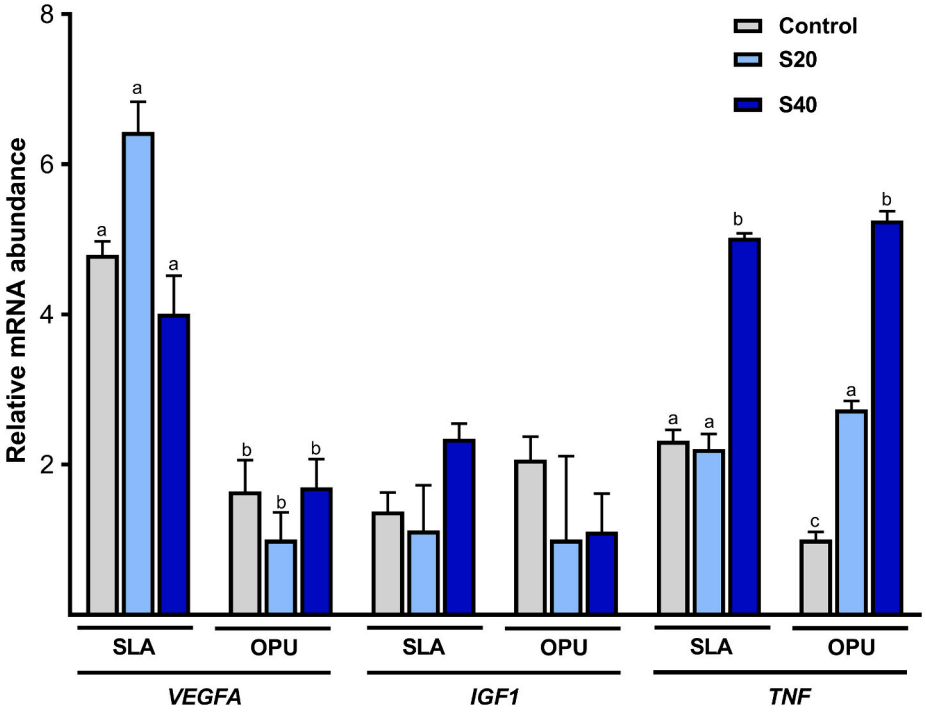

Fig. 4. Relative mRNA abundance of genes related to cytokine and growth factor presence in equine cumulus-oocyte complexes (COCs) retrieved *in vivo* (OPU) or *post-mortem* (SLA) and matured in the absence or presence of secretome retrieved from mare preovulatory follicular fluid at 20 μg/ml (S20) or 40 μg/ml (S40). The bars represent fold change, and the expression of target genes was normalized against H2A histone family member Z (*H2AFZ*), which was used as a housekeeping gene. The genes studied were vascular endothelial growth factor A (*VEGFA*), insulin-like growth factor type I (*IGF1*), and tumor necrosis factor (*hTNF*). Data are presented as the mean ± SEM of three replicates. Bars representing a single gene marked with different letters differ significantly among groups ($p < 0.05$).

overload and infertility in humans (Liu et al., 2022). In older mares, it has been demonstrated that diet supplementation with specific lipids and antioxidants increases oocytes' metabolic activity, diminishes lipid content, and increases the developmental potential of ICSI-derived embryos (Catandi et al., 2022). Hence, it could be relevant to explore if lipoproteins contained in FF secretome might be used as biomarkers to predict the success of oocyte maturation and competence in horses.

Instead of using native FF, other approaches such as the addition of EVs obtained from FF during oocyte IVM have been tried in bovine (da Silveira et al., 2017), feline (de Almeida Monteiro et al., 2020), and equine (Gabryś et al., 2022) studies. In bovine, the addition of EVs retrieved from follicles ranging from 3 to 6 mm during IVM increased blastocyst yield (da Silveira et al., 2017), but other studies demonstrated that EVs addition during IVM inhibited meiosis progression at 9 h after the beginning of IVM due to modulation of cAMP production (although blastocyst yield was not affected when compared against control) (Pioltine et al., 2020). In cats, the use of EVs did not influence oocytes' meiotic competence in fresh oocytes, but increased meiosis resumption in vitrified oocytes when warming medium was supplemented with EVs (de Almeida Monteiro et al., 2020). In horses, only compact cumulus-oocyte complexes (COCs) subjected to IVM in the presence of EVs from small follicles matured using a 2-step protocol resulted in a significant increase in their maturation rate (Gabryś et al., 2022). Although EVs can be purified from secretomes, the isolation of EVs requires expensive and time-consuming techniques such as size exclusion columns, ultracentrifugation, *etc.* In our work, we aimed to confirm the presence of EVs in the secretome using cryo-transmission electron microscopy (cryo-TEM). These experiments revealed the presence of EVs of diverse sizes enclosed in a double membrane (Fig. 2). Besides, other particles devoid of membrane were visualized, which, due to their size and previous reports in horses (Gerard et al., 2002), could correspond to lipoproteins (van Antwerpen et al., 1999).

Once the composition of the secretome was investigated, we aimed to test if its addition during IVM to equine oocytes affected their meiotic competence. In our experiments, the addition of secretome at the dosages tested did not affect the oocyte's meiotic competence. These findings contrast with those of Gabrys et al. (2022) who found an increase in MII after EVs addition to compact COCs (Gabryś et al., 2022), however, we did not separate the COCs depending upon their cumulus appearance and, considering that compact cumuli account for the 30% of the retrieved COCs (González-Fernández et al., 2018; Hinrichs, 2010), mixing compact and expanded cumuli may result in no significant differences as observed in our experiments. In our setting, maturation rates are low compared to other reports that reach a 60% MII of the COCs subjected to IVM (Hinrichs, 2018) but are coincident with other reports (47.2%) when TCM-199 was used as the base medium for IVM (Galli et al., 2007; Lewis et al., 2016). In our case, only FSH was added to the IVM medium. It is known that when other meiosis resumption promoters such as epidermal growth factor are used, higher DNA maturation rates are observed (Sánchez-Calabuig et al., 2021), in part explaining our low maturation rates. It must be noted that our OPU-retrieved oocytes were placed in maturation immediately, while *post-mortem* retrieved oocytes were held overnight, and the proportion of oocytes reaching MII was not significantly different. Significant differences could be observed in the proportion of degenerated oocytes in OPU *vs. post-mortem* groups for control (16.7% *vs.* 33.3%, respectively) and S40 (17.8% *vs.* 35.7%, respectively). These differences can be explained by the *post-mortem* degenerative changes occurring during storage of the ovaries for their transport from the slaughter facilities to the laboratory (Hinrichs, 2010). Besides, the percentage of germinal vesicle (GV) in control samples significantly differed among OPU and *post-mortem* groups (27.8% *vs.* 8.7%, respectively, $p < 0.05$). This difference could also be attributed to the use of previous holding, which has demonstrated to increase the percentage of viable GV chromatin from 53% to 70% in the same conditions and medium used in the present work (Choi et al., 2006), although statistically significant differences were not reported by the authors.

Although no significant differences were observed in nuclear maturation, the cytoplasmic maturation of the oocyte can vary, deeply influencing the quality and developmental competence of the oocytes (Watson, 2007). Therefore, in this study, oocytes were analyzed for the expression of candidate genes related with oocyte quality, developmental competence and cytokine and growth factors expression. Growth and differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are known to be oocyte-secreted factors with a crucial role in the control of ovarian function in female reproduction, modulating both the cell fate of the somatic granulosa cells and the quality and developmental competence of the oocyte (Belli and Shimasaki, 2018). Although other studies have reported differences among old and young mares (Campos-Chillon et al., 2015) and mature *vs.* immature oocytes (Scarlet et al., 2017) in horses, no differences have been found among expanded and compact COCs (González-Fernández et al., 2018), even though both genes are known to be related with cumulus expansion and enhanced meiotic competence (Kidder and Vanderhyden, 2010). In our study, the expression of *GDF9* and *BMP15* was significantly increased in OPU-derived oocytes compared to those from the slaughterhouse group. As previously stated, these differences may be related to the *post-mortem* degenerative changes during storage of the ovaries for their transport (up to 5 h in our case), which decreases initial oocyte quality. These results agree with the statistically significant rise observed in degeneration rates in *post-mortem vs.* OPU groups in this study. However, the addition of secretome *per se* did not influence the expression of *BMP15* and *GDF9* in our experimental conditions. Interestingly, secretome addition enhanced the expression of *TNFAIP6* in the *post-mortem* group at 40 μg/ml and in the OPU group for 20 and 40 μg/ml dosages. This gene has been demonstrated to be expressed in the extracellular matrix of equine follicles, as well as in COCs, and has been related with cumulus expansion and extrusion of the detached complex during ovulation in horses (Sayasith et al., 2007). Moreover, a previous study has suggested that this gene could be more reliable in predicting the equine oocyte's cumulus expansion compared to *GDF9* or *BMP15*, as its expression significantly increases in expanded COCs compared to compact COCs in horses (González-Fernández et al., 2018).

Conversely, the expression of *HSP90AA1* was not altered despite secretome addition or retrieval method (OPU *vs. post-mortem*). This gene has been demonstrated to be related to COC's developmental competence in bovine oocytes subjected to heat stress (de Souza et al., 2022) and has been linked to embryo quality in horses, being upregulated in *in vivo*-derived embryos compared to *in vitro*-obtained counterparts (Smits et al., 2011). However, to the authors' best knowledge, no report exists in which this gene has been linked to COC's developmental competence in horses, as described in bovine. Thus, we can only conclude that secretome addition does not alter its expression, at least in our experimental conditions. In addition to exploring candidate genes encoding cytokine expression and growth factors, we investigated the role of secretome addition on *VEGFA*, *IGF1*, and *TNF* expression in our experimental conditions. Cytokines are small-molecular-weight glycoproteins that act as intercellular mediators across various immune effector cells, ovarian somatic cells, and the oocyte (Prins et al., 2020). Some cytokines, such as IL1-β, have been shown to affect the developmental competence of equine oocytes (Caillaud et al., 2008). VEGFA, initially described as a vascular growth factor, is considered one of the most important key regulators of angiogenesis, with a role in primary to antral follicle selection, *corpus luteum* formation (Field et al., 2014), and its addition during oocyte IVM in bovine has been shown to increase oocyte meiotic and developmental competence (Luo et al., 2002). In our experimental setting, *VEGFA* was consistently overexpressed in the *post-mortem* group, compared to the OPU group, and secretome addition did not influence its expression. Interestingly, a prospective study reported that VEGFA concentration in FF was significantly higher when the oocyte failed to fertilize (Malamitsi-Puchner et al., 2001), and high VEGFA levels in FF have also been associated with poor embryo

morphology and low conception rates in IVF (Barroso et al., 1999; Van Blerkom et al., 1997). In addition, elevated VEGFA levels in FF are presumed to be induced by hypoxia and are considered a negative marker of oocyte quality (Revelli et al., 2009). As the *post-mortem* group was subjected to hypoxic conditions during retrieval and transport, this may have triggered the overexpression of *VEGFA*, predicting low quality of the oocytes obtained in the slaughterhouse compared to the OPU group. The mRNA of TNF, a proinflammatory cytokine known to be related with poor oocyte quality and embryo development in different species (Da Broi et al., 2018; Deb et al., 2011), was significantly lower in OPU-derived COCs compared to the *post-mortem* counterparts. Surprisingly, secretome addition induced *TNF* overexpression at 20 and 40 μg/ml in OPU-derived oocytes but only at 40 μg/ml in the *post-mortem* group. Previous reports in humans have demonstrated that FF from Polycystic Ovary Syndrome associated with metabolic syndrome showed higher TNF levels in FF compared to controls, and the percentage of top-quality embryos was decreased in these patients (Niu et al., 2017). In mares affected with metabolic syndrome, *TNF* expression is also enhanced in FF compared to controls (Sessions-Bresnahan and Carnevale, 2014), and metabolic syndrome in horses is associated with impaired fertility (Burns, 2016). Therefore, further studies are required to better understand if secretome addition impairs the oocyte's developmental competence in horses by assessing *in vitro* embryo production, subsequent development, and quality. IGF1, which regulates follicular recruitment and selection, showed no difference in expression between the OPU and *post-mortem* groups, despite secretome addition.

5. Conclusion

Secretome retrieved from equine preovulatory follicles added during IVM to equine oocytes does not affect their meiotic competence or increase their degeneration rates. However, the mRNA expression of *TNFAIP6* and *TNF* increases when secretome is added during IVM in OPU and *post-mortem* obtained oocytes. Besides, the mRNA expression of quality-related genes is different between the OPU and *post-mortem* groups, revealing divergent quality and competence depending on the oocyte source. Further research is needed to fully understand if secretome addition influences the developmental competence of equine oocytes and to validate its use during IVM clinically.

Declaration of generative AI in scientific writing

Artificial intelligence was not used to design or write the present paper.

CRediT authorship contribution statement

Marcos Luis-Calero: Formal analysis, Investigation, Methodology. **Federica Marinaro:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Pablo Fernández-Hernández:** Formal analysis, Investigation, Visualization. **José M. Ortiz-Rodríguez:** Investigation, Methodology, Resources. **Javier G. Casado:** Methodology, Visualization, Writing – original draft. **Eva Pericuesta:** Investigation. **Alfonso Gutiérrez-Adán:** Formal analysis, Funding acquisition, Methodology. **Esperanza González:** Conceptualization, Formal analysis. **Mikel Azkargorta:** Investigation, Methodology. **Ricardo Conde:** Investigation, Methodology. **Maider Bizkarguenaga:** Investigation, Methodology. **Nieves Embade:** Investigation, Methodology. **Félix Elortza:** Investigation. **Juan M. Falcón-Pérez:** Data curation, Formal analysis, Investigation, Methodology. **Óscar Millet:** Data curation, Formal analysis, Investigation, Methodology. **Lauro González-Fernández:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. **Beatriz Macías-García:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgements

Projects PID2020-112723RB-I00 and PID2021-122507OB-I00 funded by MCIN1/AEI/10.13039/501100011033 and by "ERDF A way of making Europe". José Manuel Ortiz Rodríguez was funded by PNR - DIMIVET- University of Bologna - CUP J45F21002000001, Ministry of University and Research (D.M. 737/2021). This work was supported by 'Junta de Extremadura' (Spain) and 'Fondo Europeo de Desarrollo Regional'; Reference: IB20005. Marcos Luis Calero was supported by a grant "Plan Propio de Iniciación a la Investigación, Desarrollo Tecnológico e Innovación. Acción II" from the University of Extremadura (Ref. Beca RC1). Grant FJC2021-047675-I funded by MCIN2/AEI /10.13039/501100011033 and European Union NextGenerationEU/PRTR to Federica Marinaro. We appreciate the kind help of Paula Navarrete López for proteomic figures handling. The help of the veterinary staff of the INCARSA abbatoir is greatly acknowledged. This paper is dedicated to de memory of Dr. Juan Florencio Macías Núñez who seeded the curiosity and love for research in B. M-G.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2024.105222.

References

Afedi, P.A., Larimore, E.L., Cushman, R.A., Raynie, D., Perry, G.A., 2021. iTRAQ-based proteomic analysis of bovine pre-ovulatory plasma and follicular fluid. Domest. Anim. Endocrinol. 76, 106606 https://doi.org/10.1016/j.domaniend.2021.106606.

de Almeida Monteiro, M. Melo Ferraz, Fujihara, M., Nagashima, J.B., Noonan, M.J., Inoue-Murayama, M., Songsasen, N., 2020. Follicular extracellular vesicles enhance meiotic resumption of domestic cat vitrified oocytes. Sci. Rep. 10, 8619. https://doi.org/10.1038/s41598-020-65497-w.

van Antwerpen, R., La Belle, M., Navratilova, E., Krauss, R.M., 1999. Structural heterogeneity of apoB-containing serum lipoproteins visualized using cryo-electron microscopy. J. Lipid Res. 40, 1827–1836. https://doi.org/10.1016/S0022-2275(20)34899-9.

Barroso, G., Barrionuevo, M., Rao, P., Graham, L., Danforth, D., Huey, S., et al., 1999. Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. Fertil. Steril. 72, 1024–1026. https://doi.org/10.1016/S0015-0282(99)00442-2.

Belli, M., Shimasaki, S., 2018. Molecular Aspects and Clinical Relevance of GDF9 and BMP15 in Ovarian Function. Vitamins and Hormones, vol. 107. Elsevier, pp. 317–348. https://doi.org/10.1016/bs.vh.2017.12.003.

Bonetti, T.C., Haddad, D.C., Domingues, T.S., Alegretti, J.R., Motta, E.L., Seeley, K., et al., 2019. Expressed proteins and activated pathways in conditioned embryo culture media from IVF patients are diverse according to infertility factors. JBRA Assist. Reprod. https://doi.org/10.5935/1518-0557.20190039.

Boots, C., Jungheim, E., 2015. Inflammation and human ovarian follicular dynamics. Semin. Reprod. Med. 33, 270–275. https://doi.org/10.1055/s-0035-1554928.

Brom-de-Luna, J.G., Salgado, R.M., Felix, M.R., Canesin, H.S., Stefanovski, D., Diaw, M., et al., 2021. Culture protocols for horse embryos after ICSI: effect of myo-inositol and time of media change. Anim. Reprod. Sci. 233, 106819 https://doi.org/10.1016/j.anireprosci.2021.106819.

Burns, T.A., 2016. Effects of common equine endocrine diseases on reproduction. Vet. Clin. N. Am. Equine Pract. 32, 435–449. https://doi.org/10.1016/j.cveq.2016.07.005.

Caillaud, M., Dell'Aquila, M.E., De Santis, T., Nicassio, M., Lacalandra, G.M., Goudet, G., et al., 2008. In vitro equine oocyte maturation in pure follicular fluid plus interleukin-1 and fertilization following ICSI. Anim. Reprod. Sci. 106, 431–439. https://doi.org/10.1016/j.anireprosci.2007.06.005.

Cakmak, H., Franciosi, F., Zamah, A.M., Cedars, M.I., Conti, M., 2016. Dynamic secretion during meiotic reentry integrates the function of the oocyte and cumulus cells. Proc. Natl. Acad. Sci. U. S. A. 113, 2424–2429. https://doi.org/10.1073/pnas.1519990113.

Campos-Chillon, F., Farmerie, T.A., Bouma, G.J., Clay, C.M., Carnevale, E.M., 2015. Effects of aging on gene expression and mitochondrial DNA in the equine oocyte and follicle cells. Reprod. Fertil. Dev. 27, 925. https://doi.org/10.1071/RD14472.

Catandi, G.D., LiPuma, L., Obeidat, Y.M., Maclellan, L.J., Broeckling, C.D., Chen, T., et al., 2022. Oocyte metabolic function, lipid composition, and developmental potential are altered by diet in older mares. Reproduction 163, 183–198. https://doi.org/10.1530/REP-21-0351.

Choi, Y.H., Love, L.B., Varner, D.D., Hinrichs, K., 2006. Holding immature equine oocytes in the absence of meiotic inhibitors: effect on germinal vesicle chromatin and blastocyst development after intracytoplasmic sperm injection. Theriogenology 66, 955–963. https://doi.org/10.1016/j.theriogenology.2006.01.064.

Choi, Y.-H., Velez, I.C., Macías-García, B., Riera, F.L., Ballard, C.S., Hinrichs, K., 2016. Effect of clinically-related factors on in vitro blastocyst development after equine ICSI. Theriogenology 85, 1289–1296. https://doi.org/10.1016/j.theriogenology.2015.12.015.

Claes, A., Stout, T.A.E., 2022. Success rate in a clinical equine in vitro embryo production program. Theriogenology 187, 215–218. https://doi.org/10.1016/j.theriogenology.2022.04.019.

Da Broi, M.G., Giorgi, V.S.I., Wang, F., Keefe, D.L., Albertini, D., Navarro, P.A., 2018. Influence of follicular fluid and cumulus cells on oocyte quality: clinical implications. J. Assist. Reprod. Genet. 35, 735–751. https://doi.org/10.1007/s10815-018-1143-3.

Deb, G.K., Dey, S.R., Bang, J.I., Cho, S.J., Park, H.C., Lee, J.G., et al., 2011. 9-cis retinoic acid improves developmental competence and embryo quality during in vitro maturation of bovine oocytes through the inhibition of oocyte tumor necrosis factor-α gene expression1. J. Anim. Sci. 89, 2759–2767. https://doi.org/10.2527/jas.2011-3848.

Deftu, A.T., Radu, B.M., Cretoiu, D., Deftu, A.F., Cretoiu, S.M., Xiao, J., 2020. Exosomes as intercellular communication messengers for cardiovascular and cerebrovascular diseases. In: Exosomes. Elsevier, pp. 199–238. https://doi.org/10.1016/B978-0-12-816053-4.00009-2.

Dell'Aquila, M.E., Cho, Y.S., Minoia, P., Traina, V., Lacalandra, G.M., Maritato, F., 1997. Effects of follicular fluid supplementation of in-vitro maturation medium on the fertilization and development of equine oocytes after in- vitro fertilization or intracytoplasmic sperm injection. Hum. Reprod. 12, 2766–2772. https://doi.org/10.1093/humrep/12.12.2766.

Douet, C., Parodi, O., Martino, N.A., Lacalandra, G.M., Nicassio, M., Reigner, F., et al., 2017. Exposure to follicular fluid during oocyte maturation and oviductal fluid during post-maturation does not improve *in vitro* embryo production in the horse. Zygote 25, 612–630. https://doi.org/10.1017/S096719941700048X.

Fernández-Hernández, P., Sánchez-Calabuig, M.J., García-Marín, L.J., Bragado, M.J., Gutiérrez-Adán, A., Millet, Ó., et al., 2020. Study of the metabolomics of equine preovulatory follicular fluid: a way to improve current in vitro maturation media. Animals 10. https://doi.org/10.3390/ani10050883.

Field, S.L., Dasgupta, T., Cummings, M., Orsi, N.M., 2014. Cytokines in ovarian folliculogenesis, oocyte maturation and luteinisation: cytokines in folliculogenesis. Mol. Reprod. Dev. 81, 284–314. https://doi.org/10.1002/mrd.22285.

Fu, Q., Huang, Y., Wang, Z., Chen, F., Huang, D., Lu, Y., et al., 2016. Proteome profile and quantitative proteomic analysis of Buffalo (Bubalusbubalis) follicular fluid during follicle development. IJMS 17, 618. https://doi.org/10.3390/ijms17050618.

Gabryś, J., Kij-Mitka, B., Sawicki, S., Kochan, J., Nowak, A., Łojko, J., et al., 2022. Extracellular vesicles from follicular fluid may improve the nuclear maturation rate of in vitro matured mare oocytes. Theriogenology 188, 116–124. https://doi.org/10.1016/j.theriogenology.2022.05.022.

Galli, C., Colleoni, S., Duchi, R., Lagutina, I., Lazzari, G., 2007. Developmental competence of equine oocytes and embryos obtained by in vitro procedures ranging from in vitro maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. Anim. Reprod. Sci. 98, 39–55. https://doi.org/10.1016/j.anireprosci.2006.10.011.

Galli, C., Colleoni, S., Duchi, R., Lazzari, G., 2016. Male factors affecting the success of equine in vitro embryo production by ovum pickup-intracytoplasmic sperm injection in a clinical setting. J. Equine Vet. 43, S6–10. https://doi.org/10.1016/j.jevs.2016.05.014.

Gérard, N., Fahiminiya, S., Grupen, C.G., Nadal-Desbarats, L., 2015. Reproductive Physiology and Ovarian Folliculogenesis Examined via ^{1} H-NMR Metabolomics Signatures: A Comparative Study of Large and Small Follicles in Three Mammalian Species (*Bos taurus* , *Sus scrofa domesticus* and *Equus ferus caballus*). OMICS: J. Integr. Biol. 19, 31–40. https://doi.org/10.1089/omi.2014.0097.

Gerard, N., Loiseau, S., Duchamp, G., Seguin, F., 2002. Analysis of the variations of follicular fluid composition during follicular growth and maturation in the mare using proton nuclear magnetic resonance (1H NMR). Reproduction 241–248. https://doi.org/10.1530/rep.0.1240241.

Gillespie, M., Jassal, B., Stephan, R., Milacic, M., Rothfels, K., Senff-Ribeiro, A., et al., 2022. The reactome pathway knowledgebase 2022. Nucleic Acids Res. 50, D687–D692. https://doi.org/10.1093/nar/gkab1028.

González-Fernández, L., Sánchez-Calabuig, M.J., Alves, M.G., Oliveira, P.F., Macedo, S., Gutiérrez-Adán, A., et al., 2018. Expanded equine cumulus-oocyte complexes exhibit higher meiotic competence and lower glucose consumption than compact cumulus-oocyte complexes. Reprod. Fertil. Dev. 30, 297–306. https://doi.org/10.1071/RD16441.

González-Fernández, L., Sánchez-Calabuig, M.J., Calle-Guisado, V., García-Marín, L.J., Bragado, M.J., Fernández-Hernández, P., et al., 2020. Stage-specific metabolomic changes in equine oviductal fluid: new insights into the equine fertilization environment. Theriogenology 143, 35–43. https://doi.org/10.1016/j.theriogenology.2019.11.035.

Harmati, M., Bukva, M., Böröczky, T., Buzás, K., Gyukity-Sebestyén, E., 2021. The role of the metabolite cargo of extracellular vesicles in tumor progression. Cancer Metastasis Rev. 40, 1203–1221. https://doi.org/10.1007/s10555-021-10014-2.

Hinrichs, K., 2010. The equine oocyte: factors affecting meiotic and developmental competence. Mol. Reprod. Dev. 77, 651–661. https://doi.org/10.1002/mrd.21186.

Hinrichs, K., 2018. Assisted reproductive techniques in mares. Reprod. Domest. Anim. 53, 4–13. https://doi.org/10.1111/rda.13259.

Hinrichs, K., Love, C.C., Brinsko, S.P., Choi, Y.H., Varner, D.D., 2002. In vitro fertilization of in vitro-matured equine oocytes: effect of maturation medium, duration of maturation, and sperm calcium Ionophore treatment, and comparison with rates of fertilization in vivo after Oviductal Transfer1. Biol. Reprod. 67, 256–262. https://doi.org/10.1095/biolreprod67.1.256.

Hinrichs, K., Matthews, G.L., Freeman, D.A., Torello, E.M., 1998. Oocyte transfer in mares. J. Am. Vet. Med. Assoc. 212, 982–986.

Jarkovska, K., Martinkova, J., Liskova, L., Halada, P., Moos, J., Rezabek, K., et al., 2010. Proteome mining of human follicular fluid reveals a crucial role of complement Cascade and key biological pathways in women undergoing *in vitro* fertilization. J. Proteome Res. 9, 1289–1301. https://doi.org/10.1021/pr900802u.

Kidder, G.M., Vanderhyden, B.C., 2010. Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence. Can. J. Physiol. Pharmacol. 88, 399–413. https://doi.org/10.1139/Y10-009.

Lazzari, G., Colleoni, S., Crotti, G., Turini, P., Fiorini, G., Barandalla, M., et al., 2020. Laboratory production of equine embryos. J. Equine Vet. 89, 103097 https://doi.org/10.1016/j.jevs.2020.103097.

Lewis, N., Hinrichs, K., Brison, D., Sturmey, R., White, D.G., Schnauffer, K., et al., 2016. Effect of pyruvate addition and oxygen concentration on carbohydrate metabolism during in vitro maturation of equine oocytes. J. Equine Vet. 41, 64. https://doi.org/10.1016/j.jevs.2016.04.046.

Lewis, N., Hinrichs, K., Leese, H.J., McG Argo, C., Brison, D.R., Sturmey, R., 2020. Energy metabolism of the equine cumulus oocyte complex during in vitro maturation. Sci. Rep. 10, 3493. https://doi.org/10.1038/s41598-020-60624-z.

Liu, T., Qu, J., Tian, M., Yang, R., Song, X., Li, R., et al., 2022. Lipid metabolic process involved in oocyte maturation during Folliculogenesis. Front. Cell Dev. Biol. 10, 806890 https://doi.org/10.3389/fcell.2022.806890.

Lopera-Vasquez, R., Hamdi, M., Maillo, V., Lloreda, V., Coy, P., Gutierrez-Adan, A., et al., 2017. Effect of bovine oviductal fluid on development and quality of bovine embryos produced in vitro. Reprod. Fertil. Dev. 29, 621. https://doi.org/10.1071/RD15238.

Luo, H., Kimura, K., Aoki, M., Hirako, M., 2002. Effect of vascular endothelial growth factor on maturation, fertilization and developmental competence of bovine oocytes. J. Vet. Med. Sci. 64, 803–806. https://doi.org/10.1292/jvms.64.803.

Malamitsi-Puchner, A., Sarandakou, A., Baka, S.G., Tziotis, J., Rizos, D., Hassiakos, D., et al., 2001. Concentrations of angiogenic factors in follicular fluid and oocyte-cumulus complex culture medium from women undergoing in vitro fertilization: association with oocyte maturity and fertilization. Fertil. Steril. 76, 98–101. https://doi.org/10.1016/S0015-0282(01)01854-4.

Marinaro, F., Macías-García, B., Sánchez-Margallo, F.M., Blázquez, R., Álvarez, V., Matilla, E., et al., 2019. Extracellular vesicles derived from endometrial human mesenchymal stem cells enhance embryo yield and quality in an aged murine model. Biol. Reprod. 100, 1180–1192. https://doi.org/10.1093/biolre/ioy263.

Miro, J., Catalán, J., Martínez-Rodero, I., Yánez-Ortiz, I., Mateo-Otero, Y., Flores-Bragulat, A., et al., 2022. Metabolic profiling of Preovulatory follicular fluid in jennies. SSRN J. https://doi.org/10.2139/ssrn.4184561.

Mishra, D., Banerjee, D., 2023. Secretome of stromal Cancer-associated fibroblasts (CAFs): relevance in Cancer. Cells 12, 628. https://doi.org/10.3390/cells12040628.

Niu, Z., Ye, Y., Xia, L., Feng, Y., Zhang, A., 2017. Follicular fluid cytokine composition and oocyte quality of polycystic ovary syndrome patients with metabolic syndrome undergoing in vitro fertilization. Cytokine 91, 180–186. https://doi.org/10.1016/j.cyto.2016.12.020.

Ormazabal, V., Nova-Lampeti, E., Rojas, D., Zúñiga, F.A., Escudero, C., Lagos, P., et al., 2022. Secretome from human mesenchymal stem cells-derived endothelial cells promotes wound healing in a Type-2 diabetes mouse model. IJMS 23, 941. https://doi.org/10.3390/ijms23020941.

Paes, V., De Figueiredo, J., Ryan, P., Willard, S., Feugang, J., 2020. Comparative analysis of porcine follicular fluid proteomes of small and large ovarian follicles. Biology 9, 101. https://doi.org/10.3390/biology9050101.

Pei, J., Song, R., Bao, P., Yin, M., Li, J., Zhang, G., et al., 2022. Differential proteomic analysis demonstrates follicle fluid participate immune reaction and protein translation in yak. BMC Vet. Res. 18, 34. https://doi.org/10.1186/s12917-021-03097-0.

Pioltine, E.M., Machado, M.F., da Silveira, J.C., Fontes, P.K., Botigelli, R.C., Quaglio, A.E.V., et al., 2020. Can extracellular vesicles from bovine ovarian follicular fluid modulate the in-vitro oocyte meiosis progression similarly to the CNP-NPR2 system? Theriogenology 157, 210–217. https://doi.org/10.1016/j.theriogenology.2020.06.031.

Prins, J.R., Marissen, L.M., Scherjon, S.A., Hoek, A., Cantineau, A.E.P., 2020. Is there an immune modulating role for follicular fluid in endometriosis? A narrative review. Reproduction 159, R45–R54. https://doi.org/10.1530/REP-19-0050.

Revelli, A., Piane, L.D., Casano, S., Molinari, E., Massobrio, M., Rinaudo, P., 2009. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. Reprod. Biol. Endocrinol. 7, 40. https://doi.org/10.1186/1477-7827-7-40.

Sánchez-Calabuig, M.J., Fernández-González, R., Hamdi, M., Smits, K., López-Cardona, A.P., Serres, C., et al., 2021. A high glucose concentration during early stages of in vitro equine embryo development alters expression of genes involved in glucose metabolism. Equine Vet. J. 53, 787–795. https://doi.org/10.1111/evj.13342.

Sayasith, K., Doré, M., Sirois, J., 2007. Molecular characterization of tumor necrosis α-induced protein 6 and its human chorionic gonadotropin-dependent induction in

theca and mural granulosa cells of equine preovulatory follicles. Reproduction 133, 135–145. https://doi.org/10.1530/rep.1.01200.
Scarlet, D., Ille, N., Ertl, R., Alves, B.G., Gastal, G.D.A., Paiva, S.O., et al., 2017. Glucocorticoid metabolism in equine follicles and oocytes. Domest. Anim. Endocrinol. 59, 11–22. https://doi.org/10.1016/j.domaniend.2016.10.004.
Sessions-Bresnahan, D.R., Carnevale, E.M., 2014. The effect of equine metabolic syndrome on the ovarian follicular environment1. J. Anim. Sci. 92, 1485–1494. https://doi.org/10.2527/jas.2013-7275.
Shen, X., Liu, X., Zhu, P., Zhang, Y., Wang, J., Wang, Y., et al., 2017. Proteomic analysis of human follicular fluid associated with successful in vitro fertilization. Reprod. Biol. Endocrinol. 15, 58. https://doi.org/10.1186/s12958-017-0277-y.
Sherman, B.T., Hao, M., Qiu, J., Jiao, X., Baseler, M.W., Lane, H.C., et al., 2022. DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). Nucleic Acids Res. 50, W216–W221. https://doi.org/10.1093/nar/gkac194.
da Silveira, J.C., Andrade, G.M., del Collado, M., Sampaio, R.V., Sangalli, J.R., Silva, L. A., et al., 2017. Supplementation with small-extracellular vesicles from ovarian follicular fluid during in vitro production modulates bovine embryo development. PLoS One 12, e0179451. https://doi.org/10.1371/journal.pone.0179451.
Smits, K., Goossens, K., Van Soom, A., Govaere, J., Hoogewijs, M., Peelman, L.J., 2011. In vivo-derived horse blastocysts show transcriptional upregulation of developmentally important genes compared with in vitro-produced horse blastocysts. Reprod. Fertil. Dev. 23, 364. https://doi.org/10.1071/RD10124.
de Souza, E.D., da Silva e Souza, J.F., de Oliveira Netto, P.M., de R Carvalheira, L., RITP, Batista, CCR, Quintão, et al., 2022. Inhibition of Hsp90 during *in vitro* maturation under thermoneutral or heat shock conditions compromises the developmental competence of bovine oocytes. Zygote 30, 854–862. https://doi.org/10.1017/S0967199422000387.
Spacek, S.G., Carnevale, E.M., 2018. Impact of equine and bovine oocyte maturation in follicular fluid from young and old mares on embryo production in vitro. J. Equine Vet. 68, 94–100. https://doi.org/10.1016/j.jevs.2018.04.009.
Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., et al., 2015. STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res. 43, D447–D452. https://doi.org/10.1093/nar/gku1003.
The UniProt Consortium, 2023. UniProt: the universal protein knowledgebase in 2023. Nucleic Acids Res. 51, D523–D531. https://doi.org/10.1093/nar/gkac1052.
Tjalsma, H., Bolhuis, A., Jongbloed, J.D.H., Bron, S., van Dijl, J.M., 2000. Signal peptide-dependent protein transport in *Bacillus subtilis* : a genome-based survey of the Secretome. Microbiol. Mol. Biol. Rev. 64, 515–547. https://doi.org/10.1128/MMBR.64.3.515-547.2000.
Uzbekova, S., Almiñana, C., Labas, V., Teixeira-Gomes, A.-P., Combes-Soia, L., Tsikis, G., et al., 2020. Protein cargo of extracellular vesicles from bovine follicular fluid and analysis of their origin from different ovarian cells. Front. Vet. Sci. 7.
Van Blerkom, J., Antczak, M., Schrader, R., 1997. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Hum. Reprod. 12, 1047–1055. https://doi.org/10.1093/humrep/12.5.1047.
Watson, A.J., 2007. Oocyte cytoplasmic maturation: a key mediator of oocyte and embryo developmental competence1. J. Anim. Sci. 85, E1–E3. https://doi.org/10.2527/jas.2006-432.
Wiśniewski, J.R., Zougman, A., Nagaraj, N., Mann, M., 2009. Universal sample preparation method for proteome analysis. Nat. Methods 6, 359–362. https://doi.org/10.1038/nmeth.1322.