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Original Research Article

Isolation and characterization of extracellular vesicle subsets in donkey seminal plasma

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ABSTRACT

Seminal plasma (SP), a fluid composed of secretions from the male genital tract, is rich in seminal extracellular vesicles (sEVs), nano-sized particles surrounded by a lipid bilayer membrane and loaded with functionally active molecules. Seminal EVs are secreted by functional cells of the male genital tract and play a key role in modulating reproductive processes, including sperm function and immune response in the female genital tract. The aim of this study was to isolate and characterize sEVs from donkey SP for the first time. Nine SP samples were collected from nine healthy and reproductive active donkeys. The SP samples were randomly pooled to create three pools (three SP samples per pool). The SP pools were subjected to differential centrifugation and size-exclusion chromatography to separately isolate two subsets of sEVs: small (S-) and large (L-). Orthogonal characterization of sEV samples was performed according to MISEV 2023 guidelines, including morphology (by cryogenic electron microscopy), concentration (by total protein concentration and total and CFSE positive particles by flow cytometry [FC]), particle size distribution (by dynamic light scattering), purity (by albumin assessment by FC), and specific EV protein markers (tetraspanins CD9, CD63, and CD81, and HSP70 by FC). The results showed that donkey SP is highly enriched in sEVs. Size differences were found between both sEV subsets, with S-sEVs being smaller (~160 nm) and L-sEVs larger (~290 nm). Both sEV subsets were positive for the four EV protein markers. However, the percentage of CD81-positive events was higher in S-sEV samples than in L-sEV samples ($P < 0.05$). This study is the first to isolate and characterize sEVs in donkey SP, demonstrating their heterogeneity and suggesting differences in biogenesis and function between S-sEVs and L-sEVs.

1. Introduction

For centuries, donkeys (*Equus asinus*) have been indispensable to human societies, mainly used for agriculture, transportation, and mule production. Despite a significant decline in their use in Western Europe during the 20th century due to industrialization and agricultural mechanization, donkeys still remain indispensable in developing countries, supporting rural livelihoods [1–3]. Recently, interest in donkeys has increased, driven largely due to their growing role in industries such as dairy production, cosmetics, forestry, rural tourism, and recreation,

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as well as conservation programs aimed at preserving endangered European breeds [1,2]. These trends have spurred research to understand and improve the reproductive performance of this species [2–5].

The reproductive physiology of donkeys presents specific features that distinguish them from other mammalian species. As for horses, although they are phylogenetically close to donkeys, they exhibit significant reproductive differences [6]. Their sperm differ not only in motility, morphology, and interaction with the female endometrium [7], but also in aspects such as spermatogenic efficiency, the duration of spermatogenesis [8], and the composition of their seminal plasma [9,10]. In this sense, increasing evidence suggests that male fertility potential is influenced not only by sperm, but also by seminal plasma (SP), the fluid secreted by the organs of the male genital tract that surrounds the sperm during and after ejaculation [11,12]. This fluid is loaded with a variety of active biomolecules, including inorganic ions, sugars, lipids, enzymes, metabolites and proteins that play an essential role in many physiological reproductive processes [12]. In donkeys, SP has been shown to have a significant impact on sperm function [10,13] and even on sperm preservation outcomes [5,14], underlining its essential role in male reproductive performance.

It has been shown that while many SP bioactive molecules circulate freely in the SP, others are carried by extracellular vesicles (EVs), which protect them from degradation by seminal inactivating enzymes [11]. Extracellular vesicles are nano-sized (30–1000 nm) lipid bilayer-enclosed particles (including mainly proteins, lipids and nucleic acids) that are secreted into biological fluids by all functional cells [15]. The ability of EVs to transport bioactive molecules —including mainly proteins, lipids, and nucleic acids— makes them essential mediators of cell-to-cell communication and thus involved in the modulation of both physiological and pathological processes, including reproductive ones [16,17].

Seminal EVs (sEVs) are known to be involved in modulating sperm functionality and the immune environment in the female reproductive tract to facilitate successful fertilization and embryo implantation [11]. Recent studies have shown that biomolecules carried by sEVs, mainly proteins and microRNAs (miRNAs), are associated with the fertility status in several species, including cattle [18,19], pigs [20], and humans [21]. Furthermore, studies in some mammalian species have also shown that specific sEV-miRNAs and proteins would modulate key sperm physiological processes, such as sperm maturation and motility [11]. The functional properties of sEVs would explain many of the functions attributed to SP [12]. Seminal EVs have been isolated and characterized in humans [22,23] and in many animal species [24]. These studies have shown that sEVs are a heterogeneous population with differences in size, morphology, and molecular composition. Despite the growing interest in sEVs and their role in reproductive process, the presence and characteristics of sEVs in donkeys have not been studied.

The aim of the present study was to isolate and characterize, for the first time, donkey sEVs. A size exclusion chromatography (SEC)-based procedure was used to isolate two sized subsets of sEVs: small (S-sEVs) and large (L-sEVs). The isolated sEVs were characterized according to the Minimal Information for Studies of Extracellular Vesicles 2023 (MISEV 2023) [25]. Given the involvement of sEVs in reproductive processes, the identification of sEV subsets in the donkey provides valuable insights into reproductive physiology. This study also paves the way for future research to improve reproductive strategies in this species.

2. Material and methods

2.1. Animals, ejaculates and seminal plasma samples

A total of nine healthy Catalan donkeys (aged between 5 and 15 years) of proven fertility were used as ejaculate donors. The donkeys were housed in individual boxes at the Equine Reproduction Service of the Autonomous University of Barcelona (UAB, Bellaterra, Cerdanyola del Vallès, Spain). This is a center approved by Generalitat de Catalunya (Regional Government of Catalonia), Spain, and the European Union (authorization number: ES09018) for the collection of equine semen, which operates under rigorous health and animal welfare protocols. The health status was in accordance with the health guidelines established by the Council of the European Communities (Directive 82/894/EEC of December 21, 1982) and guaranteed that the donkeys were free from equine infectious anemia, contagious equine metritis and equine viral arteritis. They were subjected to a regular semen collection regime and under the same conditions. All experiments were conducted in accordance with the ARRIVE guidelines, and since no manipulations of the animals were performed beyond semen collection, the Ethics Committee of the Autonomous University of Barcelona indicated that no additional ethical approval was necessary to carry out this study.

Nine ejaculates (one per donkey) were collected using an artificial vagina (Hannover model; Minitüb GmbH, Tiefenbach, Germany) preheated to 48°C-50 °C and equipped with a nylon filter to remove the gel fraction. After ejaculate collection, a semen aliquot (10 μL) was taken to assess sperm concentration using a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Next, each ejaculate was split into two aliquots, one for sperm quality assessment and the other for SP-harvesting.

For sperm quality assessment, the semen aliquot was diluted (1:5, v:v) in a skim milk-based extender [26]. Sperm quality was assessed for motility, morphology, and viability. Sperm motility was analyzed using a computer-assisted semen analysis (CASA), and sperm morphology and viability were assessed by microscopy (bright field optical microscope, Carl Zeiss, Göttingen, Germany) in eosin-nigrosine-stained samples at × 1000 magnification [27]. The nine ejaculates met established sperm quality thresholds of more than 70 % total motile sperm, 60 % viable sperm and more than 70 % morphologically normal sperm.

To harvest SP samples, the second aliquot of semen from each ejaculate was subjected to successive centrifugations at 1500×*g* for 10 min at 4 °C (Medifriger BL-S; JP Selecta SA, Barcelona, Spain). After each centrifugation, a sample of the resulting supernatant was examined with a phase-contrast microscope (Olympus BH-2 Europa, Hamburg, Germany) at 200 × magnification to confirm the absence of spermatozoa. The number of centrifugations varied between semen samples but was at least five to obtain sperm-free SP samples. The SP samples were stored in 5 mL tubes at −80 °C until used for isolation of sEVs.

2.2. Isolation of seminal extracellular vesicles subsets

The SP samples were thawed on ice. Two-mL aliquots of three samples were randomly mixed to produce three mixtures of SP. The sEVs were isolated in each SP mixture using the SEC-based procedure described by Barranco et al. [28] and adapted for donkey SP. First, each SP mixture (6 mL) was centrifuged at 3200×*g* for 15 min at 4 °C (Sorvall™ STR40, Thermo Fisher Scientific, Waltham, Massachusetts, USA) to remove any cellular debris. The supernatants (~4 mL) were centrifuged at 20,000×*g* for 30 min at 4 °C (Sorvall™ Legend™ Micro 21R, Thermo Fisher Scientific). The resulting supernatant and pellet were processed separately for isolation of small (S-sEVs) and large sEVs (L-sEVs), respectively. The supernatant (2 mL) was diluted in 0.22-μm filtered phosphate buffered saline (fPBS; Merck KGaA, Darmstadt, Germany; 1:2, v:v), filtered (0.22-μm Millex® syringe filters, Merck KGaA), and ultrafiltered using Amicon® Ultra-4 mL centrifugal filter 10 kDa (Merck KGaA; 3200×*g* for 60 min at 4 °C) reduced to a volume of ~1.5 mL and stored at 5 °C until SEC. The 20,000×*g* pellets (from 2 mL of SP) were resuspended in fPBS (500 μL) and stored at 5 °C until SEC.

The SEC was performed using home-made columns by loading chromatography columns (20 mL-Econo-Pac®, Bio-Rad Laboratories, Hercules, California, USA) with Sepharose CL2B® (Sigma Aldrich®, Merck KGaA; ~10 mL), followed by washing with fPBS (60 mL). The resulting 20,000×*g* pellets and supernatants were then loaded separately onto SEC columns and washed with fPBS. Twenty consecutive

500 μL-fractions were collected separately from each sample, and those enriched in sEVs (fractions 7–10) were selected and mixed (2 mL). The SEC-eluted fractions 7–10 were chosen because they were the most enriched in sEVs, as demonstrated in a preliminary experiment (non-published data). This procedure resulted in six sEV samples, three from the 20,000×*g* pellets (enriched in L-sEVs) and three from the 20,000×*g* supernatants (enriched in S-sEVs). Each resulting sEV sample was divided into four 500 μL aliquots for further characterization. The design of the experiment is shown in Fig. 1.

2.3. Characterization of seminal extracellular vesicles subsets

The characterization of sEV samples was performed using different and complementary approaches as recommended in the MISEV 2023 guidelines [25]. These include measurement of total protein concentration, particle size distribution, sEV morphology and structure, immunophenotyping of EV-specific protein markers, and analysis of the proportion of non-vesicular extracellular particles (NVEPs) to measure the purity of the samples in sEVs.

2.3.1. Total protein concentration

Total protein concentration of sEV samples was measured using the commercially available Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's guidelines. Prior to analysis, sEV samples (25 μL) were lysed by mixing with a solution of 0.1 % Triton and 0.1 % of sodium dodecyl sulfate in fPBS (1:1, v:v). The mixture was incubated at 37 °C under shaking for 30 min. After incubation, the absorbance was measured at 562 nm using a plate reader (PowerWave XS; Bio-Tek Instruments, Winooski, Vermont, USA). Total protein concentration was expressed as μg/mL.

2.3.2. Particle and sEV concentration

The concentration of particles and sEVs was measured by flow cytometry using a high-sensitivity flow cytometer (CytoFLEX S; Beckman Coulter, Indianapolis, USA) according to the protocol described by Barranco et al. [29], adapted for donkey sEV samples. The CytoFLEX S, equipped with four lasers (violet 405 nm, blue 488 nm, yellow 561 nm, and red 638 nm), was configured to measure side scatter (SSC) from the 405 nm laser (violet SSC-H). Both forward scatter (FSC) and violet SSC-H were set to logarithmic scale, while the fluorescence channels were calibrated with logarithmic gain. The SSC was calibrated using polystyrene beads with diameters ranging from 80 to 300 nm (Nanosphere™ Series 3000, Thermo Fisher Scientific). In addition, commercially available EVs expressing green fluorescent protein (SAE0193, Merck KGaA) were used to validate the ability of the flow cytometer to detect sEVs and to set the gate based on sEV size (FSC) and complexity (violet SSC-H). The events included in the sEV gate were used to calculate the concentration of particles per μL. To measure sEV concentration, 10 μL of sEV sample was incubated with 100 μL of CellTrace™ carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher Scientific) working solution (5 μL of the CFSE stock solution in 495 μL of 0.1 μm-filtered PBS) for 30 min at 37 °C in the dark. Events expressing green

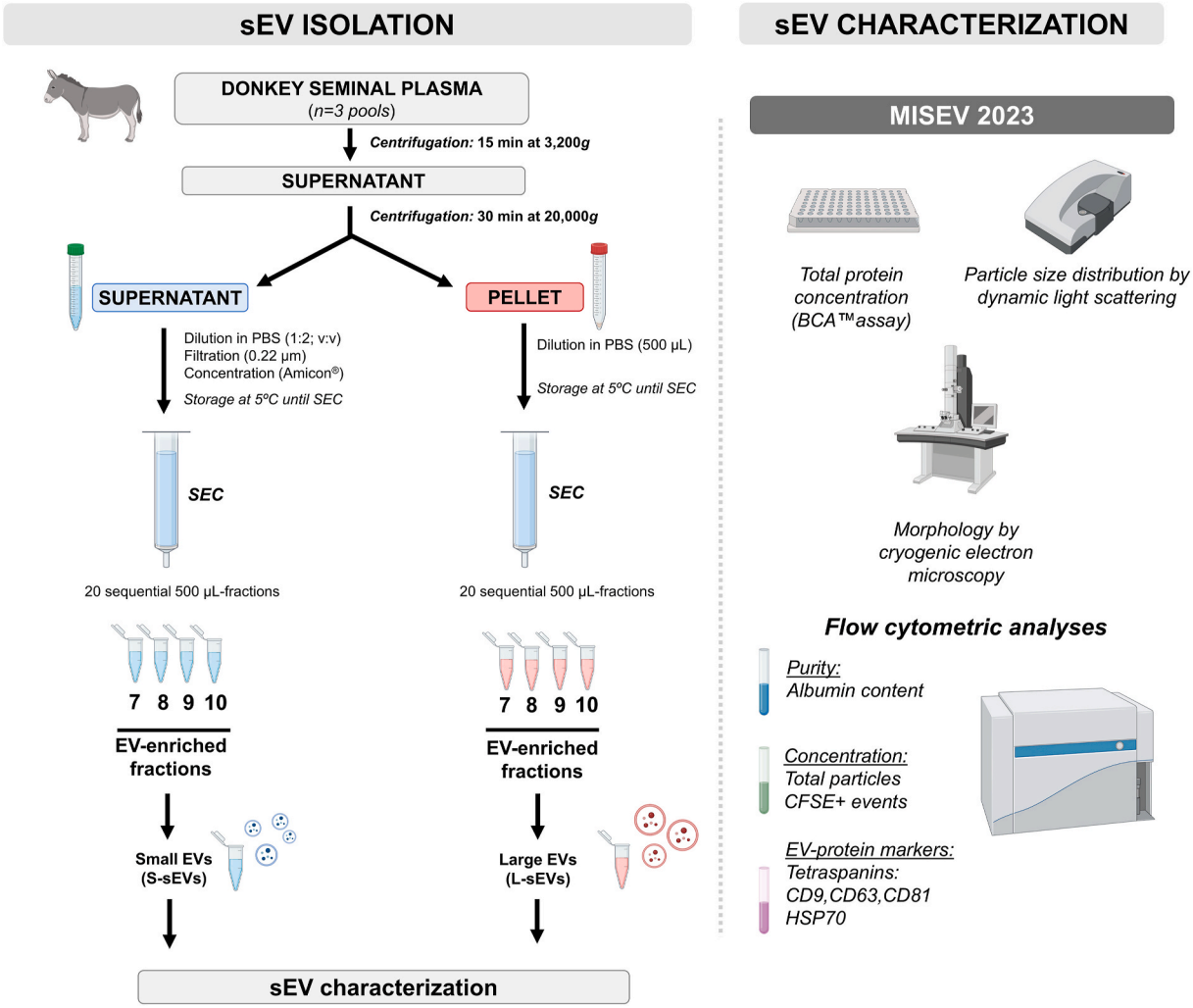

Fig. 1. Experimental design. Figure created using *Biorender.com*.

fluorescence in the sEV gate were considered with intact membrane and functional sEVs. Data were expressed as total particles/μL and particle CFSE+/μL.

2.3.3. Particle size distribution

Particle size distribution was analyzed by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS system (Malvern Panalytical, Malvern, UK). Measurements were performed by recording backscattered light at an angle of 173° and a wavelength of 633 nm. For each analysis, 50 μL of sEV sample was placed in a 10 mm pathlength cuvette and light scatter was recorded for 150 s. Each sample was measured three times to ensure accuracy. Data were analyzed using Dispersion Technology Software v.5.10 (Malvern Panalytical), which converts the DLS signal intensity into particle size distribution. The sEV diameter (nm) was calculated from the peak of the Gaussian distribution.

2.3.4. Morphology and structure of sEVs

The morphology of sEVs was analyzed by cryogenic electron microscopy (cryo-EM) according to the protocol of Parra et al. [30] with minor modifications. Briefly, each sEV sample (3 μL) was spotted onto glow-discharged 400 copper mesh Lacey Carbon TEM perforated carbon film grids (Ted Pella Inc., Redding, California, USA), blotted to a thin film and plunged into liquid ethane-N2(l) in the Leica EM GP cryo-workstation (Leica, Wetzlar, Germany). The grids were transferred to a 626 Gatan cryoholder and maintained at −179 °C. Samples were analyzed with a JEOL JEM 2011 transmission electron microscope (JEOL, Tokyo, Japan) operating at an accelerating voltage of 200 kV. Images were recorded on a Gatan Rio 16 CMOS device (CCD) camera with the Digital Micrograph 2 software package (Gatan Inc., Pleasanton, California, USA).

2.3.5. Immunophenotyping of sEVs

Immunophenotyping of sEVs was performed by flow cytometer (CytoFLEX S; Beckman Coulter, Indianapolis, USA) according to the protocol of Barranco et al. [29] adapted for donkey sEV samples.

The EV protein markers analyzed were the tetraspanins CD9, CD63 and CD81, and the transmembrane protein HSP70. The antibodies used were anti-CD9-PE (SN4 C3-3A2, 14-0098-82, Invitrogen™, Thermo Fisher Scientific), anti-CD63 (AA 120–175, antibody-online, Limerick, USA), anti-CD81 (MBS151030, MyBioSource, San Diego, USA), and anti-HSP70-APC (MA545093, Invitrogen™, Thermo Fisher Scientific). For CD63 and CD81, the secondary antibodies anti-goat HiLyte™ Fluor 647 (Jackson ImmunoResearch Labs, Baltimore Pike, USA) and anti-rabbit Alexa Fluor 633 (Thermo Fisher Scientific), were respectively used. Working solutions were prepared by diluting the antibodies with 0.1 μm-filtered PBS (1:400 for anti-CD9, anti-CD63, anti-CD81 and secondary antibodies, and 1:200 for anti-HSP70). The working solutions were centrifuged (800×*g* for 3 min; Ultrafree-MC centrifuge filters [C78142, Merck KGaA]), and the resulting filtrates were used for immunolabeling of sEVs. For flow cytometry analysis, 10 μL of sEV sample was separately incubated with the working solution of each antibody (400 μL final volume) for 30 min at room temperature.

Controls were included in the flow cytometric analysis and processed under the same conditions as the sEV-stained samples. These controls included 0.1 μm-filtered PBS, unstained sEV samples, 0.1 μm-filtered PBS with each individual antibody (including secondary antibodies), and sEV samples lysed with 0.1 % Triton and 0.1 % sodium dodecyl sulfate in fPBS and incubated with each antibody. A total of 10×10^3 events/sEV sample were recorded at a low flow rate (10 μL/min). In addition, 0.1 μm-filtered PBS was used to assess background noise, while 0.1 μm-filtered distilled water was used as the sheath fluid. Two technical replicates were performed for each sEV sample.

2.3.6. Purity of sEV samples

The purity of the samples in sEVs was assessed by measuring the amount of soluble albumin, a NVEP found in high levels in SP. Albumin was analyzed by flow cytometry using the anti-horse albumin FITC antibody (MBS524209, MyBioSource). The working solution was prepared by diluting the antibody in 0.1 μm-filtered PBS (1:200). Ten μL of sEV sample was diluted with the working solution (400 μL final volume) and incubated for 30 min at 37 °C in the dark.

2.4. Statistical analysis

Data were analyzed using the Prism 10.2.3 statistical package (GraphPad Software Inc, La Jolla, California, USA). First, the normal distribution of the data was assessed using the Shapiro–Wilk test. If necessary, an arcsine $\sqrt{x}$ transformation was applied. The data were then analyzed by unpaired *t*-test (S-sEVs *vs* L-sEVs). Differences were considered statistically significant at $P < 0.05$. Data are presented as mean ± SD.

3. Results

The total protein concentration (median, 25th–75th percentile) was similar in S-sEV (566.3, 462.4–622.5 μg/mL) and in L-sEV samples (473.9, 396.0–590.6 μg/mL; Fig. 2A). The concentrations of total particles and CFSE + particles were higher ($P < 0.05$) in L-sEV (mean ± SD; $2.97 \times 10^5 \pm 6.23 \times 10^4$ and $1.52 \times 10^5 \pm 3.70 \times 10^4$, respectively) than in S-sEV samples ($1.11 \times 10^5 \pm 5.47 \times 10^4$ and $5.31 \times 10^4 \pm 3.59 \times 10^4$, respectively) (Fig. 2B). However, the percentage of CFSE + particles was similar in S-sEV (mean ± SD; 44.87 ± 9.59 %, ranging from 34.93 to 54.06 %) and L-sEV samples (51.14 ± 4.91 %, ranging from 46.52 to 56.29 %) (Fig. 2C). Particle size distribution (sEV diameter) assessed by DLS showed that sEVs were smaller ($P < 0.0001$) in S-sEV samples (median, 25th–75th percentile; 161.7, 158.6–165.8 nm) than in L-sEV samples (median, 25th–75th percentile; 289.7, 277.1–314.4 nm; Fig. 2D; Supplementary file S1).

Cryo-EM images confirmed the presence of sEVs in both S- and L-sEV samples and showed that the population of sEVs was heterogeneous in terms of size, morphology and electron density (Fig. 3). The S-sEV samples were predominantly enriched in small sEVs, ranging from 50 to 180 nm in diameter, with spherical or ovoid morphologies (Fig. 3A). In contrast, the L-sEV samples contained larger sEVs, ranging from 100 to 500 nm in diameter, with spherical or elongated morphologies (Fig. 3B).

Immunophenotyping analysis revealed the presence of flow cytometry CFSE + positive events for the EV protein markers CD9, CD63, CD81, and HSP70 in both sEV subsets (Fig. 4). The percentage of CD81 positive was higher ($P < 0.05$) in S-sEV samples (mean ± SD; 77.80 ± 19.81 %, ranging from 55.03 to 91.15 %) than in L-sEV samples (27.80 ± 3.72 %, ranging from 25.29 to 32.07 %). No differences were found in the percentages of CD9, CD63 and HSP70-positive events between the two sEV subsets. The percentage of CD9-positive events was 35.42 ± 0.79 % (range from 34.66 to 36.24 %) and 34.98 ± 0.64 % (range from 34.44 to 35.69 %) in S-sEV and L-sEV samples, respectively. The percentage of CD63-positive events was 40.96 ± 6.46 % (range from 33.63 to 45.83 %) and 42.45 ± 2.18 % (ranging from 40.17 to 44.51 %) in S-sEV and L-sEV samples, respectively. The percentage of HSP70-positive events was 43.48 ± 4.45 % (range from 40.31 to 48.57 %) and 36.81 ± 3.70 % (range from 34.01 to 41.00 %) in S-sEV and L-sEV samples, respectively.

Flow cytometry analysis also revealed a high purity degree in sEVs in both sEV subsets, as the percentage of albumin-positive events was very low (mean ± SD; 3.09 ± 0.99 % in s-sEV samples and 2.05 ± 0.29 % in L-sEV samples).

4. Discussion

The present study shows that donkey SP contains a large phenotypically heterogeneous population of sEVs that can be isolated by SEC into two subsets that differ in size and in a specific protein marker of EVs, specifically the tetraspanin CD81. This study of donkey sEVs can be

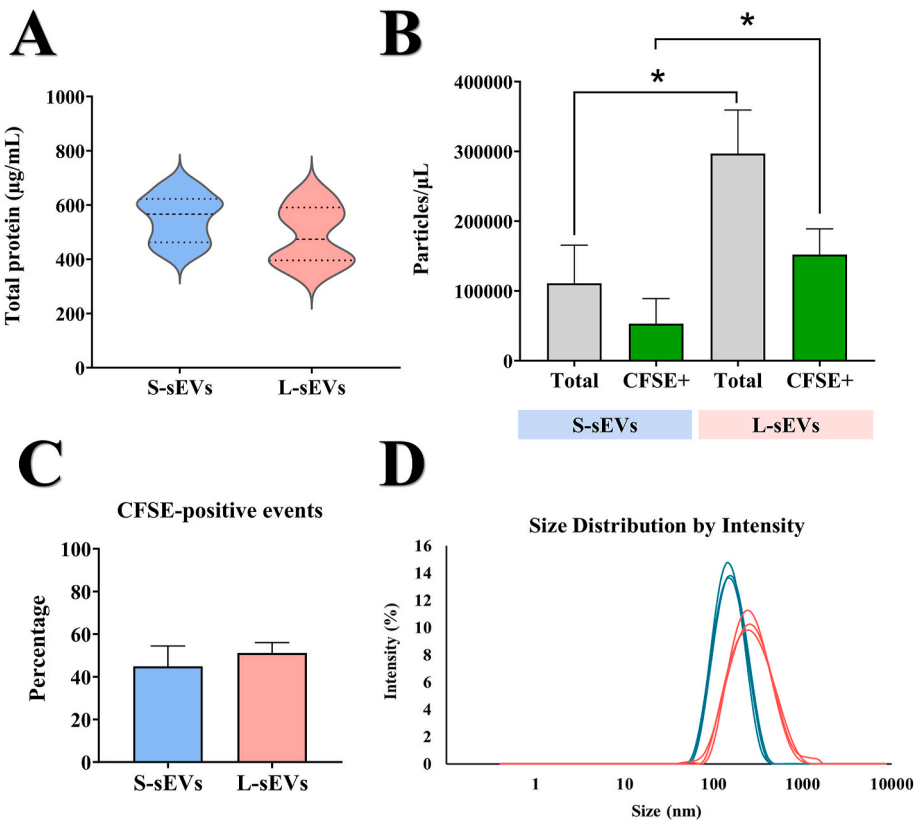

Fig. 2. **(A)** Violin plot representing the total protein concentration levels (µg/mL) in two seminal extracellular vesicles (sEVs) subset samples (small [S-sEVs, blue] and large [L-sEVs, pink]) isolated from donkey seminal plasma. Dashed line represents the median and dotted lines the 25 and 75 % quartiles. **(B, C)** Bar graphs showing the mean and standard deviation of concentrations and percentages of total particles and 5-(and 6)-Carboxyfluorescein diacetate, succinimidyl ester (CFSE)-positive particles (sEVs) in S- and L-sEV samples. **(D)** Particle size distribution by intensity (average) of sEV population in the S- (blue) and L-sEV (pink) samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

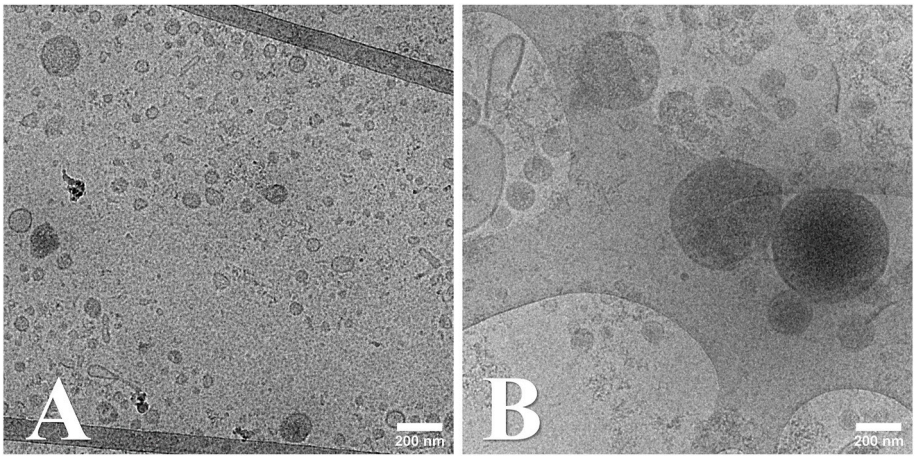

Fig. 3. Representative cryo-electron microscopy images of donkey seminal extracellular vesicles (sEVs) in **(A)** small sEV samples and **(B)** large sEV samples.

considered groundbreaking as, to our knowledge, only three studies have isolated and characterized EVs in this species, all from milk [31–33].

Mounting evidence suggests that sEVs play a key role in several reproductive processes (Fig. 5). Functional studies conducted in mammalian species have shown that sEV may be involved in modulating sperm physiological processes [11]. These include sperm capacitation, motility, acrosome reaction, lipid peroxidation and, even, sperm-zona pellucida binding [11,16,34]. In addition, sEV have been shown to modulate the immune environment in the female genital tract, facilitating embryo development and implantation [16]. Despite these findings, the specific role of sEVs in these reproductive processes requires further study, as conflicting results have been reported, possibly due to the heterogeneity of sEV subsets [11].

Given the diversity of EV subsets in a biological fluid and the uncertainties regarding their biogenesis, the MISEV 2023 guidelines suggest a classification of EVs into two size-based categories: small EVs (<200 nm) and large EVs (>200 nm) [25]. Accordingly, in our study, two size-based sEV subsets (S- and L-sEVs) were isolated using a SEC-based procedure previously described by Barranco et al. [28] and

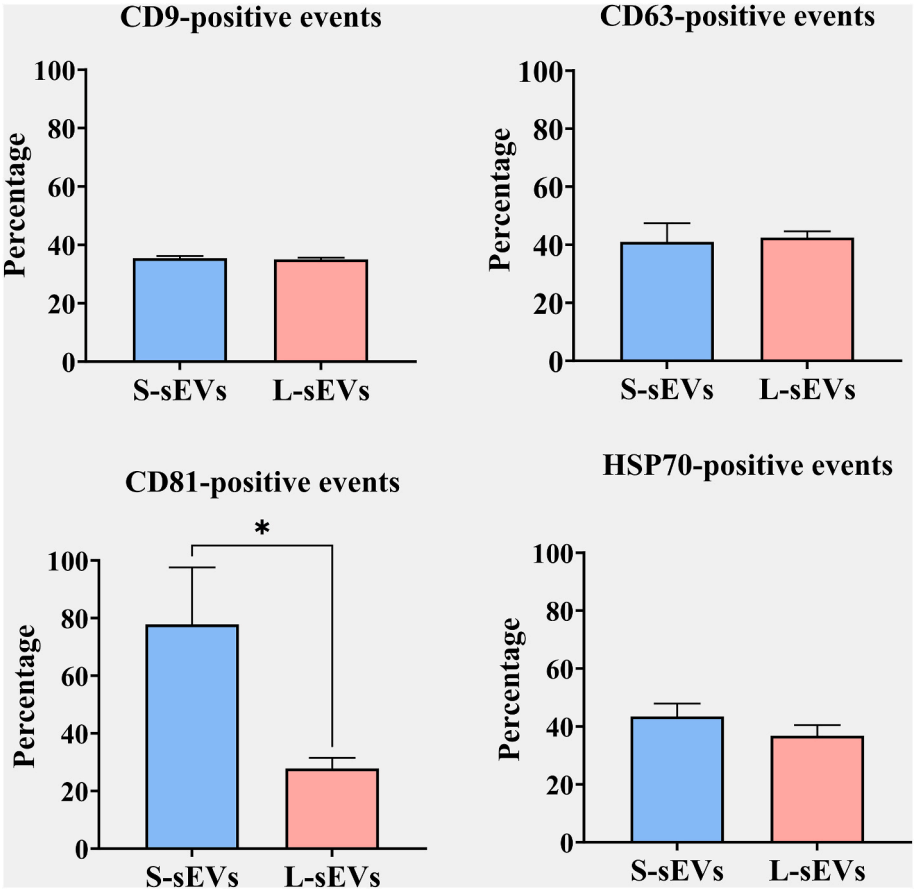

Fig. 4. Bar graphs showing the mean and standard deviation of the percentage of CFSE (5-(and 6)-Carboxyfluorescein diacetate, succinimidyl ester) positive events and also positive for CD9, CD63, CD81, and HSP70 assessed by flow cytometry in small and large seminal extracellular vesicles samples.

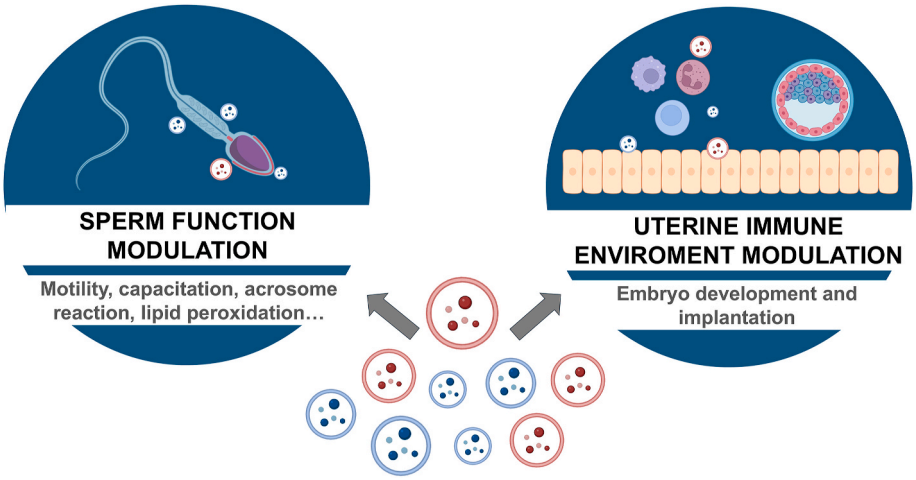

Fig. 5. Potential functions of seminal extracellular vesicles on reproductive processes. Figure created using *Biorender.com.*

adapted to donkey SP samples. Recently, this method has also been successfully used to isolate sEVs from other mammalian species, including bovine [18,35], rabbit [36] and human [37]. Previous research has shown that the EV isolation method significantly affects the number, molecular composition, functional properties, and integrity of EVs isolated from SP samples [35,38]. The only three studies of donkey EVs [31–33] used ultracentrifugation, which is considered the 'gold standard' method for EV isolation. However, SEC is increasingly preferred over ultracentrifugation for EV isolation because, in addition to a high number of isolated EVs, it is a more reproducible, efficient, scalable, cost-effective, and simple method [39,40]. In addition, the high purity of the isolated sEV samples was confirmed by the low percentage of albumin, an NVEP detected by flow cytometry. Cryo-EM images and DLS further confirmed the presence of smaller EVs in the S-sEV and larger EVs in the S-sEV and L-sEV samples. These analyses confirmed the effectiveness of the methodology used to isolate both sEV subsets.

Regarding physical sEV characterization of our sEV samples, DLS

analysis revealed that sEVs isolated from donkey SP exhibited larger sizes, in both S- and L-sEV samples, compared to those isolated from pig SP using the same protocol [28]. Similarly, sEVs isolated from other mammalian species— such as humans and rabbits —using a SEC-based procedure showed smaller sizes than those observed in our sEV samples [36,37]. It should be noted that EVs isolated from donkey milk [33] were similar in size to those of S-sEV samples. These results suggest that the size of EVs of a given body fluid would differ between species, could also lead to differences in composition and uptake. For example, smaller EVs are taken up by target cells faster than larger EVs, which leads to a more efficient transfer of their cargo [41]. Thus, the size of the sEV may have an effect on the speed of response of the target cells (sperm and/or female genital tract cells).

Another physical characterization parameter assessed in sEV samples was total protein concentration. This measure is an indirect measure of EV abundance in samples, although it may be an overestimate [25]. The concentration of total protein of sEV of donkey SP was higher than sEV samples from porcine SP isolated using the same SEC method [28]. This may suggest that donkey SP is more enriched in EVs than porcine SP. The higher number of sEVs in donkey SP would indicate that sEVs may play relevant role in the regulation of reproductive processes in this species. Interestingly, the results showed a similar total protein concentration in both sEV subset samples. This contrasts with the higher number of total particles and CFSE + particles identified by flow cytometry in the L-sEV samples. This inconsistency is often observed and highlights the low reliability of total protein concentration as an indirect measure of the abundance of sEVs in the samples. This discrepancy could be explained by the expected higher contamination by very low-density lipoproteins in S-sEV samples, as they are similar in size to the smaller EVs [42]. Another reason could be the limitations of current flow cytometers to identify the smallest EVs [29], which would represent a small proportion in S-sEV samples, as indicated by DLS results. Unfortunately, any current procedures used to characterize and quantify EVs have limitations [25]. For this reason, we have performed an orthogonal EV characterization, following MISEV 2023 guidelines, and thus minimizing potential biases.

Immunophenotypic analysis of the sEV subsets by flow cytometry showed the presence of all selected EV markers in both sEV subsets. The tetraspanins CD9, CD63, and CD81 are integral membrane widely recognized as EV markers [43]. Due to their ability to form dynamic interactions with a wide range of proteins, tetraspanins play an important role in the regulation of key cellular processes [44]. These processes include cell adhesion, modulation of cell morphology, protein trafficking and signal transduction [44]. Indeed, these interactions facilitate the organization of membrane microdomains known as tetraspanin-enriched microdomains (TEM). These TEM are thought to be involved in fundamental biological processes associated with EVs, including their biogenesis, cargo sorting, cellular targeting and uptake [43]. While most studies have focused on evaluating a single tetraspanin in EV samples to comply with the MISEV guidelines, we evaluated and verified the presence of the three more expressive EV tetraspanins, namely CD9, CD63, and CD81. These tetraspanins have also been identified in EVs isolated from porcine [45,46] and human SP [22,47]. Caballero et al. [48] highlighted the involvement of tetraspanins in the EV functionality by observing in bulls that EVs isolated from epididymal fluid and positive for CD9 were able to transfer biomolecules to maturing spermatozoa. Differences between sEV subsets were identified only in the proportion of sEVs expressing CD81, which was higher in S-sEV samples than in L-sEV samples. These results would be consistent with those of Sanchez-Mellado et al. [47], who also showed in humans that smaller sEVs (referred to as exosomes) than large sEVs (referred to as microvesicles) were positive for CD81. Barranco et al. [46] also observed a similar pattern in pigs, where S-sEV samples had a higher percentage of CD81-positive events than L-sEV samples. The differences in CD81 expression between the two sEV subsets may likely reflect differences in their biogenesis pathways, composition, and functional roles. It is widely accepted that the tetraspanin CD81 is more abundant in small EVs than in large EVs [49]. This tetraspanin would be involved in the sorting of protein and RNA cargo of EVs [50] and in the uptake of EVs by recipient cells [51]. The results of previous studies in pigs showed differences in protein, lipid, and RNA content between S-sEVs and L-sEVs [28,52,53]. These findings would support the first of the above-mentioned differential roles attributed to CD81-positive EVs. Differences between the two sEV subsets in functional roles have been demonstrated in the porcine species by Barranco et al. [54], who showed that L-sEVs have a greater impact than S-sEVs in regulating sperm metabolism. This finding highlights the functional heterogeneity of sEVs and may help to clarify the conflicting results attributed to sEVs [16,18,55], such as their putative role in sperm capacitation.

Although CD81 has been shown to play a key role in female fertility, particularly in sperm-egg fusion [56], its function in the male reproductive tract remains unclear. In some mammalian species (e.g. mouse, bovine and human), the presence of CD81 has been detected in the acrosomal area of epididymal and ejaculated spermatozoa, showing relocalization during acrosome reaction [57,58]. The CD81-enriched S-sEVs could be involved in the transfer of this tetraspanin to spermatozoa, thus influencing their functionality, e.g. in the acrosome reaction. Recent studies in donkeys have shown that SP plays an important role in modulating immune responses in the female genital tract, including regulating interactions between spermatozoa and polymorphonuclear neutrophils and promoting the formation of neutrophil extracellular traps [4,59]. Given that CD81 is a well-known regulator of immune processes [60], a plausible hypothesis is that S-sEVs, which are enriched in CD81, may mainly contribute to modulating immune environment in the female reproductive tract, facilitating sperm to reach the utero-tubal junction. Further studies are required to confirm this hypothesis.

In conclusion, this study isolated and characterized EVs from donkey SP and demonstrated that donkey SP is particularly enriched in sEVs compared to that of other mammalian species. The isolation method used, SEC, allowed the isolation of two subsets of sEVs, S-sEVs and L-sEVs, with a high degree of purity, which differ in size and protein markers, specifically CD81. This difference in CD81 EV protein marker suggests that S-sEVs and L-sEVs may have different biogenesis pathways as well as different functional roles. These results highlight the heterogeneity of EVs in donkey SP and their potential importance in the reproductive processes of this species and provide a basis for future research on sEVs.

CRediT authorship contribution statement

Jaime Catalán: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pablo Martínez-Díaz:** Methodology, Investigation. **Ana Parra:** Methodology, Investigation. **Sergi Bonet:** Methodology, Investigation. **Marc Yeste:** Writing – review & editing, Validation, Supervision, Formal analysis, Conceptualization. **Jordi Roca:** Writing – review & editing, Validation, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Isabel Barranco:** Writing – review & editing, Writing – original draft, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jordi Miró:** Writing – review & editing, Validation, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Availability of data and material

The datasets supporting the conclusions of this article are available from the corresponding author on reasonable request.

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Declaration of competing interest

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Appendix A. Supplementary data

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