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Original Research Article

Seminal extracellular vesicles alter porcine *in vitro* fertilization outcome by modulating sperm metabolism

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ABSTRACT

Porcine seminal plasma (SP) is loaded with a heterogeneous population of extracellular vesicles (sEVs) that modulate several reproductive-related processes. This study investigated the effect of two sEV subsets, small (S-sEVs) and large (L-sEVs), on porcine *in vitro* fertilization (IVF). The sEVs were isolated from nine SP pools (five ejaculates/pool) using a size-exclusion chromatography-based procedure and characterized for quantity (total protein), morphology (cryogenic electron microscopy), size distribution (dynamic light scattering), purity and EV-protein markers (flow cytometry; albumin, CD81, HSP90β). The characterization confirmed the existence of two subsets of high purity (low albumin content) sEVs that differed in size (S- and L-sEVs). *In vitro* fertilization was performed with *in vitro* matured oocytes and frozen-thawed spermatozoa and the IVF medium was supplemented during gamete coincubation (1 h at 38.5 °C, 5 % CO_2 in a humidified atmosphere) with three different concentrations of each sEV subset: 0 (control, without sEVs), 0.1, and 0.2 mg/mL. The first experiment showed that sEVs, regardless of subset and concentration, decreased penetration rates and total IVF efficiency ($P < 0.0001$). In a subsequent experiment, it was shown that sEVs, regardless of subset and concentration, impaired the ability of spermatozoa to bind to the zona pellucida of oocytes ($P < 0.0001$). The following experiment showed that sEVs, regardless of the subset, bound to frozen-thawed sperm but not to *in vitro* matured oocytes, indicating that sEVs would affect sperm functionality but not oocyte functionality. The lack of effect on oocytes was confirmed by incubating sEVs with oocytes prior to IVF, achieving sperm-zona pellucida binding results similar to those of control. In the last experiment, conducted under IVF conditions, sperm functionality was analyzed in terms of tyrosine phosphorylation, acrosome integrity and metabolism. The sEVs, regardless of the subset, did not affect sperm tyrosine phosphorylation or acrosome integrity, but did influence sperm metabolism by decreasing sperm ATP production under capacitating conditions. In conclusion, this study demonstrated that the presence of sEVs on IVF medium impairs IVF outcomes, most likely by altering sperm metabolism.

1. Introduction

The *in vitro* production (IVP) of porcine embryos has become increased interest for animal production as well as for biotechnological and biomedical research [1]. In addition, the porcine species, with its genetic, anatomical, and physiological similarities to humans, is considered an excellent animal model for human reproductive health [2]. Porcine IVP systems comprise three essential steps (i) *in vitro* oocyte maturation (IVM), (ii) *in vitro* fertilization (IVF), and (iii) *in vitro* embryo culture (IVC). Significant advances have been made in each of these steps over the past two decades, improving the quality of embryos produced [3]. However, the overall performance of porcine IVP is still far from that achieved *in vivo* [4] and is also significantly lower than that achieved in other mammalian species [5]. The efficiency of IVP in pigs is particularly hampered by the high incidence of polyspermy [5]. Several causes have been identified to explain the low IVP efficiency, including (i) suboptimal IVM, (ii) high proportions of acrosome-reacted sperm during IVF, and (iii) use of suboptimal *in vitro* culture media [5].

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Therefore, the development of strategies to overcome the low efficiency of porcine IVP remains a challenge for the scientific community.

In recent years, there has been growing scientific interest in extracellular vesicles (EVs), membrane-bound nanoparticles loaded with bioactive molecules such as proteins, nucleic acids, and lipids that are released into the extracellular environment by most body functional cells [6]. The relevance of EVs lies in their ability to serve as essential cell-to-cell messengers, transporting their cargo from origin cells to target cells where they elicit specific functional responses [6]. Thus, EVs are involved in the regulation of a variety of physiological and pathological processes [7], including those related to reproduction [8,9]. Due to the wide distribution of secretory cells throughout the body, EVs can be isolated from any bodily fluid, including those from female and male reproductive tracts [8,9]. The EVs circulating in reproductive fluids are involved in essential physiological processes as fertilization and embryo development [10–12]. *In vitro* experiments conducted in several mammalian species, including pigs, using EVs isolated from female reproductive fluids, provide the most substantial evidence implicating EVs in reproductive performance success [13–23]. These studies reported a beneficial effect on IVP outcomes when IVM, IVF and/or IVC media were supplemented with EVs isolated from oviductal, uterine or follicular fluids.

Seminal plasma (SP), a fluid of complex composition derived primarily from the accessory sex glands that surrounds spermatozoa during and after ejaculation, contains a large and heterogeneous population of EVs (sEVs) [24]. A large body of scientific evidence supports that sEVs are involved in modulating key sperm functions, including capacitation, motility, and acrosome reaction, ultimately influencing sperm fertilization capacity [25–30]. Moreover, sEVs may also be involved in modulating the immune environment of the female genital tract, which could facilitate embryo implantation and development [30,31]. However, there are conflicting results regarding the specific role of sEVs in these reproductive physiological processes [32]. In addition, the direct involvement of sEVs in fertilization has been little studied [30] and, to the best of our knowledge, there are no studies that have evaluated the effect of sEVs on the outcomes of IVF in any animal species. Only in a recent study conducted in mice, Ma et al. [33] reported improvements in embryo development when IVF medium was supplemented with epididymal EVs. A recent study from our research group also showed that large, but not small, porcine sEVs were able to modulate cumulus cell function when added to IVM medium, suggesting the ability of a specific population of sEVs to interact and modulate porcine oocyte physiology [34]. However, it is still a matter of research whether sEVs could also be effective agents for the regulation of the IVF process.

The rationale of this study was to investigate, for the first time in any animal species, the putative effect of two sEV subsets differing in size, termed small (S-sEVs) and large (L-sEVs), on porcine IVF performance. The two sEV subsets were separately isolated by size exclusion chromatography (SEC) and used to supplement the IVF medium. The involvement of sEVs in IVF outcomes was evaluated in terms of penetration rate, total fertilization efficiency and sperm binding to the zona pellucida of oocytes. Likewise, the interaction of sEVs with both gametes was also evaluated, and as a result, the influence of sEVs on the functional performance of spermatozoa was assessed.

2. Material and methods

Ethical statement

The experiments performed in this study are part of two research grants whose experiments have been approved by the Bioethics Committee of the University of Murcia (CBE codes: 367/2020 and 538/2023).

2.1. Reagents

All reagents used in this study (unless stated otherwise) were of analytical grade and provided from Merck KGaA (Darmstadt, Germany).

2.2. Boars, ejaculates, semen cryopreservation, and seminal plasma

The ejaculates were provided by AIM Iberica (Topigs Norsvin, Madrid, Spain), a company specialized in the production and marketing of semen doses for swine artificial insemination (AI). The AI-centers followed the European (ES13RS04P; July 2012) and Spanish (ES300130640127; August 2006) animal health and welfare regulations for the production and marketing of semen doses for swine AI. The boars used as ejaculate donors were of the Landrace, Pietrain and Large White breeds and were all enrolled in commercial AI-programs. Entire ejaculates were collected using the semi-automated Collectis® method (IMV Technologies, L'Aigle, France). All ejaculates included in this study met the sperm quantity/quality parameters for the production of commercial semen doses for swine AI (sperm concentration $> 200 \times 10^6$ sperm/mL, immotile sperm < 25 %, and sperm with abnormal morphology < 20 %).

Semen samples were cryopreserved according to the protocol of Hernández et al. [35]. Briefly, semen samples were centrifuged at 2400×*g* for 3 min at 17 °C (Megafuge 1.0 R, Heraeus, Hanau, Germany). Pellets containing spermatozoa were resuspended in Tris-citric-glucose medium supplemented (v/v) with 20 % egg yolk, 3 % glycerol, and 0.5 % Equex (Nova Chemical Sales, Scituate, MA, USA) to a final concentration of 1.0×10^9 spermatozoa/mL. The diluted spermatozoa were packed into 0.5 mL French straws (Minitüb, Tiefenbach, Germany) and frozen in a speed-controlled freezer (IceCube 1810, Minitüb, Germany) at an average speed of - 40 °C/min.

The isolation of sEVs was performed on nine different semen samples. Each of the samples contained semen from five ejaculates from five different boars. The semen samples were centrifuged twice at 1500×*g* for 10 min at room temperature (RT, Rotofix 32A, Hettich Centrifuge UK, Newport Pagnell, Buckinghamshire, England, UK) to collect SP. The resulting SP samples were examined microscopically (Nikon Eclipse E400; Nikon Europe BV, Badhoevedorp, Netherlands) to verify the absence of spermatozoa, stored in insulated containers and shipped at 5 °C to the laboratory, where sEV isolation was performed.

2.3. Isolation of two seminal extracellular vesicle subsets

Two subsets of sEVs of different sizes, namely S-sEVs and L-sEVs, were isolated from each of the nine SP samples using a SEC-based isolation protocol standardized for porcine SP by our research group [36]. Briefly, SP samples were centrifuged at 3200×*g* at 4 °C for 15 min (Sorvall™ STR40, Thermo Fisher Scientific, Waltham, Massachusetts, USA) to remove cell debris. The supernatants were transferred to new tubes and centrifuged at 20,000×*g* at 4 °C for 30 min (Sorvall™ Legend™ Micro 21R, Thermo Fisher Scientific). The resulting pellets and supernatants were then processed separately. Pellets, containing larger sEVs, were diluted in 0.22-μm filtered phosphate buffered saline (fPBS) to a volume of 0.5 mL, and the sample was ready for loading onto SEC columns. Supernatants, containing smaller sEVs, were diluted (1:2; v:v) in fPBS, filtered (0.22 μm; Millex® Syringe Filters) and concentrated (Amicon® Ultra-4 mL centrifugal filter 10 kDa) to a volume of 2 mL. The sample was ready for use in the SEC. Home-made columns with filter tubes (Econo-Pac® Chromatography Columns, Bio-Rad, Hercules, California, USA) and Sepharose CL2B® (10 mL) were used for SEC. Twenty eluted fractions (500 μL each) were collected in each SEC and fractions 7 to 10 were selected and pooled as they were the most enriched in sEVs. Thus, two sEV samples, one S-sEV sample and one L-sEV sample, were obtained for each of the nine SP samples. The resulting 18 samples of sEVs, nine samples of S-sEVs and nine samples of L-sEVs, were stored at -80 °C (Ultra Low Freezer; Haier Inc., Qingdao, China) until further use.

2.4. Characterization of seminal extracellular vesicles subsets

The sEV samples were characterized using multiple, combined, and complementary techniques according to the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines [37]. Specifically, the sEV samples were characterized in terms of (1) total protein concentration using a commercial kit (Micro BCA™ Protein Assay Kit; Thermo Fisher Scientific), (2) particle size distribution by dynamic light scattering (DLS) analysis, (3) morphology of sEVs by cryogenic electron microscopy (cryo-EM), (4) presence of EV-specific protein markers (CD81 and HSP90β), and (5) presence of non-EV particles by albumin content analysis. Characterizations 4 and 5 were performed by flow cytometry. Details of the characterization of sEVs are provided in Supplementary File 1.

2.5. Oocyte collection and in vitro maturation

Ovaries were collected from prepubertal gilts at a local abattoir and transported in saline solution (0.9 % w:v NaCl) at 37 °C to the laboratory within 1 h. Then, cumulus-oocyte complexes (COCs) were aspirated from 4 to 6 mm antral follicles using an 18-gauge needle attached to a 10-mL disposable syringe. Then, intact COCs (those with uniform cytoplasm with more than three layers of cumulus cells observed under a stereomicroscope) were selected for our study and placed in a Petri dish (35 mm, Nunclon, Denmark), which was prefilled with PBS (2 mL) supplemented with 0.4 % bovine serum albumin (BSA). Then, COCs were washed three times in NCSU 37 medium [38] supplemented with 5 μg/mL insulin, 1 mM glutamine, 57 mM cysteine, 10 ng/mL epidermal growth factor, 50 μM β-mercaptoethanol and 10 % porcine follicular fluid (referred to as IVM medium). COCs (groups of 50) were placed in a Nunc 4-well multidish (Thermo Fisher Scientific) which was prefilled with IVM medium (500 μL) supplemented with 1.0 mM db-cAMP and 0.12 IU/mL Pluset® (Carlier, Italy) and cultured in a humidified atmosphere of 5 % CO_2 in air at 38.5 °C for 22 h. After this period, COCs were transferred to a new Nunc 4-well multidish prefilled with fresh IVM medium (500 μL) and cultured for 22 h under the same conditions. At the end of IVM, the oocytes were denuded by gentle repeated pipetting.

2.6. In vitro fertilization

For IVF, the standard protocol routinely used in our laboratory was followed [39]. The frozen semen samples were obtained from two boars that were used in commercial AI programs. In each experiment, semen straws from both AI-boars were thawed by vigorous shaking for 20 s in a thermostatically controlled bath at 37 °C. The contents of the straws were pooled and diluted (1:3; v:v) in Beltsville Thawing Solution (BTS). The thawed semen samples were then incubated for 1 h at 37 °C, washed twice with BTS (900×*g*, 2 min at RT) and the resulting frozen-thawed sperm pellets resuspended in Brackett & Oliphant's medium [40] supplemented with 12 % heat-inactivated fetal calf serum (FCS, Gibco, Invitrogen, Italy) and 0.7 mg/mL caffeine (referred to as IVF medium). Then, *in vitro* matured oocytes were transferred to wells of a Nunc 4-well multiplate that were prefilled with IVF medium. Wells were then seeded with frozen-thawed spermatozoa (1.25×10^6 spermatozoa/mL). Gamete coincubation was performed at 38.5 °C in a humidified atmosphere of 5 % CO_2 in air for 1 h. More specific details about IVF are given in the experimental design section (2.10.1 Experiment 1: Effect of supplementing IVF medium with sEV subsets on IVF outcomes and sperm-zona pellucida binding).

2.7. Labelling of seminal extracellular vesicle subsets

To investigate the putative interaction of S- and L-sEVs with frozen-thawed spermatozoa and/or *in vitro* matured oocytes, both sEV subsets were labeled with the Vybrant DiI cell labeling solution (#V22885; Thermo Fisher Scientific), an orange-red fluorescent dye that stains membrane lipids. The staining protocol was described by Murdica et al. [41] with minor modifications. Briefly, DiI dye was diluted in fPBS (1:100; v:v) and added to the sEV sample (1:1; v:v) and fPBS (control; 1:1; v:v). The resulting samples were incubated for 20 min at 37 °C with agitation, and then ultracentrifuged at 150,000×*g* for 1 h at 4 °C (Optima L-100 XP Ultracentrifuge using a rotor SW55; Beckman Coulter, CA, USA) to remove the Dil dye not bound to sEVs. The resulting pellets containing stained sEVs were used in the Experiment 2 described below (2.10.2 Interaction of sEV subsets with *in vitro* matured oocytes and frozen-thawed spermatozoa).

2.8. Sperm parameters assessments

2.8.1. Protein tyrosine phosphorylation immunostaining

Immunolocalization of tyrosine-phosphorylated proteins was examined according to the protocol described by Spinaci et al. [42]. An aliquot of each semen sample from each experimental group was washed twice in PBS (900×*g* 2 min at RT), placed on poly-l-lysine-coated slides, and fixed with cold methanol for 15 min at −20 °C and then with acetone for 30 s at RT. Slides were then washed with PBS and non-specific binding sites were blocked with 10 % FCS in PBS (blocking solution) for 30 min. Monoclonal anti-phosphotyrosine antibody (clone 4G10; 1:150 in blocking solution) was added to the slides and incubated overnight at 4 °C. After washing with PBS, the slides were incubated with a sheep anti-mouse fluorescein isotiocyanate (FITC)-conjugated secondary antibody (BioFX Laboratories, Maryland, USA; 1:800 in blocking solution) for 1 h at RT in the dark. Slides were then washed with PBS and mounted with Vectashield mounting medium with propidium iodide counterstain for DNA. A negative control (primary antibody omitted) was processed under the same conditions. Slides were examined using an epifluorescence microscope (Nikon Eclipse E600, Nikon Europe BV) and a total of 200 spermatozoa per slide were visualized to evaluate different patterns of positivity by the same evaluator. Three different patterns of positivity were considered based on the results of Bucci et al. [43]: (i) Pattern A: spermatozoa with acrosomal and equatorial subsegment positivity (typical of non-capacitated spermatozoa); (ii) Pattern B: spermatozoa with acrosomal, equatorial subsegment, and principal piece of the tail positivity (typical of capacitated spermatozoa); and (iii) Pattern C: spermatozoa with equatorial subsegment (faint) and tail positivity. Negative spermatozoa were those without a positive signal and were not included in the results.

2.8.2. Acrosome integrity

Acrosome integrity was evaluated according to the procedure described by Spinaci et al. [42] using a FITC-conjugated lectin from *Pisum Sativum* (FITC-PSA), which labels acrosomal matrix glycoproteins. An aliquot of each semen sample from each experimental group was washed twice in PBS (900×*g* 2 min at RT) and fixed in 95 % ethanol for 30 min at 4 °C. Aliquots of semen samples were dried on slides and incubated with FITC-PSA solution (5 μg PSA-FITC/1 mL H_2O) for 20 min in the dark. The samples were then washed in PBS (900×*g* 2 min at RT) and mounted with Vectashield mounting medium containing propidium iodide. The slides were visualized under epifluorescence microscope and 200 spermatozoa per sample were analyzed. Green fluorescence in the acrosome was considered as sperm with intact acrosome, while a partial/total absence of acrosome green fluorescence was considered as sperm with acrosome disruption or acrosome reaction.

2.8.3. Sperm metabolism assessments

Oxygen consumption rate (OCR), cellular respiration index (pmoL/min) and the extracellular acidification rate (ECAR), glycolysis index (mpH/min) were measured in sperm samples from each experimental group using Seahorse XFp analyzer (Agilent, Santa Clara, CA, USA). Briefly, 1.5×10^6 frozen-thawed sperm/well were added to XFp cell culture mini-plates (Agilent, USA) previously coated with 10 μL of fibronectin (1 mg/mL in water) dried in an incubator at 37 °C for 2 h.

The mini-plates were then centrifuged at 1200×*g* for 1 min at 20 °C and the resulting supernatant was removed and replaced with 180 μL of BTS medium - standard buffer factor was 2.6 nmol/L/pH (which takes into account the amount of H^+ added to the analysis medium to change the pH level by 1 unit of pH) plus 5.56 mM glucose, 2 mM L-glutamine and 1 mM sodium pyruvate, preheated 10 min at 37 °C, and the analysis was then started. In addition, the injection ports of the XFp sensor cartridges were hydrated overnight at 37 °C with the XF calibrant and then loaded with ten times the concentration of inhibitors as indicated in the instructions for the Seahorse XFp ATP Rate assay test and the Cell Mito Stress test. For the ATP Rate Assay, final concentrations of 1.5 μM oligomycin (olig, port A) and 0.5 μM rotenone (rot) plus 0.5 μM antimycin A (AA, port B) were used. Instead, for the Cell Mito Stress Test, the final concentrations were 1.5 μM olig (port A), 4.0 μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, port B), and 0.5 μM of rot plus 0.5 μM AA (port C). Data were analyzed using the WAVE software (Agilent). Prior to analysis, the OCR and ECAR values were normalized to 1.0 × 10^6 live spermatozoa. See Supplementary File S2 and Prieto et al. [44] for more information on the sperm metabolism assessment procedures.

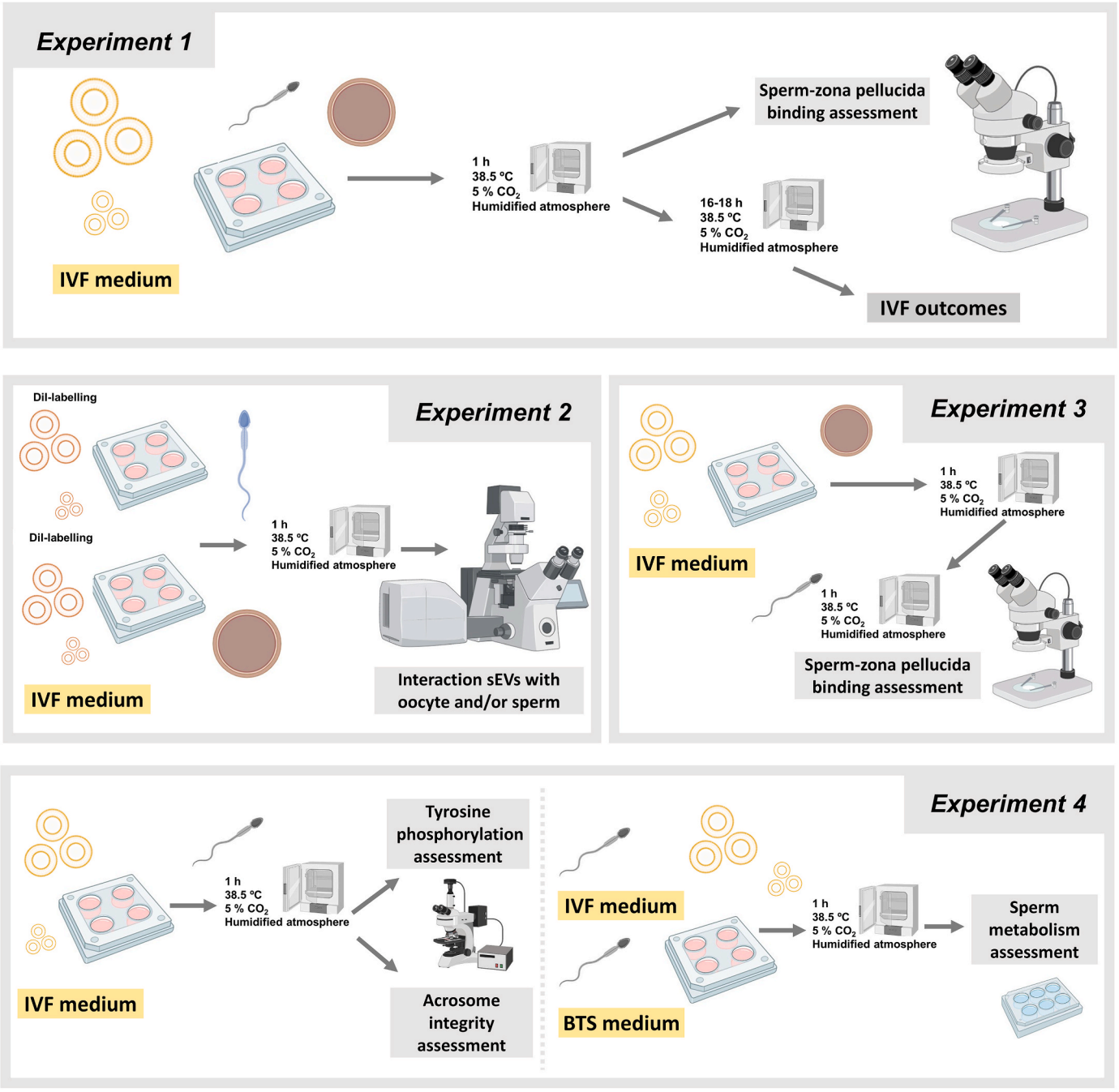

Fig. 1. Experimental design of the study. The study consisted of four experiments that were performed under *in vitro* fertilization (IVF) conditions. Experiment 1 evaluated the effect of large (L-) and small (S-) seminal extracellular vesicles (sEVs) on IVF outcomes (penetration rate and fertilization efficiency) and on the ability of sperm to bind to the zona pellucida of oocytes. Experiment 2 investigated whether L- and S-sEVs bind to frozen-thawed sperm and *in vitro* matured oocytes. Experiment 3 investigated whether oocytes coincubated with the L- and S-sEVs prior to gamete coincubation retained their fertilization potential. Experiment 4 evaluated the functionality of sperm coincubated with L- and S-sEVs in terms of capacitation, acrosome integrity, and metabolism. Created by Biorender.com.

2.9. Experimental design

Fig. 1 shows a schematic overview of the experimental design with a total of four experiments, which are described below.

2.9.1. Experiment 1: Effect of supplementing IVF medium with sEV subsets on IVF outcomes and sperm-zona pellucida binding

Three different sEV concentrations were used for supplementing IVF medium, namely 0 mg/mL (control, without sEVs), 0.1 mg/mL and 0.2 mg/mL. The sEVs (S- or L-sEVs) were placed in Nunc 4-well plates to which *in vitro* matured oocytes, frozen-thawed spermatozoa and additional IVF medium were added.

To assess penetration rate and total fertilization efficiency, the wells contained 50 *in vitro* matured oocytes and a total of 500 μL of IVF medium. After gamete coincubation period, oocytes were transferred to new Nunc 4-well plates containing fresh IVF medium and were incubated in a humidified atmosphere of 5 % CO_2 in air at 38.5 °C for 18–19 h. The oocytes were then placed on slides, covered with a coverslip, fixed in acetic acid/ethanol (1:3; v:v) for 24 h, and then stained with 1 % Lacmoid. Slides were analyzed by phase-contrast microscopy (LEITZ Diaplan). Penetration rate was the ratio of the number of oocytes penetrated to the number of oocytes inseminated. Total fertilization efficiency was the ratio between the number of oocytes containing a sperm head–male pronucleus and the number of oocytes inseminated. Immature and degenerated oocytes were not recorded. Four sets of replicates were performed.

To evaluate sperm-zona pellucida binding, the wells contained 40 *in vitro* matured oocytes and a total of 400 μL of IVF medium. After the gamete coincubation period, oocytes were washed four times in PBS with 0.4 % BSA to remove sperm slightly adhering to the zona pellucida. Oocytes were then fixed in 4 % paraformaldehyde for 15 min at RT, incubated with 8.1 μM Hoechst 33342 (H-42) for 10 min in the dark, washed twice in PBS with 0.4 % BSA, and placed individually in droplets of Vectashield (Vector Laboratories, Burlingame, CA, USA), which were placed on microscope slides and covered with a coverslip. Spermatozoa attached to the zona pellucida of oocytes were visualized by epifluorescence microscopy. Four sets of replicates were performed.

2.9.2. Experiment 2: Interaction of sEV subsets with in vitro matured oocytes and frozen-thawed spermatozoa

In vitro matured oocytes (40 per well) and frozen-thawed spermatozoa (1.25×10^6 sperm/mL) were added separately to 400-μL IVF medium wells containing DiI-labeled S-sEVs or L-sEVs at a concentration of 0.2 mg/mL or DiI-labeled PBS (control). After incubation for 1 h at 38.5 °C in a humidified atmosphere and 5 % CO_2, (1) oocytes were placed in wells with IVF medium for microscopy evaluation, (2) spermatozoa were washed in IVF medium (900×*g*, 2 min, twice), diluted in 100 μL of IVF medium, stained with 8.1 μM H-42 (10 min at 37 °C), fixed with 4 % paraformaldehyde (1:1 v:v) and 10 μL aliquot were mixed with a drop of Vectashield, mounted on a slide and cover slipped. The putative interaction of sEV subsets with oocytes or spermatozoa were analyzed using Nikon Ti-E fluorescence microscope, connected to an A1R confocal system (Nikon, Minato, Tokyo, Japan). Images were acquired using a 40 × objective at a resolution of 1024 × 1024 using a 49.81 μm diameter pinhole. All z-stacks were collected in compliance with the optical section separation (z-Interval) values suggested by the NIS-Elements AR 3.2 software (0.5 μm; 10–11 images). Three sets of replicates were performed.

2.9.3. Experiment 3. Effect of incubating in vitro matured oocytes with sEV subsets prior to gamete coincubation

In vitro matured oocytes (40 per well) were added to 400-μL of IVF medium wells containing S-sEVs or L-sEVs at a concentration of 0.1 mg/mL or 0 mg/mL (control, without sEVs) and incubated for 1 h at 38.5 °C in a humidified atmosphere and 5 % CO_2 in air. The oocytes were then washed twice in fresh IVF medium and transferred to a new well prefilled with 400 μL IVF medium containing frozen-thawed spermatozoa (1.25×10^6 sperm/mL) and incubated for 1 h under the same conditions as above. The oocytes were then fixed, stained and the sperm-zona pellucida binding was analyzed by an epifluorescence microscopy as described in Experiment 1. Three sets of replicates were performed.

2.9.4. Experiment 4. Effect of supplementing IVF medium with sEV subsets on sperm functional performance

2.9.4.1. Experiment 4.1 Effect of sEVs on tyrosine phosphorylation and acrosome integrity. Frozen-thawed spermatozoa (1.25×10^6 spermatozoa/mL) were added to 400-μL wells filled with IVF medium containing 0 mg/mL (without sEVs, control), 0.1 mg/mL and 0.2 mg/mL of S-sEVs or L-sEVs. After 1 h incubation at 38.5 °C in a humidified atmosphere and 5% CO_2 in air, spermatozoa from each well were processed for sperm tyrosine phosphorylation and acrosome integrity as described above. Five replicates were performed for tyrosine phosphorylation and three replicates for acrosome integrity.

2.9.4.2. Experiment 4.2 Effect of sEVs on sperm metabolism. Frozen-thawed sperm samples were divided into two aliquots that were washed twice (900×*g*, 2 min), one with IVF medium and the other with BTS. The sperm pellet obtained from the IVF medium was diluted in IVF medium (capacitation medium) to a final sperm concentration of 20×10^6 spermatozoa/mL and divided into three aliquots to which 0.4 mg/mL or 0 mg/mL (control IVF, without sEVs) of S-sEVs or L-sEVs were added to a final volume of 225 μL. Spermatozoa diluted in BTS to a volume of 225 μL and a sperm concentration of 20×10^6 spermatozoa/mL were considered as non-capacitated spermatozoa (control BTS). Samples were incubated for 1 h at 38.5 °C in a humidified atmosphere and 5 % CO_2 in air. Sperm metabolic assays were then performed as described above. Three sets of replicates were performed.

2.10. Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 9.3.0 (GraphPad Software, Inc., La Jolla, CA, USA; https://www.graphpad.com/). First, the Shapiro-Wilk test was used to test whether data followed normal distribution. One-way ANOVA was performed to analyze the effect of sEV (subsets and concentrations) on IVF variables and on sperm functional parameters of tyrosine phosphorylation and acrosome integrity. Tukey's test was used for multiple comparisons. Sperm metabolism was analyzed by one-way ANOVA and the Newman-Keuls test was used when F-values were significant at $P < 0.05$. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Characterization of sEVs subsets

Total protein concentration (mean ± SD) differed ($P < 0.0001$) between sEV subsets, being higher in S-sEV (88.95 ± 24.15 μg/mL) than in L-sEV (47.35 ± 16.75 μg/mL) samples. The size distribution of the particles (median and 25^{th}-75^{th} interquartile range) differed ($P < 0.0001$) between S-sEV (133.35 nm; 127.02–161.09 nm) and L-sEV (264.36 nm; 235.39–281.13 nm) samples (Fig. 2A). Cryo-EM confirmed that sEVs were heterogeneous in size, shape, and electron density. Small sEV samples were enriched in small, rounded, and low electron density sEVs, whereas L-sEV samples were enriched in large sEVs with elongated or ovoid shapes and high electrodensity (Fig. 2B). Flow cytometry showed that the majority of identified events were CFSE positive, with similar percentages (mean ± SD) in the S-sEV (83.03 ± 6.58 %) and L-sEV (87.28 ± 6.34 %) samples. The percentage (mean ± SD) of CD81-positive events was similar in S-sEV (39.61 ± 18.03 %) and L-sEV (34.77 ± 11.49 %) samples, and the percentage (mean ± SD) of

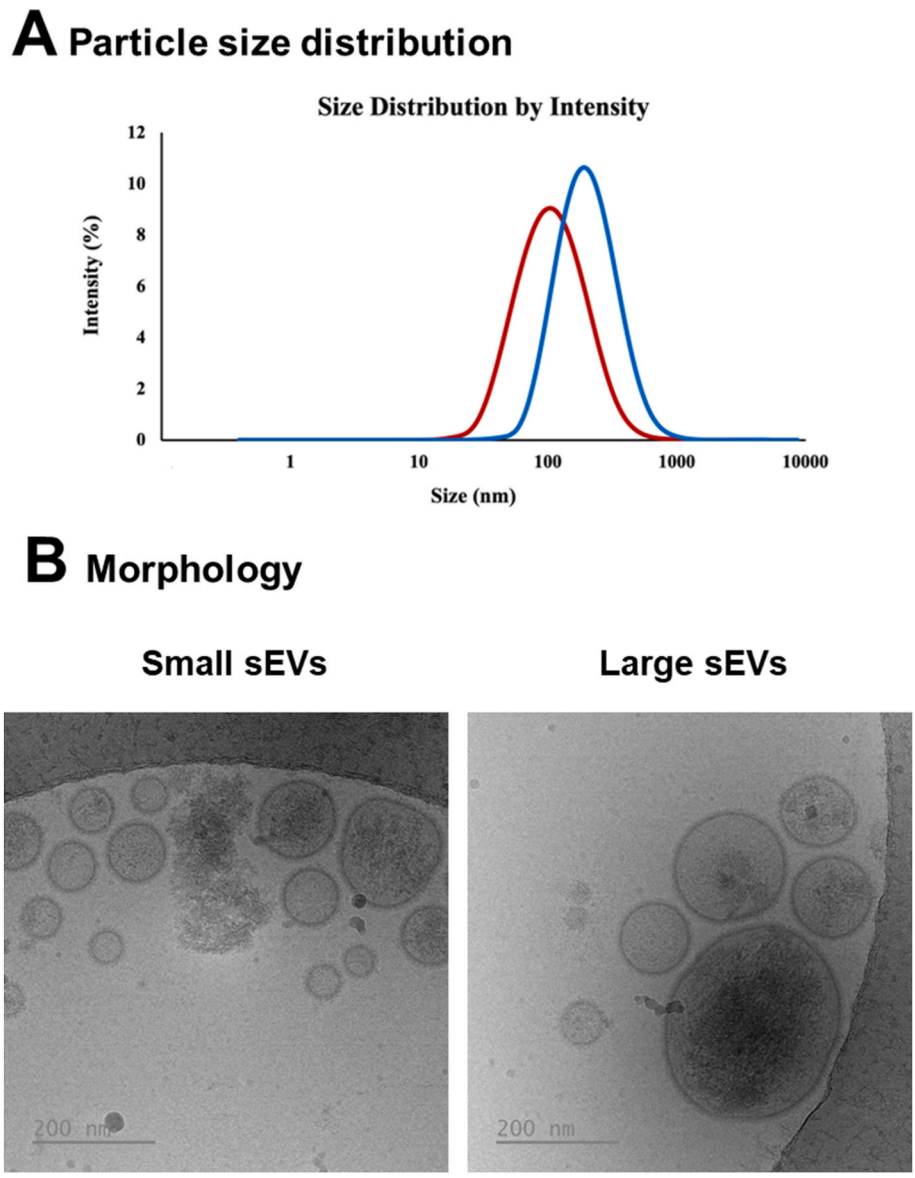

Fig. 2. Characterization of seminal extracellular vesicles (sEVs) isolated from porcine seminal plasma using a method based on size exclusion chromatography. (A) Particle size distribution determined by dynamic light scattering (red line: small sEVs; blue line: large sEVs). (B) Representative images of the morphology of small and large sEVs assessed by cryogenic electron microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

HSP90β-positive events was also similar in S-sEV (90.29 ± 6.64 %) and L-sEV (90.45 ± 5.40 %) samples. Flow cytometry also showed that both subsets of sEVs had less than 10 % albumin, indicating a low level of free protein contamination. Specifically, the percentage of albumin (mean ± SD) was 5.34 ± 2.02 in the S-sEV samples and 3.60 ± 1.06 in the L-sEV samples ($P < 0.05$).

3.2. The presence of sEVs in the IVF medium has a negative effect on IVF outcomes

The addition of S-sEVs or L-sEVs to the IVF medium during gamete coincubation impaired ($P < 0.0001$) the IVF outcomes compared to the control, regardless of the concentration of sEVs added (Fig. 3). The penetration rate was less than 20 % in the presence of sEVs while it was close to 60 % in the control (Fig. 3A). This affected the total fertilization efficiency, which was less than 15 % in the presence of sEVs while it was more than 45 % in the control (Fig. 3B).

3.3. The ability of spermatozoa to bind to the zona pellucida of oocytes is impaired when sEVs are added to the IVF medium

The possible effect of the presence of sEV subsets in the IVF medium on sperm-zona pellucida binding was investigated to determine why sEVs reduce IVF outcomes. The results showed that when sEVs were added to the IVF medium, the number of sperm attached to the zona pellucida was indeed lower ($P < 0.0001$), regardless of the subset of sEVs and the concentration added. Specifically, the mean number of bound spermatozoa ranged from 9 to 12 in the presence of sEVs, compared to more than 25 in the control (Fig. 4).

3.4. Seminal EVs bind to frozen-thawed spermatozoa but not to in vitro matured oocytes during IVF

Confocal microscopy images showed that both S- and L-sEVs bind to head and midpiece of the tail of frozen-thawed spermatozoa (Fig. 5A). However, no sEVs were observed to bind to *in vitro* matured oocytes

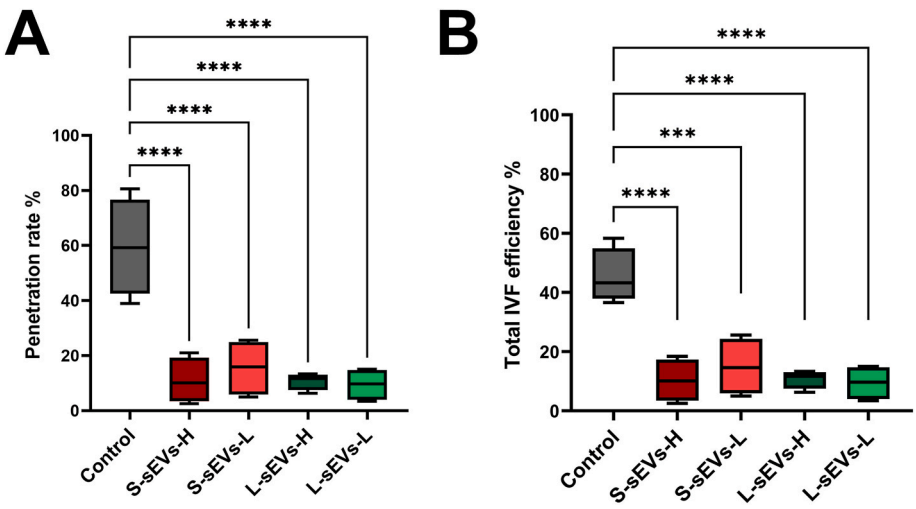

Fig. 3. Effect of porcine seminal extracellular vesicles (sEVs) on *in vitro* fertility (IVF) outcomes. Box-whisker plot showing the (A) penetration rate (number of oocytes penetrated per number of oocytes inseminated) and (B) total IVF efficiency (number of oocytes with a single sperm head or a single male pronucleus per number of oocytes inseminated). The concentration of sEVs added to the IVF medium during gamete coincubation was indirectly calculated from the total protein concentration and was 0.2 mg/mL (High, H) and 0.1 mg/mL (Low, L) for both small (S-) and large (L-) sEVs. The control consisted of the same IVF conditions except that no sEVs were added to IVF medium. Coincubation was performed for 1 h at 38.5 °C in a humidified atmosphere and 5 % CO_2 in air. Four replicates were performed with 50 oocytes per experimental group and replicate. The boxes enclose the 25th-75th percentiles, the line represents the median, and the whiskers extend to the 5th-95th percentiles. **** and *** indicate significant differences among samples at $P < 0.0001$ and $P < 0.001$, respectively.

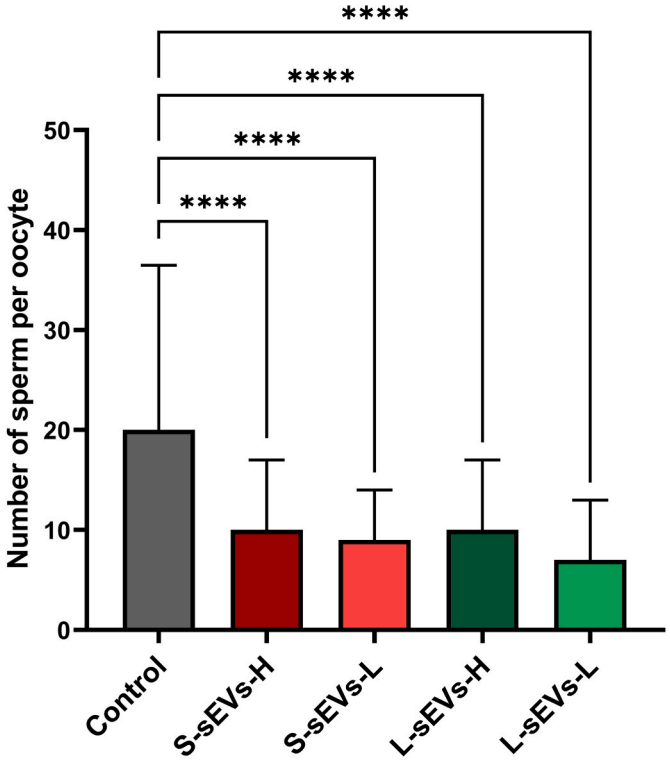

Fig. 4. Bar graph showing the mean and SD of the number of spermatozoa bound to the zona pellucida of *in vitro* matured oocytes in response to the presence of sEVs in the *in vitro* fertilization (IVF) medium during gamete coincubation. The concentration of sEVs added to the IVF medium during gamete coincubation was indirectly calculated from the total protein concentration and was 0.2 mg/mL (High, H) and 0.1 mg/mL (Low, L) for both small (S-) and large (L-) sEVs. The control consisted of the same IVF conditions except that no sEVs were added to IVF medium. Coincubation was performed for 1 h at 38.5 °C in a humidified atmosphere and 5 % CO_2 in air. Four replicates were performed with 40 oocytes per experimental group and replicate. **** indicates significant differences among samples at $P < 0.0001$.

(Fig. 5B). Negative controls (*in vitro* matured oocytes or sperm in IVF containing DiI labeled-PBS) confirmed the absence of red fluorescence staining (Fig. 5).

3.5. Sperm-zona pellucida binding is not affected by incubation of in vitro matured oocytes with sEVs prior to gamete coincubation

In vitro matured oocytes were incubated with sEVs prior to gamete coincubation to rule out any negative effect of sEVs on the ability of the zona pellucida to allow sperm attachment. No differences in the number of spermatozoa attached to the zona pellucida were observed between oocytes incubated with sEVs and control (oocytes not incubated with sEVs) (Supplementary Fig. S1).

3.6. Sperm capacitation was not affected by the addition of sEVs to the IVF medium

Sperm protein tyrosine phosphorylation was analyzed to test whether the addition of sEVs to the IVF medium could affect sperm capacitation. The immunolocalization of tyrosine phosphorylated sperm proteins was not affected by the presence of sEVs in the IVF medium, regardless of the subset of sEVs and the concentration added (Fig. 6A). Similarly, the percentage of sperm with damaged acrosome was not affected by the presence of sEVs in the IVF medium, regardless of the subset of sEVs and the concentration added (Fig. 6B).

3.7. Addition of sEVs to IVF medium affected sperm metabolism

Under basal metabolic conditions, ATP synthesis by mitochondrial oxidative phosphorylation system (OXPHOS) and glycolysis was determined by measuring OCR and ECAR to obtain mitoATP and glycoATP production rates (the rate of ATP production correlated with the conversion of glucose to lactate in the OXPHOS and glycolytic pathways), respectively (Fig. 7). Control BTS spermatozoa showed the highest ATP production rate based on glycolysis and OXPHOS. Surprisingly, in control IVF spermatozoa, the glycolytic pathway was disabled, and the energy metabolism was supported only by OXPHOS. However, although control IVF spermatozoa had a higher rate of mitoATP than control BTS spermatozoa, the total ATP production of IVF spermatozoa, either

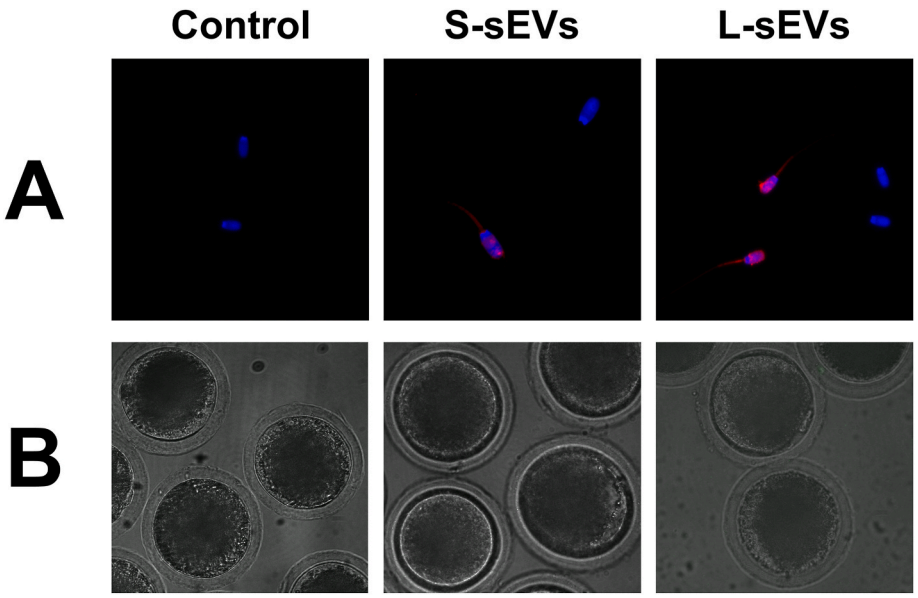

Fig. 5. Interaction of porcine seminal extracellular vesicles (sEVs) with either *in vitro* matured oocytes or frozen-thawed spermatozoa under *in vitro* fertilization (IVF) conditions. Representative images of (A) spermatozoa and (B) *in vitro* matured oocytes after incubation with small (S-) and large (L-) sEVs (0.2 mg/mL). Seminal EVs labeled with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) were added to IVF medium and incubated with *in vitro* matured oocytes or frozen-thawed spermatozoa at 38.5 °C, 95 % humidity, and 5% CO_2 in air for 1 h. Spermatozoa were stained with Hoechst 33342 after the incubation period. The control was oocytes or sperm incubated under the same conditions but in IVF medium supplemented with Dil-labeled phosphate buffered saline only. Three replicates were performed in each experiment.

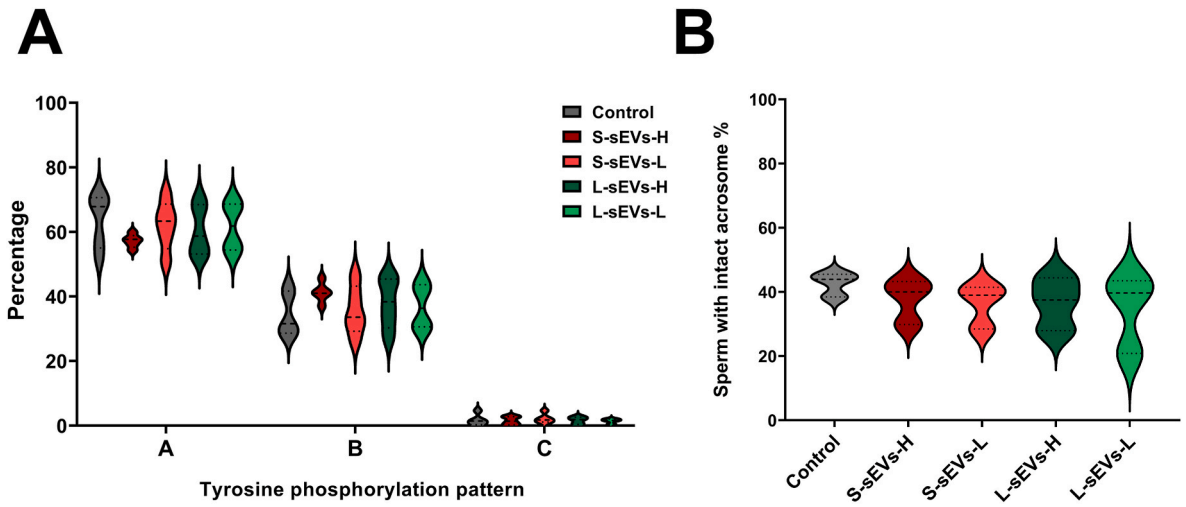

Fig. 6. Influence of seminal extracellular vesicles (sEVs) on functional traits of spermatozoa incubated under *in vitro* fertilization (IVF) conditions (1 h at 38.5 °C in a humidified atmosphere and 5 % CO_2 in air). Violin plots showing the percentage of (A) sperm displaying tyrosine phosphorylation pattern A (acrosomal and equatorial subsegment positivity), pattern B (acrosomal, equatorial subsegment, and principal piece of the tail positivity), or pattern C (equatorial subsegment weak positivity and tail positivity); and (B) sperm with intact acrosome. The concentration of sEVs added to the IVF medium during gamete coincubation was indirectly calculated from the total protein concentration and was 0.2 mg/mL (High, H) and 0.1 mg/mL (Low, L) for both small (S-) and large (L-) sEVs. The control consisted of the same IVF conditions except that no sEVs were added to the IVF medium. Five and three biological replicates were performed for tyrosine phosphorylation and acrosome integrity assessments, respectively. Dashed line indicates the median and dotted lines indicate 25–75% interquartile range.

coincubated with sEVs or not, was lower than that of control BTS spermatozoa ($P < 0.05$). IVF spermatozoa incubated with L-sEVs showed the lowest ATP production rates among the IVF spermatozoa (Fig. 7A). The sperm energy map confirmed aerobic energy metabolism with a more active OXPHOS pathway in the IVF than in the BTS spermatozoa (Fig. 7B).

In a pilot experiment, S-sEVs and L-sEVs were coincubated with control BTS spermatozoa to verify the causes of the glycolytic pathway deactivation. The results showed that the metabolic shift towards full OXPHOS would be due to the IVF medium, as no differences in total ATP, mitoATP, and glycoATP production rates were found between spermatozoa diluted in BTS alone or spermatozoa diluted with BTS supplemented with S-sEVs or L-sEVs (Supplementary Figs. S2–A). Consequently, spermatozoa diluted in IVF medium in the presence or absence of S-sEVs or L-sEVs showed a mitoATP/glycoATP ratio greater than one unit, revealing a prevailing oxidative phenotype (Supplementary Figs. S2–B).

Sperm mitochondrial respiration expressed as OCR from all sperm samples is shown in Fig. 8. The kinetic profile obtained using serially injected mitochondrial inhibitors, namely olig, FCCP, and rot + AA, was

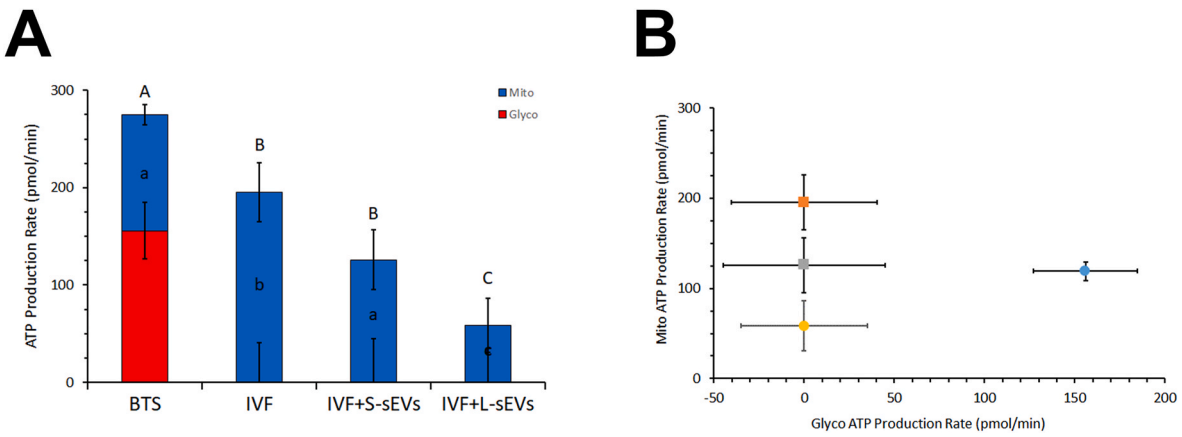

Fig. 7. Effect of porcine seminal extracellular vesicles (sEVs) on the metabolism of frozen-thawed spermatozoa during *in vitro* fertilization (IVF). Real-time ATP production rate of basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in sperm samples diluted in Beltsville Thawing Solution (control, BTS), IVF medium (control, IVF), and IVF medium supplemented with small sEVs (IVF + S-sEVs), or large sEVs (IVF + L-sEVs). (A) Quantification of ATP production by mitochondrial oxidative phosphorylation (blue bar; Mito) or by the glycolytic pathway (red bar; glucose conversion to lactate; Glyco). (B) Energy map with OCR vs ECAR of control BTS (blue), control IVF (orange), IVF medium with S-sEVs (gray), and IVF medium L-sEVs (yellow) are plotted. Data expressed as columns (A plot) and points (B plot) graph represent the mean ± SD (vertical and horizontal bars) of data from three biological replicates. Different letters indicate significant differences ($P < 0.05$) between experimental samples within the same parameter. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

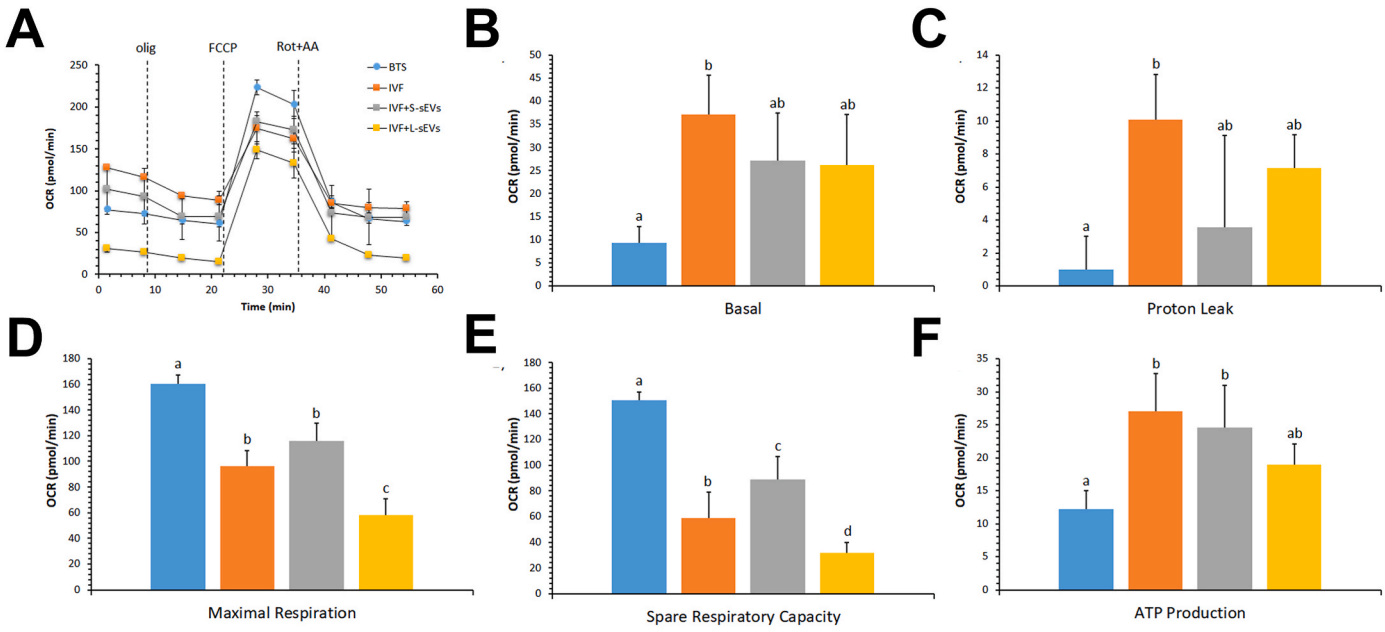

Fig. 8. Effect of porcine seminal extracellular vesicles (sEVs) on the bioenergetic profile of frozen-thawed spermatozoa during *in vitro* fertilization (IVF). Sperm samples were diluted in Beltsville thawing solution (control, BTS), IVF medium (control, IVF), and IVF medium supplemented with small sEVs (IVF + S-sEVs) or large sEVs (IVF + L-sEVs). (A) The mitochondrial respiration profile was obtained from the basal oxygen consumption rate (OCR) on BTS (blue), IVF (orange), IVF + S-sEVs (gray), and IVF + L-sEVs (yellow) under basal respiration conditions and after the addition of 1.5 μM oligomycin (olig), 4.0 μM of carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and a mixture of 0.5 μM rotenone plus antimycin A (rot + AA). Inhibitor injections are indicated by dotted lines. (B–F) Bioenergetic parameters of sperm respiration of BTS (blue bar), IVF (orange bar), IVF + S-sEVs (gray bar), and IVF + L-sEVs (yellow bar) are shown in plots B (basal), C (proton leak), D (maximal respiration), E (spare respiratory capacity), and F (ATP production). Data expressed as points (plot A) and column plots (plots B, C, D, E, and F) charts represent the mean ± SD (vertical bars) of three replicates. Different letters indicate significant differences ($P < 0.05$) among samples within the same parameter. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

similar in all sperm samples (Fig. 8A). However, differences in bioenergetic parameters were observed ($P < 0.05$). Both basal respiration and proton leaks were increased in the control IVF sample, and activation was partially but not significantly reversed in IVF samples supplemented with S-sEVs or L-sEVs (Fig. 8-B, C). In contrast, maximal respiration and spare respiratory capacity were inhibited in the control IVF and supplementation with S-sEVs or L-sEVs did not reverse the negative effect on mitochondrial respiration (Fig. 8-D, E). Consistently with the oxidative phenotype stimulated by IVF medium (Fig. 7), mitoATP production was significantly ($P < 0.05$) increased in spermatozoa from in IVF samples (Fig. 8-F).

4. Discussion

To the best of our knowledge, this is the first study in an animal species to evaluate the putative effect of supplementing the IVF medium

with two different sized sEV subsets on IVF outcomes. Seminal EV supplementation, regardless of sEV subset, resulted in impaired IVF outcomes, specifically penetration rate and overall IVF efficiency. The IVF impairment was presumably due to sEVs binding to spermatozoa and preventing them for binding to the zona pellucida of oocyte. The results also showed that sEVs were able to bind to the head and midpiece of the tail of frozen-thawed spermatozoa, but not to *in vitro* matured oocytes, during IVF. Although sEVs did not affect sperm capacitation, measured in terms of protein tyrosine phosphorylation, and acrosome integrity, they were able to alter sperm metabolism, which may be the cause affecting the ability of sperm to bind to the zona pellucida during IVF.

The two sEV subsets used to supplement the IVF medium were isolated from porcine SP using a SEC-based method, which has been shown to be effective in isolating two different sizes of sEVs, namely small and large, with high purity degree [36]. While differential ultracentrifugation is still the most common method for EV isolation, SEC is becoming increasingly popular due to its high efficiency, reproducibility, low cost, and simplicity, isolating a more pure and functional EV population than ultracentrifugation [45,46]. Consistent with MISEV 2018, the isolated sEV subsets were characterized by a combination of multiple and complementary approaches, including total protein concentration, cryo-EM, DLS and flow cytometry, which confirmed the high purity of the isolated sEVs and that the size of sEVs differed between the two subsets.

Recent studies conducted in livestock species have reported that supplementation of IVM, IVF and/or IVC media with EVs isolated from female reproductive fluids had a positive effect on embryo development and quality [13–23]. Regarding to IVF, Alcântara-Neto et al. [15]. demonstrated that the presence of porcine oviductal EVs during sperm-oocyte coincubation increased the monospermy rate compared to the control. However, to the best of our knowledge, only one study conducted in mice showed a positive effect of supplementing IVF medium with EVs isolated from epididymal fluid on embryo development [33]. These findings, together with the fact that SP modulates several reproductive processes [47] and is highly enriched in sEVs [32,48], led us to evaluate whether sEVs would have effects on IVF outcomes when added during gamete coincubation. The two sEV subset were evaluated separately because they have compositional differences [36] and differentially affect porcine granulosa cell function during IVM [34].

Given the lack of similar studies involving sEVs in porcine IVF or in other mammalian species, we chose the standard porcine IVF protocol routinely used in our laboratory [39]. The two sEV concentrations tested were chosen based on previous studies demonstrating beneficial effects of female reproductive EVs on IVP [15,16,23,49]. The results showed that supplementation of IVF medium with any of the sEV subsets decreased penetration rates and overall fertilization efficiency, regardless of the concentration of sEVs added. Alcântara-Neto et al. [15] also observed that supplementation of IVF medium with porcine oviductal EVs decreased the penetration rate, but did not observe that this affected differences in total fertilization efficiency. On the other hand, Ma et al. [33], who supplemented IVF medium with epididymal EVs in mice, observed no effect, either negative or positive, on fertilization rates. The decrease in both fertilization rate and average IVF efficacy observed in our study would be due to the reduced ability of sperm to bind to the zona pellucida of oocytes. Our results would be consistent with those of Piehl et al. [26] who also found a decreased ability of porcine sperm coincubated with sEVs to bind to the zona pellucida of oocytes. These researchers suggested that the inability of spermatozoa to bind to the zona pellucida would be related to the protein load that the sEVs transfer to the spermatozoa. In this regard, in a recent proteomic study of porcine sEVs, we identified several proteins involved in the ability of spermatozoa to interact with the zona pellucida [36]. Among the proteins identified were the spermadhesins PSP-I and PSP-II, which have been shown in *in vitro* studies to impair the ability of porcine spermatozoa to bind to the zona pellucida of oocytes [50]. The quantitative proteomic analysis of Barranco et al. [36] also revealed that both spermadhesins were in similar abundance in the two sEV subsets, which may help explain why no differences were found between the two sEV subsets in the spermatozoa-zona pellucida binding response.

The biological activity of EVs is based on their ability to interact and/or fuse with target cells where they elicit specific functional responses [51]. In our study conducted under IVF conditions, sEVs were able to bind to spermatozoa, specifically in the head and midpiece of the tail, but not to oocytes. Focusing first on oocytes, the fact that sEVs were unable to bind to them was not surprising, as this would be consistent with a recent study, also conducted in pigs, showing that sEVs were unable to interact with oocytes during IVM [34]. Similarly, other studies have reported that EVs isolated from female reproductive biofluids (follicular and oviductal fluids) were unable to bind to oocytes [17,52–54]. With respect to spermatozoa, the fact that sEVs were able to bind to spermatozoa was also not particularly surprising, as numerous studies had already demonstrated this ability of sEVs in several species [41,55–57], including in pigs [28]. These studies also show that sEVs can bind to any of the three major segments of spermatozoa, namely the head, midpiece and tail. Alcantara-Neto et al. [23] and Ferraz et al. [58] reported that EVs isolated from oviductal fluid bind to similar segments in porcine and feline spermatozoa, respectively. Taken together, these results would indicate that the binding site of EVs to spermatozoa does not differ between EVs from different reproductive fluid. Thus, whether EVs bind to one sperm segment or the other may depend more on the molecular composition of the EVs than on their cellular origin [32]. Our study would be the first to demonstrate that sEVs were able to bind to frozen-thawed spermatozoa. Rab family proteins, that are present in frozen-thawed porcine spermatozoa [59], would be the main receptors for sEV sperm binding [32]. Since sEVs can bound to spermatozoa, it is reasonable to assume that this binding affects sperm functionality. In the experiments conducted in the present study, sEVs interacted with frozen-thawed spermatozoa undergoing *in vitro* capacitation. Accordingly, we assessed tyrosine phosphorylation, a hallmark of sperm capacitation, and acrosome integrity to evaluate putative effects of sEVs on sperm functional responses. However, sEVs did not affect either of these sperm functional responses. Previous studies have reported an inhibitory effect of sEVs on tyrosine phosphorylation in human [60,61] and porcine [26] spermatozoa. However, other studies such as the Aalberts et al. [62] did not find a relevant effect of sEVs on tyrosine phosphorylation in stallion spermatozoa, which would be consistent with our results. Differences in the composition of sEVs may explain these conflicting results. Murdica et al. [41] reported that the effect of sEVs on tyrosine phosphorylation in human spermatozoa differed between men with a normal spermiogram and men with asthenozoospermia or azoospermia. Regarding to acrosome response, while some studies reported that sEVs stimulate the acrosome reaction [63], others reported the opposite [61,64,65]. Our results would be consistent with those reported by Piehl et al. [26] in pigs, who showed no effect of sEVs on acrosome integrity. Differences in the sEV isolation procedure used, with consequent cross-contamination by free proteins, in the experimental conditions, and in the diversity of sEV subpopulations with clear differences in molecular cargo between them may explain, at least in part, the discrepancies between studies about the effect of sEVs on sperm functional traits. In addition, it cannot be ruled out that the semen donor may contribute to the discrepancies between the studies mentioned above. Male differences in sperm quality and fertility have been widely demonstrated in humans [66] and livestock species [67], including in pigs [68]. It is plausible, but not yet proven, that there are differences between males in the phenotypic and compositional characteristics of sEVs. Therefore, it would be interesting to conduct studies analyzing the male influence on the composition of sEVs and how this affects sperm functionality and fertility.

In addition to capacitation and acrosome traits, our study also assessed the metabolic status of spermatozoa incubated with sEVs, which would in itself is relevant as it has not been previously assessed, at least to our knowledge. The first interesting finding was that frozen-

thawed pig spermatozoa were shown to rely primarily on glycolysis. This would be consistent with the results of our previous study where porcine semen preserved at 17 °C showed a switch in metabolic pattern after 24 h of preservation compared to fresh ejaculated semen [44]. In fact, as previously reported by Nesci et al. [69], freshly ejaculated sperm rely on OXPHOS for energy production and motility, whereas in preserved sperm there is an increase in the glycolytic pathway for energy production. These results agree with what we observed in this study. In fact, the metabolism of frozen-thawed spermatozoa switched toward glycolysis, which allowed us to assume that the mitochondria were at least partially shut down. This metabolic situation was modified by capacitating condition of IVF medium since the source of ATP was only OXPHOS. This metabolic scenario was not altered by supplementation the IVF medium with S- or L-sEVs. The significant change caused by sEVs was in the total amount of ATP produced by the spermatozoa, which was lower than in the IVF control spermatozoa. This finding may explain the poor IVF outcomes of sperm incubated with sEVs. In addition to the possible steric hindrance, there was also a decrease in energy production. The results on mitochondrial parameters confirmed the findings that there was a shift towards oxidative metabolism in sperm incubated in IVF medium. The increase of basal respiration and ATP production was the effect of mitochondrial activation to boost energy to sustain the capacitation of sperm. In contrast, the decrease in maximal respiration and spare respiratory capacity highlighted the lack of flexibility of OXPHOS machinery and the inability of spermatozoa to respond to changes in energetic demand.

Some of these *in vitro* results could also occur in an *in vivo* context, either after natural mating or AI. One of the most important findings of the present study is that sEVs interact with spermatozoa and not with oocytes. In the *in vivo* context, sEVs would interact with sperm during their transit through the female genital tract, but prior to fertilization time in the oviduct. In this regard, Aalberts et al. [62] proposed in a study in stallions that sEVs would bind to spermatozoa in uterus and fuse with the sperm membranes when the sperm reach the oviduct. Whether or not free sEVs reach the oviduct is currently unknown. Therefore, it is not known whether sEVs interact with oocytes in oviduct. The only existing evidence comes from an *in vitro* study showing that sEVs interact with cumulus cells [34].

5. Conclusion

The present study demonstrated, for the first time in a livestock species, that coincubation of gamete with sEVs during IVF has a negative effect on IVF outcome. This negative effect would be evidenced by the inability of spermatozoa to bind to zona pellucida of *in vitro* matured oocytes. Such a negative effect would be similarly caused by small and large sEVs. This study also showed that both sEV subsets were able to bind to frozen-thawed spermatozoa, but not to *in vitro* matured oocytes. Binding of sEVs to spermatozoa during IVF would alter sperm metabolism by decreasing ATP production, which would be one of the reasons for poor IVF outcomes.

Data availability statement

The data supporting the conclusions of this study will be made available by the corresponding author.

CRediT authorship contribution statement

Isabel Barranco: Writing – original draft, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Marcella Spinaci:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Investigation, Conceptualization. **Salvatore Nesci:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Yentel Mateo-Otero:** Writing – review & editing, Visualization, Methodology, Investigation. **Vito Antonio Baldassarro:** Writing – review & editing, Methodology, Investigation. **Cristina Algieri:** Methodology, Investigation. **Diego Bucci:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Jordi Roca:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors do not have any conflicts of interest to declare.

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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.2024.02.024.

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