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Pig Seminal Plasma Extracellular Vesicles Enhance Motility During Preservation of Epididymal Spermatozoa but Impair Capacitation and IVF Parameters

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ABSTRACT

Background: Extracellular vesicles (EVs) produced by accessory sex glands play a key role in sperm functionality, influencing motility, viability, acrosome integrity, metabolic activity, and capacitation. Most studies on the effects of EVs on spermatozoa have focused on ejaculated spermatozoa, which have already been exposed to their own native EVs. This prior exposure may contribute to the variability in reported effects of EVs on sperm function.

Objectives: To evaluate the role of EVs on the function of pig epididymal spermatozoa and their potential use for improving sperm preservation and in vitro fertilization (IVF).

Materials and Methods: EVs were isolated from ejaculates of boars with proven fertility using ExoQuick ULTRA and subsequently characterized according to the guidelines of the International Society for Extracellular Vesicles (ISEV). Epididymal spermatozoa were collected from the cauda epididymis and diluted in MR-A medium before being incubated with EVs for 72 h. Sperm motility, viability, acrosome integrity, and mitochondrial activity were assessed at 0, 24, 48, and 72 h. These parameters were also evaluated at 24 and 48 h after sperm incubation at 38.5°C for 5 h. To evaluate the role of EVs on sperm function, PKA activity (pPKA) and tyrosine phosphorylation (Tyr-P) were assessed in capacitated spermatozoa by Western blot. IVF assays were also performed at 0 and 48 h of storage to evaluate sperm quality. The IVF parameters evaluated were oocyte penetration rate, sperm count per oocyte, and sperm adhesion to the zona pellucida.

Results: Incubation of spermatozoa with EVs improved motility, but impaired acrosome integrity and viability. Incubation of spermatozoa with EVs also caused a reduction in pPKA activity. Regarding Tyr-P, it was observed that EVs prevented the phosphorylation of 20 kDa proteins. IVF parameters decreased when fertilization was performed with EV-incubated spermatozoa.

Conclusions: Supplementation with EVs improves sperm motility, modulates capacitation by reducing protein phosphorylation, and reduces fertilization parameters.

1 | Introduction

The ejaculate is a mixture of spermatozoa and seminal plasma (SP), which is a complex fluid produced by the accessory sex glands. In boars, these accessory sex glands include vesicular glands, prostate, and bulbourethral glands and along with the testes and epididymis, form the liquid fraction of the ejaculate [1]. SP performs several functions associated with sperm

metabolism: it provides a transport medium, maintains sperm viability, and regulates sperm capacitation [2]. SP also protects spermatozoa from the female immune system, and acts on the female reproductive tract by establishing a favorable environment for the embryo [2]. The ejaculate is composed primarily of inorganic ions, hormones, proteins, peptides, cytokines, enzymes, cholesterol, DNA, and RNA. Some of these molecules have been observed protected inside membranous structures called extracellular vesicles (EVs), which prevent their degradation [3].

EVs play crucial roles in sperm function and fertility. These nanovesicles have a diameter of 40–150 nm in small EVs and 100–1000 nm in large EVs [4]. In the male reproductive tract, many types of cells are involved in the generation of EVs, which can interact with spermatozoa through mechanisms like lipid-raft domain-mediated endocytosis [5, 6]. Proteomic analyses have revealed that EVs are involved in both the delivery and removal of proteins from sperm membranes, particularly those related to vesicle biogenesis, metabolism, and membrane adhesion [7]. In boars, SP EVs are produced by the epididymis and accessory sex glands [8]. EVs interact with spermatozoa, modulating their activity and influencing several functional aspects like total motility and progressive motility (MotP). Boar EVs also improve viability, prevent premature capacitation, regulate oxidative stress, and provide antioxidant effects [9].

The need to preserve spermatozoa for use in in vitro fertilization (IVF) or artificial insemination has increased due to advancements in assisted reproductive techniques. For these applications, boar spermatozoa are stored for several days at 15°C–20°C using an insemination extender, which facilitates the storage of semen doses and their subsequent distribution [10]. More recently, the supplementation of spermatozoa with SP EVs has been proposed as a strategy to enhance sperm preservation. However, most studies have focused on evaluating the effects of SP EVs on ejaculated spermatozoa. These sperm cells have already been exposed to secretions from the accessory sex glands and may already be influenced by them due to the process of ejaculation. During ejaculation, sperm cells mix with prostatic secretions and EVs derived from the accessory glands. In the present study, spermatozoa from the cauda epididymis have only been exposed to epididymal secretions and EVs (epididymosomes). Consequently, the study of the impact of accessory gland EVs on epididymal spermatozoa could provide a clearer understanding of their specific functions. In addition, the potential use of SP EVs as supplements in insemination doses is considered a promising strategy to enhance sperm preservation. For these reasons, the present study was conducted to assess the effects of EVs from accessory glands on mature epididymal spermatozoa, which have only been in contact with epididymal EVs.

2 | Materials and Methods

2.1 | Ethics

All procedures in this study were approved by the Ethical Committee of Animal Experimentation of the University of Murcia on June 1, 2020 (PID2019-106380RB-I00) and by the Animal Production Service for the Agriculture Department of the Region of Murcia (Spain) (A13230106) in compliance with the Spanish Policy for Animal Protection RD53/2013, which aligns with the European Union Directive 2010/63/UE. The animals were handled with care to minimize stress throughout the experiments. All experiments were conducted in accordance with relevant guidelines and regulations.

2.2 | Reagents

Unless otherwise stated, all chemicals used in this study were obtained from Merck Life Science S.L.U. (Madrid, Spain) or Thermo Fisher Scientific (Waltham, MA, USA).

2.3 | Isolation of EVs From SP

Five ejaculates from boars with proven fertility were collected using the gloved-hand method. The criteria for selecting boar ejaculates were a sperm concentration exceeding 200×10^6 spermatozoa/mL, with at least 80% of the spermatozoa exhibiting total motility, 60% showing MotP, and over 75% normal morphology. A volume of 10 mL of semen was centrifuged twice at 1500 g for 10 min to eliminate cellular components from SP. The supernatant was stored at −80°C.

To isolate EVs, the SP samples were thawed at room temperature and processed with the ExoQuick ULTRA EV Isolation Kit (System Biosciences, Palo Alto, CA, USA) in accordance with the manufacturer's instructions. In brief, 250 µL of SP was combined with 67 µL of ExoQuick solution and incubated for 30 min at 4°C. After incubation, the samples were subjected to centrifugation at 3000 g for 10 min at 4°C. The final pellet was resuspended in 200 µL of buffer B. The protein concentration of EVs was determined using a Bradford assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). After, 200 µL of buffer A was added to resuspend the remaining EVs, and the resulting volume was passed through a resin column and centrifuged at 1000 g for 30 s to remove the storage buffer. Aliquots of 50 µL of purified EVs were stored at −80°C until further analysis.

2.4 | Characterization of EVs

The isolated EVs were characterized using multiple complementary approaches in line with the Minimal Information for Studies of Extracellular Vesicles (MISEV 2023) guideline [11].

2.4.1 | EV Morphology Visualization

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used to evaluate the presence of EVs, their morphology, and their binding to spermatozoa.

The procedure used to evaluate the morphology of EVs was based on the protocol established by Toledo-Guardiola et al. [12]. Specifically, 10 µL of the EV suspension was applied to a formvar-carbon-coated grid and allowed to adsorb for 30 s at room temperature. Afterward, the grid was rinsed once with distilled water for 1 min and subsequently stained with 10 µL of 2% uranyl acetate for 1 min to achieve negative staining. The grids were then

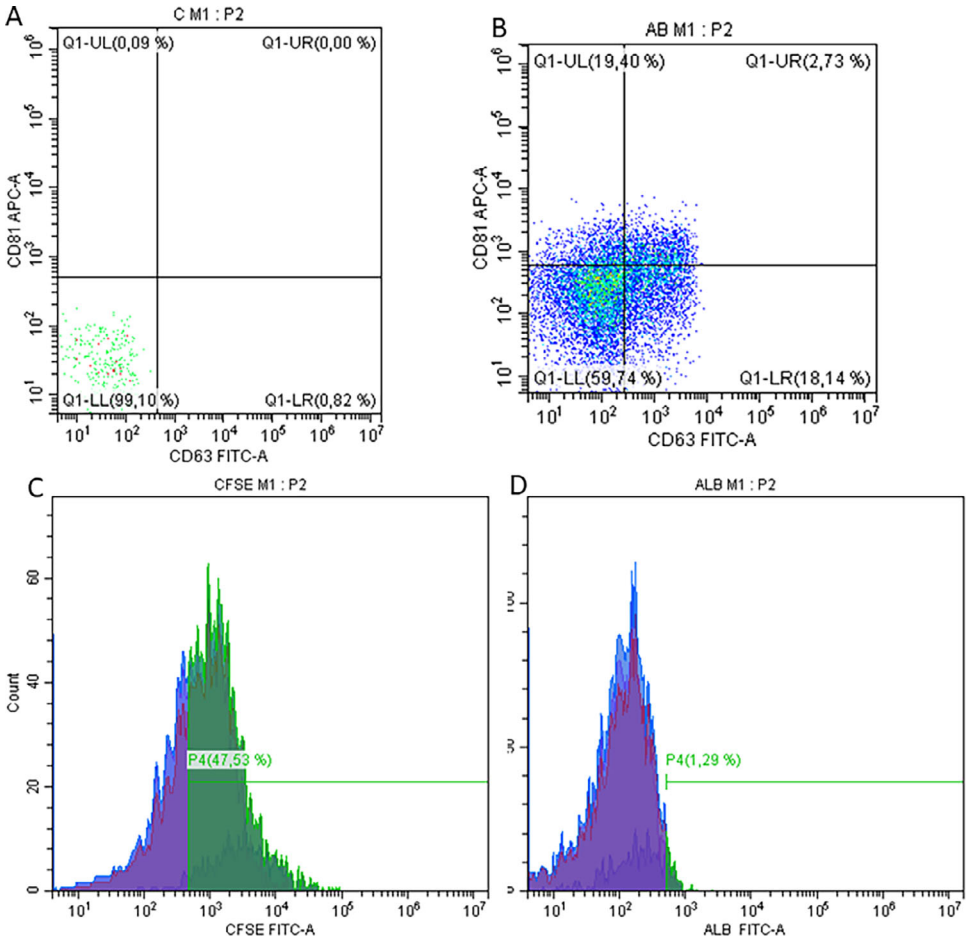

FIGURE 1 | Experimental flow cytometry analysis of M1 sample. (A) Dotplot of unstained EVs used as control for tetraspanin analysis. (B) Dotplot of EVs stained with anti-CD81-APC and anti-CD63-FITC, showing different sub-populations, double positive, double negative, and single positive for each antibody. (C) CFSE staining showing functional EVs. (D) Albumin staining to detect sample lipoprotein contamination.

air-dried for 20 min at room temperature. Imaging was performed using a JEOL1011 transmission electron microscope operating at 80 kV (Jeol, Japan). Digital images were taken at 59,000–97,000 magnification.

To evaluate the presence and morphology of EVs bound to spermatozoa, and prior to examination via SEM, it was necessary to pre-treat the sperm samples with the vesicles attached. The sperm preparation was executed in accordance with the protocol outlined by Korneev et al. [13], with some modifications. Epididymal sperm samples were diluted with TALP medium to a concentration of 3×10^6 spermatozoa/mL. Samples were incubated with EVs for 2 h. Subsequently, the samples were centrifuged at 400 g. The supernatant was discarded, and the remaining pellet was diluted in 0.1 M sodium cacodylate buffer. The sample was then centrifuged and immediately diluted in 3.5% glutaraldehyde in 0.1 M sodium cacodylate buffer to prevent drying. The cells were fixed for 15 min and then washed with 0.1 M sodium cacodylate buffer. Samples were dehydrated by immersing them in a series of graded ethanol solutions of increasing concentrations (30%, 50%, 70%, 90%, 96%, and 100%) for 5 min each. The ethanol residues were removed with a critical point dryer. The samples were then visualized by SEM, with a metal support coated with conductive carbon paint and a 5-nm thick carbon layer applied. An Apreo 2 S field emission scanning electron microscope (FE-SEM, Thermo Fisher Scientific) was used to visualize the samples.

2.4.2 | EV Total Protein Concentration

The analysis of protein concentration was carried out via Bradford protein assay in compliance with the manufacturer's instructions. The assay was performed using a 96-well microplate and the absorbance was quantified using a microplate reader (Apollo 11 LB913, Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) at a wavelength of 595 nm. Each sample was analyzed in triplicate. Quantitative results were obtained by plotting the sample readings on the standard curve, which was generated from the standard samples of bovine serum albumin (BSA). The results were expressed as µg/µL.

2.4.3 | EV Particle Size and Concentration

Particle concentration and size were determined via nanoparticle tracking analysis (NTA) on a NanoSight NS300 system (Malvern Panalytical, Malvern, UK) equipped with a 405 nm laser, a syringe pump system, and a scientific-grade CMOS camera. The EVs were resuspended and diluted with PBS (sterile-filtered, suitable for

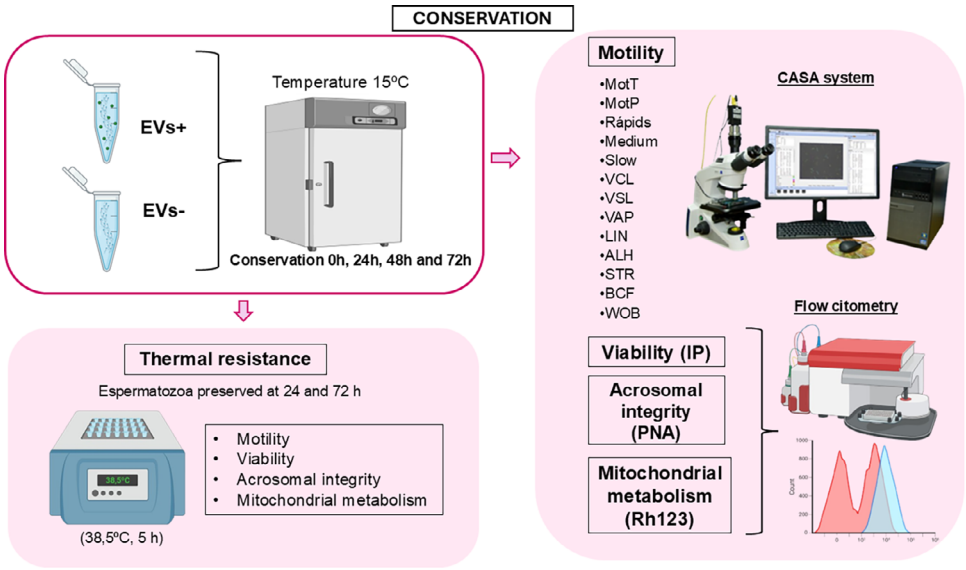

FIGURE 2 | Experimental design of preservation studies. Epididymal spermatozoa were diluted with EVs (EVs+) or without EVs (EVs−) in an MR-A boar semen extender and stored at 15°C. Preservation analysis was performed at 0, 24, 48, and 72 h. The following motility parameters were evaluated using the computer-assisted sperm analysis (CASA) system: total motility (MotT), progressive motility (MotP), percentage of rapid, medium and slow sperm, curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), sperm linearity (LIN), lateral head displacement (ALH), straightness index (STR), beat-cross frequency (BCF), and lateral head wobble (WOB). In addition, viability was assessed using propidium iodide (PI), acrosome integrity was assessed using Arachis hypogaea lectin (PNA), and mitochondrial activity was measured using rhodamine 123 (Rh123) by flow cytometry. Thermal resistance tests were also performed at 24 and 72 h, where the spermatozoa were exposed to a temperature of 38.5°C for a period of 5 h. The above parameters were then evaluated.

cell culture, Sigma-Aldrich) to achieve a concentration range of 10^9 particles/mL. The dilution was then subjected to analysis. Particles were automatically tracked and sized based on the principles of Brownian motion and the diffusion coefficient. The camera was set to level 15, and five 30-second videos were recorded at 30 frames per second. Data were processed using NTA software (version 3.3; dev build 3.3.104) to determine the concentration and size of the measured particles, as well as the corresponding standard error.

2.4.4 | EV Tetraspanin Identification

EV markers were examined using a high-resolution flow cytometer (CytoFLEX S; Beckman Coulter, Life Sciences Division Headquarters, Indianapolis, USA) equipped with red (638 nm), blue (488 nm), yellow (561 nm), and violet (405 nm) lasers. The functionality of the flow cytometer was verified using a standard calibration kit (Nanobead Calibration Kit, Bang Laboratories Inc., Indiana, USA), consisting of microspheres with diameters of 50–100 nm and an internalized fluorescent dye. The beads were employed to establish the EV input and counting. The optical configuration was set up to use side scatter (SSC) data from the 405-nm laser (violet-SSC-A). The forward scatter (FSC) and violet-SSC-A were adjusted on a logarithmic scale, and the fluorescence channels adjusted on a logarithmic gain. The analysis was restricted to events with size (FSC) and complexity (violet-SSC-A) characteristics specific to EVs (Figure 1).

Samples were analyzed using low flow settings (5–10 µL/min) and at least 10,000 events were acquired for each sample. The sheath fluid used was distilled water (filtered at 0.1 µm), while 0.1-µm-filtered PBS was employed before sample acquisition to remove background noise. The concentration of EVs was calculated based on the number of positive CFSE (carboxyfluorescein succinimidyl ester) events and the dilution rate. CFSEs diffuse freely through vesicle membrane, being hydrolyzed esterases. Esterases can only work inside vesicle membranes. Once hydrolyzed, CFSEs can bind covalently to proteins and emit fluorescence when excited at 488 nm.

The EVs were characterized cytometrically in accordance with the recommendations of the International Society of Extracellular Vesicles (MIFlowCyt-EV) [14]. A 10-µL aliquot of each EV sample was incubated with CellTrace CFSE (Thermo Fisher Scientific) to identify intact EVs and distinguish them from non-EV structures, including membrane fragments. Subsequently, the EV sample was divided into three portions, which were incubated with anti-HSP70β-PE, anti-CD63-FITC, anti-CD81-allophycocyanin or anti-Albumin-FITC at room temperature for 30 min. Finally, the samples were diluted in 0.1-µm-filtered PBS to a volume of 300 µL and analyzed in a CytoFLEX S high-resolution flow cytometer (Figure S1).

2.5 | Epididymal Sperm Collection

Epididymides were collected from boars slaughtered at the local abattoir and transported to the laboratory within 30 min. Upon arrival, they were washed twice in saline solution and the epididymal fluid was collected from the cauda epididymides following the protocol described by Soriano-Úbeda et al. [15]. A pool was prepared by mixing epididymal fluid from different boars. After epididymal collection, concentration was determined

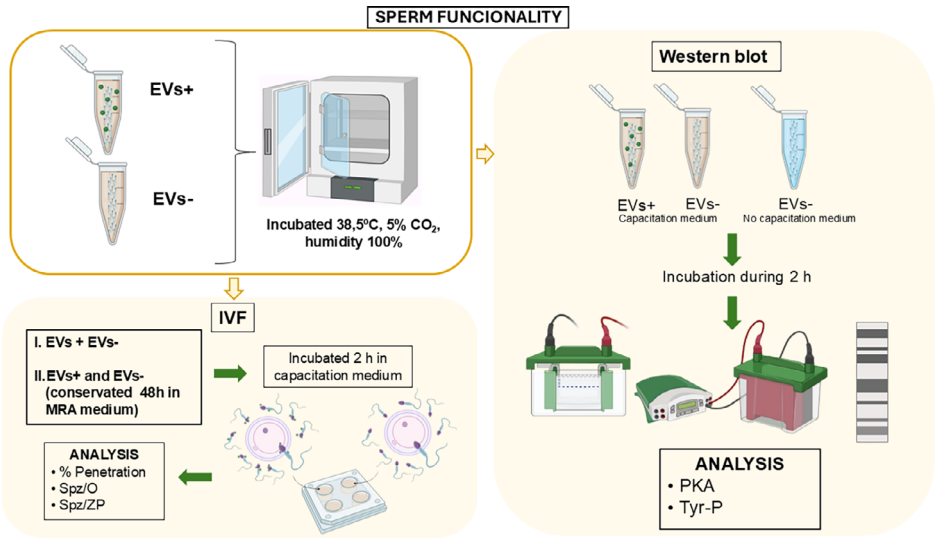

FIGURE 3 | Experimental design for sperm functionality assays: epididymal spermatozoa were incubated with EVs (EVs+) or without EVs (EVs−). The experimental groups, along with a control group (non-capacitated spermatozoa without EVs), were incubated for 2 h. Subsequently, a Western blot analysis was performed to evaluate protein kinase A activity (pPKA) and tyrosine phosphorylation (Tyr-P). In addition, functionality was evaluated through in vitro fertilization conducted at 0 h and after 48 h of sperm preservation, followed by a 2-h incubation in capacitation medium (TALP). The parameters analyzed included the percentage of oocytes penetrated (% Penetration), the number of spermatozoa penetrating each oocyte (Spz/O), and the number of spermatozoa bound to the zona pellucida (Spz/ZP).

using a calibrated sperm spectrophotometer (Spermacue Minitub, Tiefenbach, Germany) and then diluted with an MR-A boar semen extender (Kubus S.A., Madrid, Spain) to achieve a final concentration of 30×10^6 spermatozoa/mL.

2.6 | Analysis of Epididymal Spermatozoa

2.6.1 | Motility Analysis by CASA

Sperm motility and kinetic parameters were determined by computer-assisted spermatozoa analysis (CASA) system (PROISER R+D S.L., Valencia, Spain). For this purpose, a 4-μL drop of the samples was placed in a prewarmed (38.5°C) 20 μm SpermTrack chamber (STP-21006, PROISER I+D S.L., Valencia, Spain). The acquisition parameters were 25 frames per second and particles between 10 and 80 μm^2. Five images per sample were captured, with data collected from at least 200 spermatozoa per sample.

The percentage of spermatozoa with MotP (%), curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), linearity of the curvilinear path (LIN, ratio of VSL/VCL, %), straightness of the average path (STR, ratio of VSL/VAP, %), wobble coefficient (WOB, %), amplitude of lateral head displacement (ALH, μm), and beat-cross frequency (BCF, Hz) were analyzed. Spermatozoa with VAP < 20 μm/s were considered immotile, while those with VAP ≥ 25 μm/s and STR of at least 45% were considered progressively motile.

2.6.2 | Viability, Acrosome Status, and Mitochondrial Activity

To evaluate the status of the acrosome and viability, the peanut *Arachis hypogaea* lectin linked to fluorescein isothiocyanate (FITC-PNA) and propidium iodide (PI) were used. The fluorochrome used to assess mitochondrial activity was rhodamine 123 (Rh123; R-8004, Merck). To study fluorescence measurement, a Guava easyCyte 6-2 flow cytometer (Millipore, California, USA) was used. The 488 nm blue laser was used for excitation, while the FL-1 sensor with a band-pass filter of 525 nm detected the PNA-FITC and Rh123. The FL-2 sensor with a 650 nm band-pass filter detected the PI. An FSC and SSC gate was applied to exclude aggregates and contaminants from the subsequent analysis [16]. Data from 3000 events were analyzed, with two measurements recorded for each sample.

For evaluation, samples of epididymal spermatozoa were prepared at a concentration of 200,000 spermatozoa/mL in PBS. Samples were incubated with 2.5 μg/mL of PI and with 2 μg/mL of PNA-FITC for 10 min at room temperature before measurement [16]. To prepare Rh123 for mitochondrial activity, samples were incubated with 0.53 mM of Rh123 in PBS, and then incubated at 37°C for 10 min, followed by centrifugation at 1000 g for 3 min. Flow cytometry data were analyzed using Expo32ADC software (Beckman Coulter Inc.). A fluorescence compensation procedure was performed to correct for spectral overlap, identifying four subsets of spermatozoa in terms of viability and acrosomal status: viable spermatozoa without acrosomal damage (PI and FITC-PNA-negative), dead spermatozoa without acrosomal damage (PI positive and FITC-PNA negative), dead spermatozoa with acrosomal damage (PI and FITC-PNA positive), and viable spermatozoa with acrosomal damage (PI negative and FITC-PNA positive) [17]. Regarding mitochondrial activity two distinct subsets of spermatozoa were identified: the fluorescence-positive spermatozoa were identified as those with mitochondrial activity, whereas the fluorescence-negative spermatozoa were identified as those without activity.

2.7 | Sperm Capacitation Analysis

To determine the sperm capacitation status, pPKA and Tyr-P were evaluated.

2.7.1 | Determination of pPKA and Tyr-P

Total isolated proteins from 3×10^6 spermatozoa samples were extracted and analyzed using Western blot according to the methodology previously described by Soriano-Úbeda et al. [15]. Electrophoresis was performed on a 12% sodium dodecyl sulfate polyacrylamide gel (Mini-Protean TGX gels, Bio-Rad) at a current of 40 mAmp/gel. Membrane transfer was then performed at 250 mAmp/gel.

Membrane unspecific unions were blocked with BSA (5%) in Tris-Buffered Saline Tween 20 (TBST) and incubated overnight at 4°C with the primary polyclonal antibodies anti-PKA substrate (9624; Cell Signaling Technology; 1:10,000) and anti-Tyr-P (05-321X; Merck; 1:10,000), with anti-β-tubulin (T0198; Merck; 1:5000) as a housekeeping protein. Subsequently, the membranes were incubated with goat-anti mouse and goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000) for 1–2 h at room temperature.

Blots were visualized using Pierce ECL Plus Western Blotting Substrate, and the chemiluminescence was captured using an Amershan Imager 600 (GE Healthcare). The relative amount of signal in each membrane was quantified using the ImageQuant TL v8.1 software (GE Healthcare, Life Sciences, Buckinghamshire, UK).

2.8 | In Vitro Sperm Penetration Test

2.8.1 | Oocyte Collection and In Vitro Maturation

Porcine oocytes were isolated from ovaries obtained from prepuberal gilts at a local abattoir transported to the laboratory at 38.5°C in a saline solution. Cumulus–oocyte complexes (COCs) were collected from antral follicles (3–6 mm in diameter). Only COCs exhibiting complete and dense cumulus oophorous were cultured in groups of 50 in 4-well dishes (Nunc, Roskilde, Denmark) containing 500 μL of maturation medium NCSU-37 [18]. The COCs were matured for 40–42 h as previously described by Funahashi et al. [19].

2.8.2 | In Vitro Fertilization

After in vitro maturation (IVM), the COCs were gently pipetted to remove the cumulus cells and groups of 50 oocytes were transferred to 500 μL of TALP medium [20]. The oocytes were inseminated at concentration of 125,000 spermatozoa/mL for 18 h at 38.5°C under 5% CO_2 in a humidified atmosphere. After 18 h of co-culture, putative zygotes were fixed and evaluated as previously described by Matás et al. [17]. Putative zygotes containing one or more decondensed sperm heads or male pronuclei with one or more detached sperm tails were considered penetrated. Oocytes exhibiting degenerative changes, those that were immature, and those with atypical cytoplasmic features were excluded from the count. The IVF parameters evaluated were percentage of penetrated oocytes (Pen, %; defined as no. oocytes penetrated/total no. inseminated), number of sperm penetrating each oocyte (SPZ/O; defined as no. pronuclei or decondensed sperm heads observed minus one pronucleus from female oocyte), and number of spermatozoa bound to the zona pellucida (SPZ/ZP; defined as those remaining after oocytes had been washed through multiple passages with an automatic pipette and several transfers into different PBS-containing wells).

2.9 | Experimental Design

The aim of this study was to evaluate the role of EVs from SP on the functionality of epididymal spermatozoa.

2.9.1 | Experiment 1: Effect of EVs on Epididymal Sperm Preservation

Samples of 30×10^6 spermatozoa/mL were supplemented with 400×10^6 EVs/mL (EVs+) as described previously by Lange-Consiglio et al. [21], or without them (EVs−) in an MR-A extender and stored at 15°C for up to 72 h (Figure 2). The following analyses were performed at 0, 24, 48, and 72 h:

i. Sperm motility was assessed using CASA, and acrosome integrity, viability, and mitochondrial activity were assessed by flow cytometry. This experiment was performed in seven replicates.

ii. Thermal resistance was used as an indicator of boar semen quality, as spermatozoa are subjected to temperature changes in the female genital tract, affecting the viability and storage of sperm acrosomes [22]. Assays were performed after 24 and 72 h of storage, and samples were incubated for a further 5 h at 38.5°C. After these 5 h of incubation, motility, viability, acrosome integrity, and mitochondrial activity were determined. This experiment was performed in six replicates.

2.9.2 | Experiment 2: Effect of EVs on Epididymal Sperm Functionality

2.9.2.1 | Effect of EVs on Sperm Capacitation. A total of 3×10^6 spermatozoa were diluted in 1 mL of capacitation medium (TALP) supplemented or not with EVs (EVs+ and EVs−, respectively). Samples were incubated for 2 h at 38.5°C, 5% CO_2, and 100% humidity. Spermatozoa diluted in an MR-A extender were used as non-capacitated. After incubation, the proteins were extracted, and pPKAs and Tyr-P were determined (Figure 3). Five replicates were performed.

2.9.2.2 | Effect of EVs on Gamete Interaction and Sperm Penetration. In vitro matured oocytes were inseminated with 125,000 spermatozoa/mL supplemented or not with EVs (EVs+ or EVs−, respectively) in TALP medium for 2 h. After 16–18 h of co-culture, IVF parameters were evaluated (Figure 3). Seven replicates were performed.

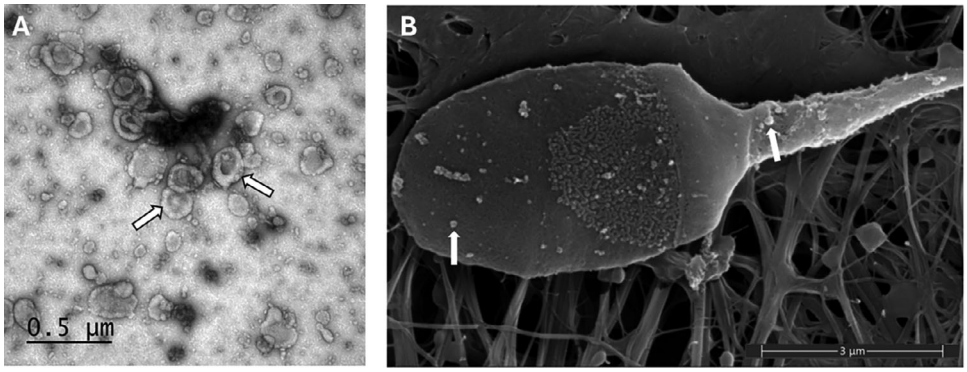

FIGURE 4 | Detection of EVs in seminal plasma by transmission electron microscopy and scanning electron microscopy. (A) Vesicles examined by transmission electron microscopy appeared as roughly round and enclosed by double membranes embedded in abundant amorphous material, which occasionally included short, flattened membranous structures. Most vesicles exhibited a clear internal space. Arrows indicate EVs. (B) Vesicles attached to spermatozoa as captured by scanning electron microscopy. Samples were incubated in TALP, fixed, and examined to evaluate the morphology and adhesion of EVs to spermatozoa. EVs are bound to the head and the midpiece of the flagellum. Arrows indicate EVs.

2.10 | Statistical Analysis

Statistical analysis was performed using the IBM SPSS Statistics 24.0 software package (IBM SPSS Inc., Chicago, USA; version 28). Motility, kinetic parameters, cytometry results, and IVF parameters are presented as the mean ± standard error.

Normality of the variables was assessed by the Shapiro–Wilk test, and the homogeneity of variance was assessed by the Levene test. One-way ANOVA and post hoc Tukey tests were performed for the preservation data. The Student's *t*-test for independent samples was used to analyze the IVF results. A statistically significant difference was considered at $p \leq 0.05$.

3 | Results

3.1 | Characterization of EVs

TEM and SEM visualizations confirmed the presence of EVs in SP (Figure 4A,B . EVs were identifiable from their cup-shaped membrane-surrounded vesicles in their native stage (Figure 4A). NTA analysis revealed a mean particle size in the sample of around 168.0 ± 2.7 nm and a concentration of $58.4 \times 10^7 \pm 1.36 \times 10^7$ particles/mL (Figure 5). The percentage of EVs markers by flow cytometry was 21.7% for CD63, 22.10% for CD81, and 63.8% for HSP70. Furthermore, the high purity of the samples was confirmed by the detection of albumin contamination (< 10%).

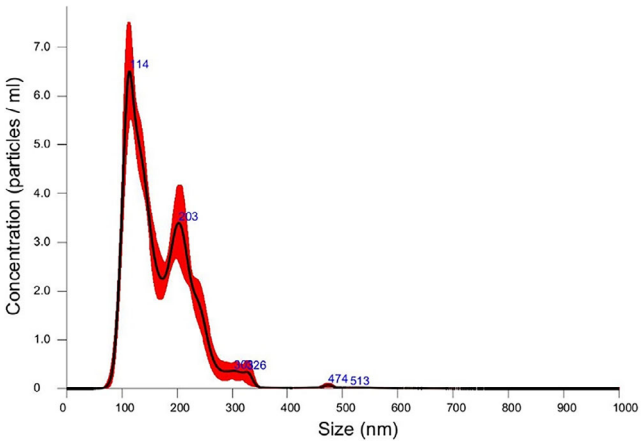

FIGURE 5 | Characterization of seminal plasma EVs. Nanoparticle tracking analysis (NTA) of boar SP EVs. Mean particle concentration (particles/mL) and size distribution (nm) of EVs.

3.2 | Experiment 1: Effect of EVs on Epididymal Sperm Preservation

3.2.1 | Sperm Motility

Total motility and MotP as well as velocity parameters were influenced by the presence of EVs during the preservation process. Both total motility and MotP remained at higher levels throughout the experiment, but statistically significant differences ($p < 0.05$) were only observed at 24 (62.4 ± 4.1% vs. 46.7 ± 4.9%) and 48 h (35.1 ± 5.8% vs. 17.3 ± 5.6%) for total motility and at 48 h for MotP (11.4 ± 2.5% vs. 4.1 ± 2.5%) (Figure 6A,B). The proportion of spermatozoa that exhibited medium velocity at 0, 24, and 48 h were higher when the medium was supplemented with EVs ($p < 0.05$). However, the percentage of slow spermatozoa increased at 48 h in the EVs+ group (28.4 ± 7.9% vs. 4.1 ± 1.0%). The percentage of rapid spermatozoa remained unchanged (Table S1).

VSL, VAP, and ALH also were affected by the presence of EVs. VSL and VAP decreased in the presence of the EVs at time 0 (29.6 ± 1.8 µm/s vs. 38.6 ± 3.5 µm/s, and 40.7 ± 2.4 µm/s vs. 50.9 ± 3.4 µm/s, respectively). However, from time 24 h, VSL and VAP began to converge. The value of ALH increased at 72 h in spermatozoa supplemented with EVs (2.1 ± 0.6 µm vs. 0.4 ± 0.2 µm) (Table S2) but remained stable at all other times. No statistically significant differences were observed for other motility parameters throughout the study (Table S3).

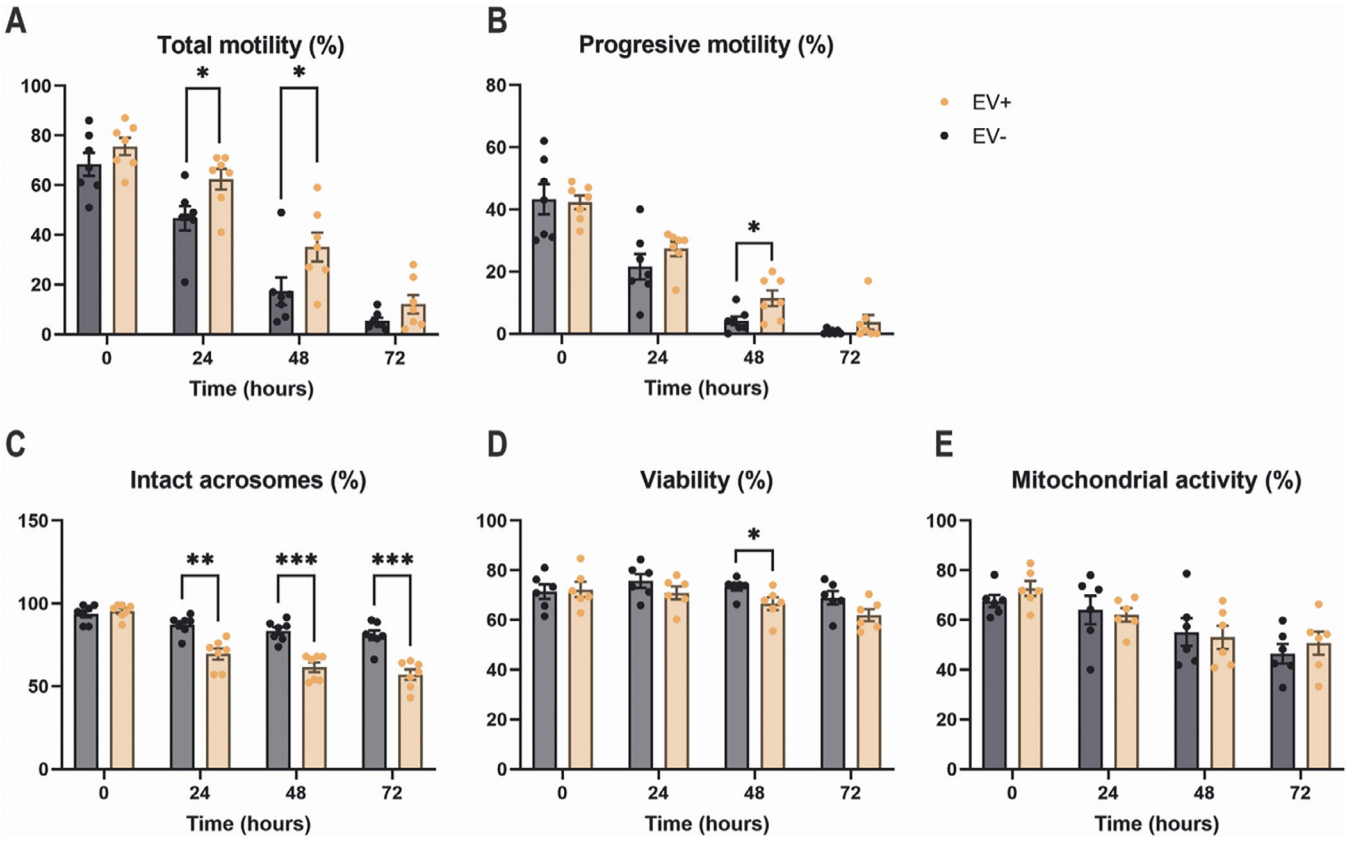

FIGURE 6 | Effect of EVs on epididymal sperm functionality. Sperm parameters evaluated in epididymal spermatozoa stored at 15°C with or without EVs. Evaluations were performed at 0, 24, 48, and 72 h after incubation with EVs (EVs+) or without EVs (EVs−). Parameters evaluated by computer-assisted sperm analysis (CASA): (A) total motility, (B) progressive motility. Parameters evaluated by flow cytometry: (C) intact acrosomes: percentage of spermatozoa with intact acrosomes, (D) viability: percentage of viable spermatozoa, and (E) mitochondrial activity: percentage of spermatozoa with high mitochondrial activity. EVs+: Epididymal spermatozoa supplemented with EVs. EVs−: Epididymal spermatozoa without supplementation. Significant differences were represented with an asterisk.

3.2.2 | Sperm Viability, Acrosomal Integrity, and Mitochondrial Activity of Spermatozoa

Sperm viability was affected by the presence of EVs. At 48 h, the percentage of viable spermatozoa decreased in the supplemented group ($p < 0.05$) (Figure 6D). Moreover, the percentage of spermatozoa with intact acrosomes remained significantly lower in the EVs+ group throughout the experiment ($p < 0.05$), from 24 to 72 h (Figure 6C). No statistically significant effects on mitochondrial activity were observed throughout the study (Figure 6E).

3.2.3 | Sperm Thermal Resistance

The results for total motility and MotP showed no significant differences after the thermal resistance experiment, although the EVs+ group had higher total motility and MotP at both time points (24 and 72 h) (Figure 7A,B). In addition, although the percentage of intact acrosomes was greater in the EVs− group over time, there were no significant differences in viability between the experimental groups (Figure 7C,D). Finally, no differences in mitochondrial activity were observed between the experimental groups over time (Figure 7E).

3.3 | Experiment 3: Effect of EVs on Epididymal Sperm Functionality

3.3.1 | Effect of EVs on Sperm Capacitation

The Western blot of pPKA revealed a decrease of intensity when spermatozoa were incubated in capacitation media supplemented with EVs. Western blot analysis of Tyr-P showed no differences in phosphorylation pattern, but a 20 kD band was only present in the capacitated spermatozoa group in the absence of vesicles (Figure 8A).

3.3.2 | Effect of EVs on Gamete Interaction and Sperm Penetration

The IVF results after 2 h of sperm incubation and after 48 h of preservation are presented in Figure 8B,C. When insemination was performed using spermatozoa that had been preincubated with EVs, a significant decrease was observed in all evaluated parameters ($p < 0.05$). Specifically, there was a reduction in the percentage of oocyte penetrated by spermatozoa, a lower number of spermatozoa penetrated per oocyte, and a decrease in the number of spermatozoa bound to zona pellucida in both conditions.

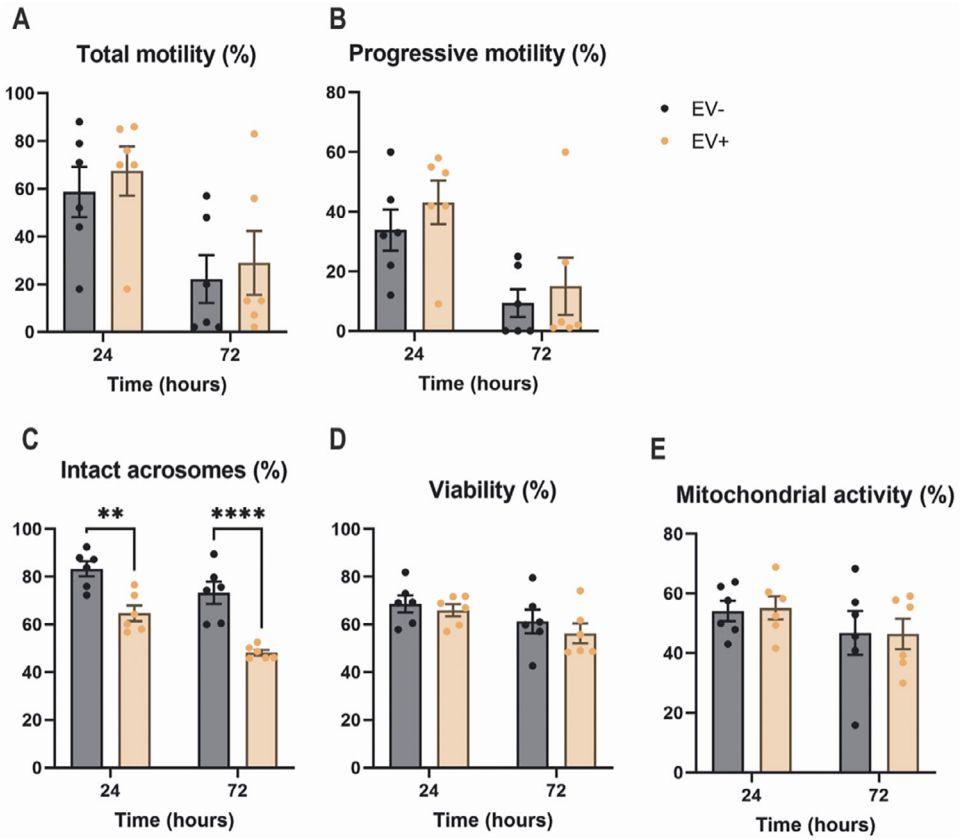

FIGURE 7 | Effect of EVs on epididymal sperm thermal resistance. The thermal resistance assay was conducted after 24 and 72 h of storage with EVs (EVs+) or without EVs (EVs−) with samples being incubated for an additional 5 h at 38.5°C. Following 5-h incubation parameters were evaluated by computer-assisted sperm analysis (CASA): (A) total motility, (B) progressive motility; and by flow cytometry: (C) intact acrosomes: percentage of spermatozoa with intact acrosomes, (D) viability: percentage of viable spermatozoa, and (E) mitochondrial activity: percentage of spermatozoa with high mitochondrial activity. EVs+: Epididymal spermatozoa supplemented with EVs. EVs−: Epididymal spermatozoa without supplementation. Significant differences were represented with an asterisk.

4 | Discussion

Spermatozoa from the cauda epididymis have only been exposed to epididymal secretions and EVs. During ejaculation, they mix with secretions and EVs from the accessory glands. Most studies have examined the effects of SP on ejaculated spermatozoa, which may already be influenced by these secretions. Therefore, studying the impact of accessory gland EVs on epididymal spermatozoa offers a clearer understanding of their specific functions. This study investigates the effect of supplementation of epididymal spermatozoa with SP EVs on sperm preservation and functionality.

The results of this study showed that the addition of EVs to the sperm preservation medium improved both the total motility and the MotP over time. While the effects on total motility were evident at time 0, they did not reach statistical significance until 24 h. These results are similar to other previous studies on ejaculate preservation, which also reported an improvement in sperm motility when EVs were incorporated under optimal conditions [9, 23]. Other studies, such as those conducted by Guo et al. [24], which were performed under non-capacitive conditions, and Lange-Consiglio et al. [21], which included incubation in a capacitation medium (TALP), showed that EVs primarily enhanced MotP. Thus, our results suggest that EVs positively influence sperm motility in both functional contexts. The underlying mechanism may be explained by the findings of Park et al. [25], who observed that prostasomes, which are EVs secreted by the prostate, contain Ca^{2+} and the necessary machinery to regulate the signaling pathway, that is, crucial for maintaining motility. Regarding sperm velocity, it was observed that spermatozoa of average and slow velocities were affected by EVs. At 48 h, the decrease in average velocity was less pronounced, while a significant increase in slow velocity was observed in the supplemented spermatozoa. This suggests that EV supplementation helps mitigate the reduction in velocity, making the decline less severe. However, more research is required to confirm this.

A reduction in both lineal velocity and VAP was observed in the supplemented group at time 0. While these values fluctuated over time, they did not reach statistical significance. This result could be attributable to a diluent effect, since the study of Lange-Consiglio et al. [21] found that the highest adhesion of EVs to spermatozoa occurs after 1 h, whereas this assessment of these parameters occurred at time 0. Regarding other parameters, EVs could maintain the ALH for a period of 72 h.

Sperm viability and acrosomal integrity were affected by the presence of EVs in the medium during the analyzed time period.

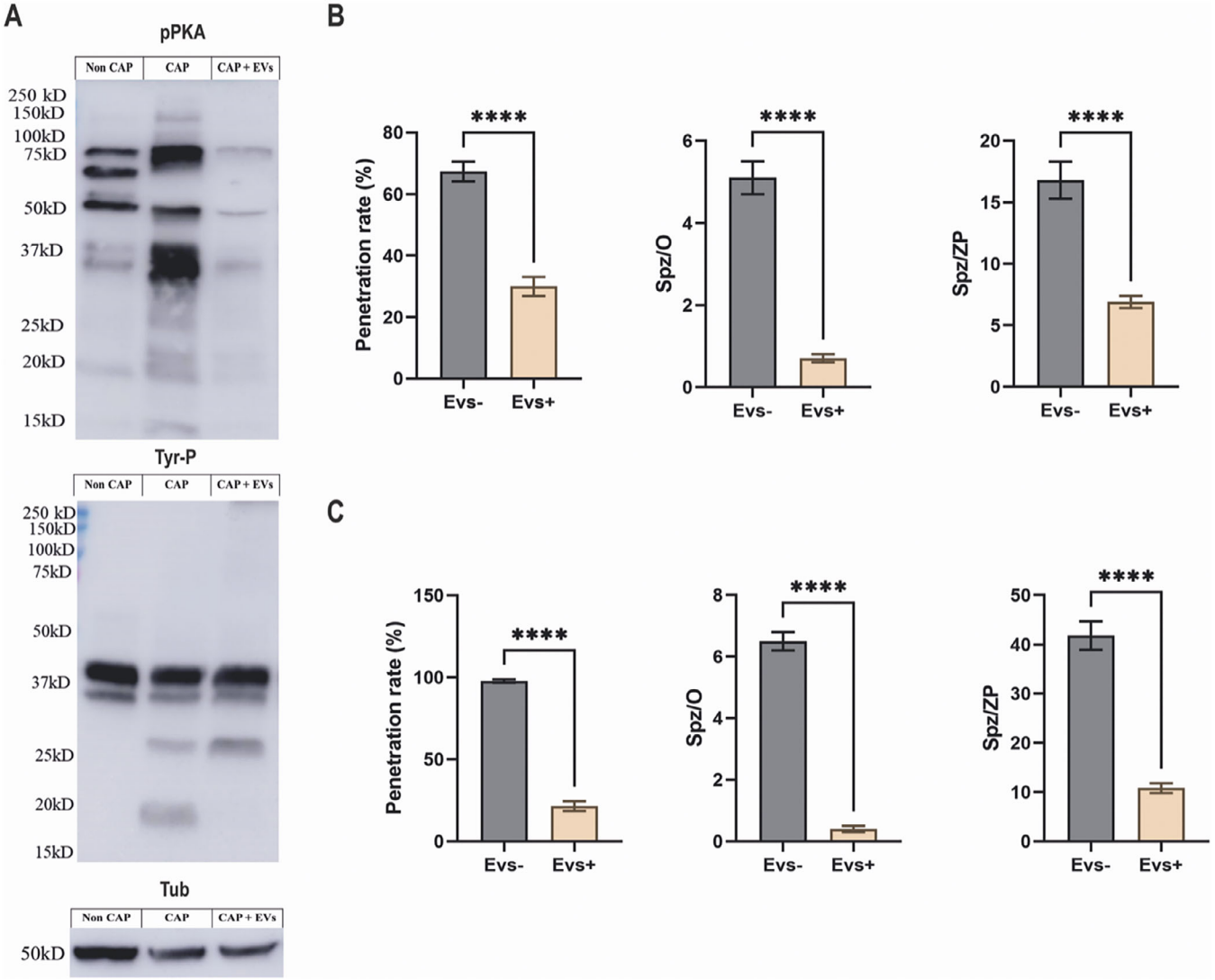

FIGURE 8 | Effect of EVs on epididymal sperm capacitation and penetration capacity. (A) Capacitation status of epididymal spermatozoa assessed through protein kinase A activity (pPKA) and tyrosine phosphorylation (Tyr-P). Tubulin was used as a loading control. Non-CAP: Non-capacitated epididymal spermatozoa without EV supplementation. CAP: Capacitated epididymal spermatozoa without EV supplementation. CAP+EVs: Capacitated epididymal spermatozoa supplemented with EVs. (B) Effect of EVs on IVF with epididymal sperm after 2 h of incubation. EVs+: Epididymal spermatozoa supplemented with EVs. EVs−: Epididymal spermatozoa without supplementation. Results were shown as penetration rate (percentage of oocyte fertilized), Spz/O (mean of spermatozoa penetrated per oocyte), and Spz/ZP (mean of spermatozoa binding to zona pellucida). Data were represented as the mean and SEM of each experimental group. (C) Effect of EVs on IVF with epididymal sperm stored for 48 h. EVs+: Epididymal spermatozoa supplemented with EVs. EVs−: Epididymal spermatozoa without supplementation. The same parameters described in Figure 6B were evaluated. Significant differences were represented with an asterisk.

These results contrast with those observed by Du et al. [9]. and Wang et al. [23]. in ejaculated spermatozoa, where supplementation with EVs improved the plasma membrane integrity, likely due to the antioxidant properties of EVs. However, a comprehensive review of the extant literature did not reveal any relevant studies that evaluated the acrosomal integrity and viability of epididymal spermatozoa.

It is noteworthy that despite the observed alterations in motility, no differences were observed between groups in terms of mitochondrial activity. Some studies have demonstrated the capacity of EVs to increase mitochondrial activity [26, 27]. However, the influence of EVs on mitochondrial activity in epididymal spermatozoa remains to be elucidated.

Until they reach the site of fertilization, spermatozoa are exposed to temperatures of 38°C in the female genital tract for extended periods. This thermal stress causes only in spermatozoa with functional metabolism, to remain motile and capable of fertilization. Therefore, the ability of spermatozoa to endure extended storage periods combined with incubation at 38°C can serve as an indicator of semen quality in boars [22]. Accordingly, an objective of this study was to investigate the effect of EVs on spermatozoa under thermal stress. The results demonstrated that there were no differences in sperm motility or viability. However, a notable increase in the percentage of spermatozoa exhibiting acrosomal damage was observed in the EVs+ group at both time points. These findings indicate that EVs may exert a negative impact on the acrosome of epididymal spermatozoa.

EVs inhibit capacitation-dependent cholesterol efflux, membrane fluidity increase, and premature acrosome reaction by stabilizing the sperm membrane. Components such as the spermadhesins AWN and PSP-1 (key porcine seminal proteins involved in preventing premature capacitation delivered via vesicles) are also implicated in this process [26]. Cholesterol and phospholipids play a crucial role in maintaining membrane integrity by balancing their physiological ratio. These vesicles act early during capacitation to modulate sperm functionality and enhance fertilization potential [9]. The findings indicated that EVs reduced pPKA in capacitated spermatozoa. The results were similar to those of Soriano-Úbeda et al. [27] who observed that ejaculated spermatozoa exhibited lower pPKA than epididymal spermatozoa. Based on these results as well as the present work, we suggest that a significant part of the effect of SP on capacitation is due to the action of EVs. This EV-mediated effect could also extend to the fertility potential of spermatozoa.

The tyrosine phosphorylation results showed that the band indicating proteins of approximately 20 kD did not appear when spermatozoa were incubated in a capacitation medium with EVs. This indicates that EVs regulate protein phosphorylation on tyrosine residues of this molecular weight. Other studies have shown significant differences in band intensity in this context and have linked the presence of EVs with the inhibition of cholesterol loss, increased membrane fluidity, and the disappearance of a 14 kD band in tyrosine phosphorylation [26]. It is noteworthy that the authors of these studies focused on ejaculated spermatozoa, which exhibit a different phosphorylation pattern than epididymal spermatozoa do [27].

The IVF results showed that the presence of EVs for 2 h or after 48 h of storage decreased all fertilization parameters. These findings indicate that EVs play a pivotal role in regulating sperm capacitation and, consequently, fertilization, with these effects persisting over time. Although, as with the previous experiments, no studies employing epididymal spermatozoa were identified for direct comparison with the present results, studies using ejaculated spermatozoa [28, 29] have similarly demonstrated that EVs reduce the acrosome reaction and in vitro fertility. Furthermore, researchers have demonstrated that EVs can impair sperm adhesion to the zona pellucida, a phenomenon that was also observed in this study [28]. Perhaps, this effect may be attributed to the influence of EVs on sperm adhesins, as in vitro studies have demonstrated that EVs alter the binding capacity of porcine spermatozoa to the zona pellucida of the oocyte [30].

In conclusion, this study shows that the addition of EVs to epididymal spermatozoa improves motility but negatively affects viability and acrosomal integrity. Regarding sperm capacitation, a reduction in pPKA activity was observed in the presence of EVs, accompanied by an inhibition of Tyr-P in proteins of approximately 20 kD. Exactly why the 20 kDa proteins do not appear to be phosphorylated when EVs are present in the capacitation medium remains unclear. Finally, a decrease in IVF parameters was observed when fertilization was performed with spermatozoa that had been incubated with EVs. It seems reasonable to conclude that EVs play a role in these functions, preventing premature capacitation and promoting preservation through the maintenance of sperm motility over time. Nevertheless, further research is required to investigate the multiple effects of EVs on epididymal spermatozoa.

Author Contributions

Ana Moya-Fernández: writing – review and editing, writing – original draft, validation, software, methodology, investigation, formal analysis, data curation. **Santa María Toledo-Guardiola**: writing – review and editing, writing – original draft, validation, software, methodology, investigation, formal analysis, data curation. **Peter Silke**: writing – review and editing, validation. **Sergio Navarro-Serna**: writing – review and editing, validation, software, investigation, formal analysis, data curation. **Carmen Matás**: writing – review and editing, writing – original draft, validation, software, methodology, investigation, formal analysis, data curation, visualization, supervision, resources, project administration, funding acquisition, conceptualization.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The original contributions presented in the study can be found in the article and Supporting Information and further inquiries can be directed to the corresponding authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary Figure S1: Initial forward scatter (FSC) and side scatter (SSC) flow gating plots for EVs. (A) Detection of functional EVs by staining with CFSE (carboxyfluorescein succinimidyl ester) and gating by FSC. SSC-H: Side Scatter—Height. FITC-H: Fluorescein Isothiocyanate – Height. (B) Detection of functional EVs, by staining with violet SSC of functional EVs. SSC-A: Side Scatter—Area. FITC-A: Fluorescein Isothiocyanate – Area. To delimit the functional EVs region, unstained EVs were run beforehand. The EVs region was used to gate EVs for the analysis of antibodies. **Table S1** (2): Sperm velocity from epididymal samples preserved with or without EVs and stored at 15°C for 72 h. Data are expressed as the mean ± S.E.M.**Table S2** (3): Motility parameters (VCL, VSL, VAP, and ALH) of epididymal spermatozoa with or without EVs supplementation, stored at 15°C for up to 72 h. Data are presented as mean ± S.E.M. **Table S3** (4): Motility parameters (LIN, STR, WOB, and BCF) of epididymal spermatozoa with or without EVs supplementation, stored at 15°C for up to 72 h. Data are presented as mean ± S.E.M.