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Exosomes in specific fractions of the boar ejaculate contain CD44: a marker for epididymosomes?

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Abstract

Seminal plasma (SP) is a complex fluid containing proteins, peptides, enzymes, hormones as well as extracellular vesicles (EVs). The SP interacts with spermatozoa and the inner cell lining of the female genital tract, adsorbing proteins and exosomes that modulate sperm functions and female immune responsiveness. In the present study, boar sperm-free SP was studied using flow cytometry (FC) after membrane tetraspanins (CD9, CD63 and CD81) and membrane receptor CD44 marking of non-enriched (whole SP) or gradient fractions enriched through two-step discontinuous KBr-density-gradient ultracentrifugation, in whole ejaculate or in selected ejaculate fractions. The results, evaluated by transmission electron microscopy, confirmed the presence of exosomes in all fractions of the pig SP. Noteworthy, these pig SP-exosomes were CD44-bearing when analyzed by FC, with bands detected by western blotting (WB) at the expected 85 kD size. The two-step discontinuous KBr-density-gradient ultracentrifugation enriched the population of exosomes in two specific gradient fractions, indicating exosomes (either prostasomes or epididymosomes) could be separated from low-density lipoprotein (LDL) but they co-sediment with the high-density lipoprotein (HDL)-bearing fraction. The findings pave for the selective isolation of exosomes in functional studies of their function when interacting with spermatozoa, the oocyte and/or the female genitalia, including hyaluronan-CD44 interplay.

Key words: seminal plasma, ejaculate fractions, gradient ultracentrifugation, exosomes, lipoproteins, tetraspanins, western blot, CD44, pig.

1. Introduction

Seminal plasma (SP), the extracellular medium where mammalian spermatozoa are suspended in, derives from the testis, epididymis and accessory sexual glands [1,2]. The SP has a complex composition with important functions, from modulating sperm transport, affecting sperm motility and function [1,3] including fertilizing capacity [4], to the induction of gene regulation of the immune system of the female towards tolerance of the foreign paternal spermatozoa and accompanying proteins [5,6]. Hormones, as well as hundreds of proteins and peptides (including antioxidative enzymes and cytokines [7-10], are present [10,11] to influence fertility, apparently for their interaction with the female immune function [5,12] *via* post-translational or post-transcriptional effects on proteins respectively RNA of the female; changes that would continue evolving along the female transcriptome and proteome to render further responses [13]. The mammalian SP also contains extracellular vesicles (EVs), spherical membrane-coated structures released as part of the genital cell secretome [14]. The EVs are classified by size, as either exosomes (30-100 nm in diameter) or microvesicles (100-1000 nm diameter) [14-18]. In SP, exosomes are also classified by point of origin, as epididymosomes, prostasomes or even vesiculosomes [19-21].

Exosomes are considered to maintain homeostasis, and their modulating function in the SP has been related to the transfer of proteins but also other molecules including small regulatory non-coding RNAs to the sperm surface [21, 26]. This transfer affects sperm function in pigs, from sperm maturation to zona pellucida binding [22-25], apparently by infiltration into the sperm membrane [26]. Exosomes isolated from the sperm-rich fraction (SRF) of the pig ejaculate induced expression changes in immune-related genes when applied to uterine epithelial cells *in vitro,* similar to the changes observed in the endometrium of mated sows [21]. Such changes could presumably be exerted via

exosomal low-density (LDL) or high-density (HDL) lipoproteins and their load of proteins, cytokines/chemoquines, and microRNAs (miRNAs) [27-30].

Isolation and enrichment of EVs, exosomes in particular, has been cumbersome, despite the several techniques used: ultracentrifugation, density-gradient separation, immunoaffinity capture, microfluidic systems, sequential filtration, and combinations of several techniques [14,29-33]. Of all, the immunoaffinity precipitation and capture provided by several commercial kits (i.e. Exo-Quick-TC, System Biosciences, Mountain View, CA, USA) is probably the easiest method. However, due to the low density and small size of the exosomes, purity is poor and demands additional confirmation by western blotting of exosomal markers, as hsp70 [21]. Gradient ultracentrifugation is more accurate, provided contamination with lipoproteins (LDL/HDL) is considered. Of particular relevance is the knowledge that the HDL also carries miRNAs [29,30,34], requiring differential identification of exosomes and HDL.

Exploration of SP-exosomes has been extensive in human presumably because most exosomes are derived from the prostate gland, whose secretion can be specifically retrieved by rectal palpation [2] but research has also been relevant in other mammalian species, including pig [24]. Use of protein markers, as the highly conserved transmembrane-4 superfamily tetraspanins has dominated, associated to the use of ultrafiltration or ultracentrifugation. Tetraspanins CD9 and CD81 can build fusion-competent sites together with integrins, paying a role in cell-to-cell communication [35]. While CD9 is common to all EVs, CD63 is most often related to microvesicles and CD81 is overrepresented in exosomes, including the in SP of pigs [36]. Tetraspanins as CD9, CD63 and CD81, having a broad tissue distribution are included in most commercial kits to isolate and identify EVs. Other markers, as CD44, the cell-surface glycoprotein receptor for hyaluronan depicts -through multiple CD44 isoforms-

an ample, diverse biological activity, related to numerous functions and pathologies [37]. The CD44 has until now, however, only been present in EVs / exosomes derived from mesenchymal stem cells [38], related to extracellular matrix [39] and tumoral exosomes [40-42], in relation to the local production/presence of the ligand hyaluronan. CD44 is expressed by numerous cell types including pig spermatozoa [43] but also in the epididymis and prostate [44]. CD44 is yet in pig SP, a complex biofluid, where the possibility of co-isolating exosomes with other vesicles or proteins is very high [33,45]. The present study aimed detection of exosomes in pig seminal plasma using FC of EV-markers (tetraspanins CD9, CD63, CD81), the receptor CD44, western blotting and electron microscopy. Since the pig ejaculates in sequential fractions [1], these were further characterized for exosome and lipoprotein isolation via a two-step discontinuous KBr-density-gradient ultracentrifugation. The location of CD44 was a particularly goal since this is yet to be explored in seminal fluid/SP despite being present in epididymal and prostate secretions which accompanies the emitted spermatozoa, and could thus act as a specific putative biomarker.

2. Material and methods

2.1. Ethics statement

Animal husbandry and experimental handling were performed in compliance with the European Community (Directive 2010/63/EU) and current Swedish legislation (SJVFS 2017:40). The experiments were approved in advance by the “Regional Committee for Ethical Approval of Animal Experiments” (Linköpings Djurförsöksetiska nämnd) in Linköping, Sweden (permits no. 75-12 and no. ID1400).

2.2. Animals

Young mature boars of Swedish Landrace breed (9-11 months, n= 5) with proven sperm quality (concentration, motility and morphology) were recruited from a controlled breeding farm. The animals were individually kept in separate pens at the Translational Medicine Centre (TMC/CBR-3) of Linköping University under controlled temperature and light regimes (12h: 12h light/dark cycle). Pigs were fed with commercial feedstuff (Lantmännen, Stockholm, Sweden) according to national standards [46] provided with water *ad libitum* and receiving the same management. Throughout all experiments, animals were handled carefully and in such a way to avoid any unnecessary stress.

2.3. Semen collection, evaluation and harvesting of seminal fluid/plasma (SP)

To achieve efficient manual collection of semen from the boars, they were properly trained prior to the experiment to mount a stainless steel-made dummy. Both whole ejaculates (5 per boar) and specific fractions of the ejaculate, e.g. the sperm-peak fraction (first 10 mL portion of the sperm-rich fraction, SRF), the rest of the SRF and the post-SRF (5 fractionated collections per boar) were manually collected using the gloved-hand method and transferred to pre-warmed plastic tubes. The semen was assessed for sperm concentration and motility (velocity and forward progressive motility) using a light microscope (Axio Scope, Carl Zeiss, Stockholm, Sweden) equipped with a thermal plate (Temp Controller 2000-2, Pecon GmbH, Erbach, Germany) kept at 38 °C, positive phase contrast optics (10x objective), a Charge Coupled Device (CCD) camera (UI-1540LE-M-HQ, Ueye, IDS Imaging Development Systems GmbH, Obersulm, Germany), and the Qualisperm® Software (Biophos SA,

Lausanne, Switzerland); a high throughput system (usually 4 fields per minute), capable of analyzing >2 000 spermatozoa/field [47]. Ejaculates with at least 70% motile and 75% morphologically normal-looking spermatozoa immediately after collection were used for the experiments.

The samples of the various ejaculate fractions were centrifuged at 10 000 × g at 4 ˚C for 10 min (Centrifuge 5424R, Eppendorf AG, Hamburg, Germany). The supernatants (SP) were harvested, checked for absence of spermatozoa, boar- and fraction-wise pooled and stored at -80 ^{0}C until analysed.

2.4. Experimental design

Seminal plasma samples were examined for exosome contents, identified via transmission electron microscopy, Exo-FLOW™ Exosome purification beads linked to different antibodies against CD9, CD44, CD63 and CD81 markers (System Biosciences (SBI), 265 North Whisman Rd., Mountain View, CA 94043) and western blotting (WB) using a pig-specific monoclonal antibody (anti-CD44 mouse antibody 60224-1-Ig; Nordic BioSite, Proteintech Europe, Manchester, UK) [43]. Samples examined were those originally centrifuged (see above), following buffer-XE elution or a two-step discontinuous KBr-density-gradient ultracentrifugation procedure [48,49] to harvest three gradients with differential densities of <1.063 (F1, coincident with isolation of very low/low density lipoprotein (VLDL/LDL), 1.16-1.21 (F2, for exosomes and high-density lipoprotein, HDL) respectively 1.21-1.26 g/mL (F3, for exosomes) [50].

2.4.1. Elution of SP

After a further centrifugation (16 000 × g at 4°C, 10 min), the SP samples were filtered (0.8 μm syringe filters; Millipore® Millex®-AA, cat. no. SLAA033SB), mixed 1:1 (v/v) with Buffer XBP and the mix placed into the exoEasy spin column (exoRNeasy Serum/Plasma Midi Kit, Cat No./ID: 77044; Qiagen, Sollentuna, Sweden). The column was spun at 500 x g. for 1 min at rt. The flow-through was discarded, Buffer XWP (3.5 mL) replaced and spun at 5 000 × g for 5 min to remove residual volume from the column. After discarding the flow-through again, Buffer-XE (1 mL, Cat No./ID: 76214; Qiagen, Sollentuna, Sweden), was added to the membrane, incubated for 1 min., re-centrifuged at 500 × g for 5 min to collect the eluate which was assessed with the Exo-FLOW™ Exosome purification beads, as described below.

2.4.2. Two-step discontinuous density-gradient ultracentrifugation of seminal plasma

Exosome fractions, with densities of <1.063 (F1), 1.16-1.21 (F2) and 1.21-1.26 g/mL (F3), were isolated from SP using two-step discontinuous KBr-density-gradient ultracentrifugation, as described before [49]. Seminal plasma (4.5 mL) was mixed with 0.5 mL of EDTA (10 mg/mL) and sucrose (5 mg/mL). The 5 ml solution was mixed with 2.072 g KBr to a final density of 1.24 g/ml and transferred to an ultracentrifugation tube. The solution was overlayered with a KBr/PBS solution with the density 1.063 g/mL. Tubes were sealed and ultracentrifugation was performed at 290 000 × *g* at 15 °C for 4 h in a Beckman Coulter Ti 70.1 rotor. Exosome fractions, with densities of <1.063 (F1), 1.16-1.21 (F2) and 1.21-1.26 g/mL (F3), were aspirated from the tubes with syringes and kept at -80°C until analysis.

2.4.3. Exosome identification and analysis

Exosomes were identified using the Exo-FLOW™ Exosome purification beads (System Biosciences, SBI, Mountain View, CA, USA) linked to biotinylated capture antibody antibodies against the tetraspanins CD9, CD63 and CD81 (EXOFLOW15OA-1), and against the receptor CD44 (EXOFLOW21OA-1) following the protocols of the manufacturer. In brief, 40 μL streptavidin magnetic bead slurry were placed in 1.5 mL tubes on a magnetic stand, washed with Bead Wash buffer, attaching the beads on the side of the tube with the magnetic stand. Biotinylated capture antibodies (CD9, CD81, CD63 and CD44) were added, kept on ice for 2h, then washed with Bead Wash buffer, to be re-attached to the magnetic stand to remove the supernatant and keep the beads now bound with the biotinylated capture antibodies. Concentrated, isolated exosomes (100 μL) were added to each bead tube and incubated on a rotating rack at 4°C overnight for capture. The samples were placed again on the magnetic stand for 2 min to carefully remove the supernatant, followed by washing with Bead Wash buffer and a flick to mix. Exosome Stain Buffer (240 μL) and 10 μL of Exo-FITC exosome stain were added, placing the tubes on ice for 2 hours. Thereafter, the supernatant was removed after replacing tubes for 2 min on the magnetic stand. Following a new wash, samples resuspended in 300 μL Bead Wash buffer, were examined by flow cytometry (FC) using a GalliosTM (Beckman Coulter, Bromma, Sweden) instrument equipped with standards optics, violet laser (405 nm) 2 colours, argon laser (488 nm) 5 colours and HeNe-laser (633 nm) 3 colours. Filter configuration: Blue: FL1 550SP 525BP (FITC). The instrument is controlled with Navios software (Beckman Coulter, Bromma, Sweden). Analyses of acquired data were performed using the Kaluza software (Beck- man Coulter, Bromma, Sweden) on a separate PC. In all cases we assessed 25,000 events per sample, with a flow rate of 200 particles/s. Bead flow separation data for the various capture antibodies coupled with Exo-FITC staining

resulted from forward scatter (Height-linear) versus FITC intensity (Average-log), after a primary gating on the majority bead singlets by use of Side Scatter (Average-log) vs. Forward Scatter (Average-linear).

2.4.4. Western Blot

Samples of pooled seminal plasma/fluid (1 mL) were centrifuged (2 000 × *g* at 5 °C for 30 min) and supernatants harvested. Then, 200 µL of Total Exosome Isolation Reagent (Invitrogen™, Waltham, MA USA, Product code: 13355394) were added. After vortexing and refrigeration at 5 °C for 30 min, samples were centrifuged again (10,000 × *g* at 5 °C for 10 min) prior to extraction of exosome proteins by incubation in 200 µL of RIPA buffer (Sigma-Aldrich). After sonication (Amplitude 60 W, 10 s, 2 cycles), samples were kept at 4˚C for 40 min. The extracted samples were centrifuged at 13 000 ×*g* for 10 min, and proteins quantified using a DC Protein assay kit (Bio Rad, Hercules, CA, USA), following manufacturer's instructions. Protein suspensions (0.625 µg protein/µL) were denatured by heating at 70˚C for 10 min. Aliquots (10 µl) of each protein suspension were loaded into 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (BIORAD). Electrophoresis was performed at 150 V for 45 min, followed by transfer of the proteins to Nitrocellulose membranes (LI-COR Biosciences, Lincoln, NE, USA) at 100 V for 60 min. The membranes were blocked at room temperature for 60 min with Blocking solution (LI-COR Biosciences, Lincoln, NE, USA) and washed in phosphate-buffered saline (PBS) (ThermoFisher Scientific, Waltham, MA, USA) containing 0.1% Tween-20 (Sigma-Aldrich) (PBST). After three washes of 10 min in PBST, one membrane was incubated at 4˚C overnight with the primary monoclonal (1:500 dilution, anti-CD44 antibody 60224-1-Ig; mouse monoclonal antibody to CD44; species specificity: pig; Nordic BioSite, Proteintech Europe, Manchester, UK). The

specificity of the monoclonal antibody was tested in another membrane by previous co-incubation (RT with agitation for one min) of the primary antibody in excess presence (1:5 ratio) of its specific blocking peptide (CD44 Fusion Protein Ag7633, Nordic BioSite, Proteintech Europe, Manchester, UK). The day after, the membrane was washed 3 times in PBST and incubated for 60 min with a dilution 1:15,000 of the secondary antibody (goat anti-mouse IRDye 800 CW (925-32210, LI-COR Biosciences, Lincoln, NE, USA) followed by extensive washing in PBST. The membranes were scanned using the Odyssey CLx (LI-COR Biosciences, Lincoln, NE, USA), and images of the blots were obtained using the Image Studio 4.0 software (LI-COR Biosciences, Lincoln, NE, USA).

2.4.5. Ultrastructure of exosome gradient fractions

Aliquots (5 µL) of the gradient fractions (F1-F3) obtained by two-step discontinuous KBr-density-gradient ultracentrifugation were fixed at room temperature in a 0.1% (w/v) paraformaldehyde solution (50 µL) for at least 18 hours. After washing in distilled water, a five (5) µl drop of the fixed fractions were deposited on 200-mesh EM copper grids with formvar coating, the excess fluid removed by blotting and incubated for 7 minutes at room temperature. The grids were thereafter transferred to 2% uranyl acetate (w/v) drops for negative staining [51]. Electron micrographs were obtained using a transmission electron microscope (EM JEM 1230, JEOL Ltd., Tokyo, Japan), operated at 100kV. Two-dimensional data was collected and images were processed for assessing the size of the EVs using Image J (https://imagej.nih.gov/ij/) and RStudio (https://www.rstudio.com) for illustration of the collected summarized data.

3. Results

The flow cytometric sorting procedure was able to clearly separate the distinct particular surface markers for the EVs, see **Figs 1-2**. Both the pools of uncentrifuged (bulk) and the eluted Buffer XE pooled boar SP showed positive binding/staining to the three tetraspanins tested (CD9, CD63, CD81) and the hyaluronan receptor CD44 (see **Fig 2**, **Table 1**). Moreover, this marking was present in each one of the fractions of the boar ejaculate (Sperm-peak/P1, rest of the SRF and the post-SRF) (**Table 1**). However, the percentages of positive staining were variable among fractions, with the SRF-P1 being the one with the lowest proportion of positive stating, and the P1 having the highest, irrespective of considering tetraspanins or CD44 (**Table 1**).

When the same fractions of the boar ejaculate were further explored assaying the three density fractions obtained using the two-step discontinuous KBr-density-gradient ultracentrifugation (see **Table 2**) it became evident that neither the three tetraspanins (CD9+CD63+CD81) nor the CD44 could be detected in the F1-gradient fraction (<1.063 g/mL) in any of the ejaculate fractions tested (P1, SRF-P1 and Post-SRF). The other two gradient fractions (F2: 1.16-1.21g/mL, F3: 1.21-1.26 g/mL) showed positive EXO-FLOW results, indicating the presence of exosome-like EVs in all three fractions of the boar ejaculate. Moreover, not only these EVs could be identified as exosomes, but they also consistently marked for CD44.

The ultrastructure of the gradient fractions obtained using the two-step discontinuous KBr-density-gradient ultracentrifugation confirmed the screening performed by the EXO-FLOW mapping (**Figs 2 and 3 a)** are representative views of tetraspanin and CD44 positive samples (gradients F2 and F3) from the P1-sperm peak ejaculate fraction depicting round or cup-shaped membrane surrounded vesicles containing a content with varying electron-density. An amorphous, often filamentous structures (300-400

nm long) were present in various amounts among samples, compatible with protein aggregates, and larger than eventual HDL (usually between 7-13 nm size). The analysis of the size of the EVs revealed they had an asymmetric size distribution, ranging between 20 and 200 nm in diameter, with the majority being between 50 and 60nm, e.g. indicative of exosomes (**Figs 3b and d**). In comparison (data not shown), the F1-gradient, negative to all tetraspanins and even to CD44, depicted mostly electron-lucent vesicles of very small size, probably corresponding to low-density lipoproteins (LDL), as expected for this particular gradient fraction (<1.063 g/mL).

The Western Blot (WB) using the porcine specific anti-CD44 monoclonal antibody (60224-1-Ig) detected the receptor CD44 in the pig seminal plasma from all ejaculated fractions (L1: P1, L2: SRF-P1 and L3: Post-SRF) identifying expected bands at 85 kDa, indicative of a full-size CD44 (Lane 1-3, **Fig 4a**). The bands showed a gradient of relative intensity (L1-3), being most intense in P1 (L1). The co-incubation of the primary antibody with its specific blocking peptide (CD44 Fusion Protein Ag7633) at 1:5 ratio, neutralized the detected CD44 bands (**Fig 4b)**, confirming the specificity of the monoclonal antibody and the presence of CD44 in pig seminal plasma.

4. Discussion

In the present study, sperm-free boar seminal plasma was studied using flow cytometry of EXO-FLOW™ Exosome purification beads incubated with antibodies against a battery of membrane tetraspanins (CD9, CD63 and CD81) and the membrane receptor CD44. The samples examined were either centrifuged bulk SP from three well-characterized fractions of the boar ejaculate (P1, SRF-P1 and Post-SRF) or enriched gradient fractions obtained through a two-step discontinuous KBr-density-gradient

ultracentrifugation procedure. The gradient fractions had three densities (<1.063 g/mL, 1.16-1.21g/mL, 1.21-1.26 g/mL) were further examined with transmission electron microscopy. The results confirmed the presence of exosomes in all ejaculate fractions of the boar SP. The two-step discontinuous KBr-density-gradient ultracentrifugation enriched the population of exosomes in two specific gradient fractions, indicating exosomes (either prostasomes or epididymosomes) could be separated from LDL but co-sedimented with the HDL-bearing fraction.

Many methods for isolation of EVs (microvesicles and/or exosomes) have been applied in different fluids and species (reviewed by [29, 51-52]). These techniques include among others ultracentrifugation (serial, stepwise, etc [53]), density-gradient separation, immunoaffinity precipitation and capture, microfluidic systems, sequential filtration etc. Precipitation with following capture is probably the easiest method, commercially offered, easy and quick [21]. However, due to the low density and small size of the exosomes, purity is low-to-poor and demands further screening of exosome surface markers (as hsp70). Ultracentrifugation is more accurate, provided contamination with LDL/HDL is avoided, relevant when isolation/enrichment pursues further studies of the miRNA cargo of the cell vesicles [29,30]. In the present study, we studied samples that were only centrifuged for sperm separation from the SP. Moreover, SP derived from specific fractions of the pig ejaculate were further subjected to a two-step discontinuous KBr-density-gradient ultracentrifugation [49]. The procedure yielded good results, separating very low/low-density lipoprotein (VLDL/LDL) in F1 (<1.063 g/mL), from the F2 (1.16-1.21 g/mL) where we expected exosomes to be present alongside high-density lipoprotein (HDL) which appeared to co-sediment in F3 (1.21-1.26 g/mL). The ultrastructure confirmed the findings, samples being somewhat heterogeneous, i.e. containing both exosomes and microvesicles, with a dominance of

the first named. The examination of the size of the vesicles and liproproteins confirmed the EVs were within the expected ranges [54].

The finding that while all SP-fractions in pigs were positive for CD9, CD63 and CD81, and even to CD44; and that the P1 (the sperm-peak fraction of the pig ejaculate [1]) was the one yielding the most positive staining, compared to the SRF-P1 substantially disagrees with the results reported by Bai [21] where they, using only Exo-Quick-TC exosomal isolation kit concluded that exosomes were only present in the sperm-rich fraction of the boar ejaculate. The reason for these differences might relate to the type of exosome isolation, which is far from less accurate than the one used hereby. In the present study we further used a more accurate collection of samples, collecting in separate the sperm-peak portion (P1), which represents the bulk of the epidydimal secretion, containing exosomes (epididymosomes). The rest of the SRF, containing more prostate secretion, contained also exosomes (prostasomes). Although we could not determine whether the P1 staining corresponded to epididymosomes or if the SRF-P1 had more prostasomes; the distinction is irrelevant, since these are probably similarly carrying the same tetraspanins and even the CD44. Further efforts are needed to made a deeper mapping of the kind of dominating structure and the cargo present, a matter that it is apparently not solvable at present [26,36,55].

Newer flow cytometers, such as those with high resolution and reduced angle flow cytometers allow now for better identification of particles at the nanometre level (i.e. below 40 nm) [56]. A very recent study using this instrumentation to examine the SP of pigs (bulk ejaculate) yielded results confirming the EVs present were of heterogenous size, still not allowing for an accurate enrichment of either exosomes or MVs [36]. However, this particular study demonstrated not only that the pig SP contained more exosomes than MVs, but also the existence of some degree of

selectivity for the tetraspanins among the EVs; i.e. exosomes had more CD9+CD81 compared to MVs, with a predominance of CD81 [36]; yet showing an overlapping of the marking [14]. Further development of discriminative protocols is pre-requisite if studies of the load of these EVs is to be functionally tested [35]. We already know what general cargo they carry, including ncRNAs, and specific proteins [34]. However, not only the EVs carry these molecules; lipoproteins are also carriers with similar delivery capacity for miRNAs, both LDL and particularly HDL [34]. The capacity of the two-step discontinuous KBr-density-gradient ultracentrifugation to clearly separate the LDL fraction (F1) is a major advantage in this respect. However, the apparent co-sedimentation of the F2-F3 (exosomes, MVs and HDL) needs to the further separated, a matter to be solved before we can continue with examination of the role their respective cargo may play on spermatozoa (protective role[4]) and the female genital tract (signalling for already documented changes in gene expression, including immune process genes [5,6,21]). Since exosomes are constituted to a large extent of membrane lipids, further studies are needed to determine their role and to aid in the more accurate isolation and classification of the different EVs [33,45].

Noteworthy was the detection of CD44 in the EVs of the the pig SP, basically in all fractions, and the EV- and HDL- bearing gradient fractions. The CD44 is present in the female and male genital tract, as well as in the cumulus-oocyte complex and the early embryo, often in relation to its character of membrane receptor for the ligand hyaluronan (rev by [57]). It is particularly expressed by the epididymis and the prostate [58], and it is also present in the pig spermatozoon [43]. The presence of CD44 in the EVs of the porcine sperm-free SP would therefore not be a surprise, considering that prostasomes are recruited by and attached to spermatozoa in response to early capacitation events in the uterus and oviduct, and stimulate secondary pathways related

to hypermotility and acrosome reaction, when approaching the oocyte [49]. However, which specific role the CD44 receptor plays in EVs and or HDL is yet unknown. The CD44 defines action via the ligands involved, as with hyaluronan, but relevant evidence exists coupling the cellular adhesion molecule (CAM)-CD44 with secondary signalling complexes involved in wound healing, angiogenesis and modulation of the immune system [59]; the latter crossing over to pathological status, as development of neoplasias and metastases [37,60], particularly in those where hyaluronan presence or production is confirmed [59,61]. EVs containing HA in their surface, often derived from mesenchymal-derived tumours contain CD44, as one of the present CAMs [62], most likely to retain the EV-associated hyaluronan during transport in body fluids (rev by [59,61]). The findings of the present study relied initially on using the FC/EXO-FLOW platform to identify the presence of CD44 in EVs (microvesicles/exosomes) in specific fractions of the boar ejaculate, with variations in between. We therefore performed a Western blotting to confirm the CD44 presence via a pig-specific monoclonal antibody previously tested, including co-incubation with the immunogenic peptide used to prepare the antibody [43]. The WB confirmed CD44 is present in exosomes in the pig SP, with a variation in relative intensity between the three fractions explored, decreasing from the P1 (most intense) towards the Post-SRF (less intense).

What would then be the meaning of having CD44 present in EVs of the porcine SP? As mentioned before, EVs particularly those in blood plasma and in mesenchyma-derived tumours carry surface-associated hyaluronan probably attached via surface present CD44 [59]. Moreover, this hyaluronan coating of EVs could also regulate interactions of EVs with target cells, for instance the CD44-bearing epithelium of the female genital tract [63]. In the case of the SP, it contains hyaluronan [57] which appears to influence *in vitro* fertilization of pig oocytes [64], modulating sperm capacitation *in vitro* [65]

and *in vivo* [66], with presence of hyaluronan and CD44 in the oviduct [57]. Such interactions are not surprising, considering surface molecules on EVs would determine the uptake and biological functions of EVs. In this particular case, the presence of CD44 would provide the SP-EVs with a relevant player for interacion with cells of the female genital tract epitelial lining and other molecules in the fluid present in the uterus and oviduct, which is rich in glycosaminoglycans (GAGS), including hyaluronan [67]. EVs from tumoral origin carry sulfated GAG molecules that could bear specific cytokines/chemoquines [59], including TGF-ß, a tolerogenic chemoquine detected in the pig-SP [6] whose gene expression is increased after mating [5]. Such cytokine signalling by the SP would be behind the improved fertility obtained after AI with frozen-thawed semen exposed to SP during freezing or post-thaw [68-71]. Further studies are foreseen to determine the cargo of SP-EVs in relation to their sperm protective function and signalling to the female immune system towards the establishment of a status of maternal immune tolerance to paternal antigens. Of particular interest is the gradient of relative intensity depicted by the WB along the three ejaculate fractions explored. The P1 is the fraction containing most cauda epididymal fluid accompanyting the peak of spermatozoa ejaculated (25% of the total sperm numbers in a pig ejaculate [1]). The rest of the SRF contains increasing secretions of particularly the prostate, emission that increases in the Post-SRF, where most of the ejaculate volume is represented by secretion of the prostate and the seminal vesicles, alongside a dramatic decrease of sperm numbers and, per definition the lowest amounts of epididymal fluid. Under these facts, it is pertinent to argument for the possibility that the CD44 relative intensity differing in a similar fashion among the lanes depicted in the WB, might be indicative that the CD44 mainly identifies

epididymosomes, rather than prostasomes, or even vesiculosomes. Further studies are, however, needed to test this hypothesis.

Conclusion

EVs, particularly exosomes carrying CD44 are present in the seminal plasma of boars, with differences among specific ejaculate fractions. The two-step discontinuous KBr-density-gradient ultracentrifugation enriched the population of exosomes/MVs in two specific gradient fractions, indicating exosomes (either prostasomes or epididymosomes) could be separated from LDL but co-sedimented with the HDL-bearing fraction. CD44 appears as possible biomarker for the presence of epididymosomes in pig seminal plasma.

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Author Contributions

M.A-R., H.K. and H.R-M. designed the experiments. M.A-R. and S.A.L. executed the experiments. M.A-R. performed analyses of data and wrote the first draft of the

manuscript. All authors read, contributed and approved the final manuscript. M.A-R. and H.R-M. secured funding.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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Figure 1. Density plots (flow cytometry/EXO-FLOW) of tetraspanins CD9, CD63 and CD81 in the seminal plasma of specific fractions of the boar ejaculate (P1: first 10 mL of the sperm rich-fraction (SRF); SRF-P1: the rest of the SRF; Post-SRF: the rest of the ejaculate). The dotted line marks the limit between negative (left hand side) and positive (right hand side, %) plot allocation.

Figure 2. Density plots (flow cytometry/EXO-FLOW) of the CD44 receptor in the sperm-free seminal plasma (SP) of specific fractions of the boar ejaculate (P1: first 10 mL of the sperm rich-fraction (SRF); SRF-P1: the rest of the SRF; Post-SRF: the rest of the ejaculate). Control: samples not ultracentrifuged, F1-F3: gradients obtained after two-step discontinuous KBr-density-gradient ultracentrifugation (F1: <1.063 g/mL, coincident with isolation of very low/low density lipoprotein (VLDL/LDL), F2: 1.16-1.21 g/mL, for exosomes and high-density lipoprotein, HDL) and F3: 1.21-1.26 g/mL (for epididymosomes, co-sedimenting with F2). The dotted line marks the limit between negative (left hand side) and positive (right hand side, %) plot allocation.

Figure 3 a-d. Transmission electron micrographs of extracellular vesicles (arrows) isolated by two-step discontinuous KBr-density-gradient ultracentrifugation in the gradient fractions F2 (1.16-1.21 g/mL, **a**), and F3 (1.21-1.26 g/mL, **c**) of boar seminal plasma from the sperm-peak P1 fraction (first 10 mL portion of the sperm-rich fraction, SRF), bar= 200 nm. The accompanying histograms in **b** and **d**, depict frequencies of size intervals, indicating the EVs present in the respective fractions were exosomes.

Figure 4 a-b. Western Blot (WB) detection of the receptor CD44 in pig seminal plasma from three porcine ejaculate fractions (L1: P1, L2: SRF-P1 and L3: Post-SRF). **a**. the porcine specific anti-CD44 monoclonal antibody (60224-1-Ig) identified expected bands at 85 kDa for CD44 in all ejaculated boar fractions (L1-L3). **b.** Co-incubation of the primary antibody with its specific blocking peptide (CD44 Fusion Protein Ag7633) (1:5 ratio) neutralized the detected bands in L1-L3.

Table 1. Proportions of flow cytometric FITC-staining (EXO-FLOW) for various extracellular vesicle (EV)-markers (tetraspanins CD9, CD63, CD81 and the receptor CD44) in pooled boar semen fractions (P1: the sperm-peak fraction (first 10 mL portion of the sperm-rich fraction, SRF), SRF-P1: the rest of the SRF and the post-SRF), testing either the Eluted Buffer XE (CD9) or bulk fractions (CD9, CD63, CD81, CD44). *EXO-FLOW Result: exosome presence +/Absence -.

EV-surface marker	Sample type	SP fraction tested	FITC- (%)	FITC+ (%)	EXO-FLOW result*
CD9, CD63, CD81, CD44	Control, no EVs	-	**99.99**	0.12	**-**
CD9	Eluted Buffer XE	Boar P1	0.1	**99.9**	+
		Boar SRF-P1	0.25	**99.75**	+
		Boar Post-SRF	0.06	**99.94**	+
CD9	Bulk boar SP	P1	0.06	**99.94**	+
		SRF-P1	36.23	63.67	**(+)**
		Post-SRF	0.37	**99.63**	+
CD63	Bulk boar SP	P1	0.03	**99.97**	+
		SRF-P1	44.25	55.75	**(+)**
		Post-SRF	19.69	80.31	+
CD81	Bulk boar SP	P1	0.03	**99.97**	+
		SRF-P1	25.77	74.23	+
		Post-SRF	6.58	**93.42**	+
CD44	Bulk boar SP	P1	0.07	**99.93**	+
		SRF-P1	40.43	59.57	**(+)**
		Post-SRF	11.63	**88.37**	+

Table 2. Proportions of flow cytometric FITC-staining (EXO-FLOW) for various extracellular vesicle markers (tetraspanins CD9, CD63, CD81 and the receptor CD44) in the three density fractions (F1: <1.063, F2: 1.16-1.21, F3: 1.21-1.26 g/mL) obtained using the two-step discontinuous KBr-density-gradient ultracentrifugation of boar seminal plasma from three specific ejaculate fractions (P1: the sperm-peak fraction (first 10 mL portion of the sperm-rich fraction, SRF), SRF-P1: the rest of the SRF and the post-SRF, n=3). *EXO-FLOW result: exosome presence +/Absence -.

EV-surface marker	Ejaculate fraction	KBr-density-gradient	FITC- (%)	FITC+ (%)	EXO-FLOW result*
CD9, CD63, CD81, CD44	Control	-	**99.9**	0.1	-
CD9	P1	F1	**99.67**	0.33	-
		F2	0.05	**99.95**	+
		F3	0.02	**99.98**	+
	SRF-P1	F1	**99.67**	0.33	-
		F2	0.08	**99.92**	+
		F3	0	**100**	+
	Post-SRF	F1	**99.51**	0.49	-
		F2	0.08	**99.92**	+
		F3	0.02	**99.98**	+
CD63	P1	F1	**98.54**	1.35	-
		F2	0.09	**99.91**	+
		F3	0.01	**99.99**	+
	SRF-P1	F1	**99.9**	0.1	-
		F2	16.51	**83.49**	+
		F3	0.27	**99.73**	+
	Post-SRF	F1	**99.67**	0.33	-
		F2	0.11	**99.89**	+
		F3	0.09	**99.91**	+
CD81	Pool P1	F1	**99.91**	0.09	-
		F2	0.22	**99.78**	+
		F3	0.01	**99.99**	+
	SRF-P1	F1	**99.84**	0.16	-
		F2	0.03	**99.97**	+
		F3	0	**100**	+
	Post-SRF	F1	**99.17**	0.83	-
		F2	0	**100**	+
		F3	0	**100**	+
CD44	P1	F1	**98.79**	1.21	-
		F2	0.09	**99.91**	+
		F3	0.04	**99.96**	+
	SRF-P1	F1	**99.61**	0.39	-
		F2	0.16	**99.84**	+
		F3	0.01	**99.99**	+
	Post-SRF	F1	**99.54**	0.46	-
		F2	0.28	**99.72**	+
		F3	0.03	**99.97**	+

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Figure 1.

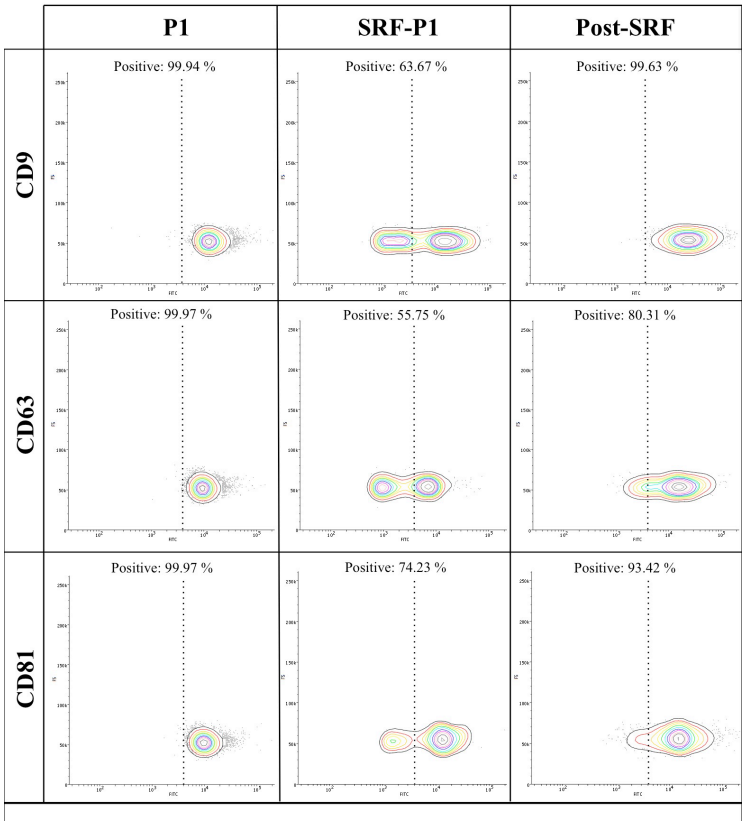

Figure 2.

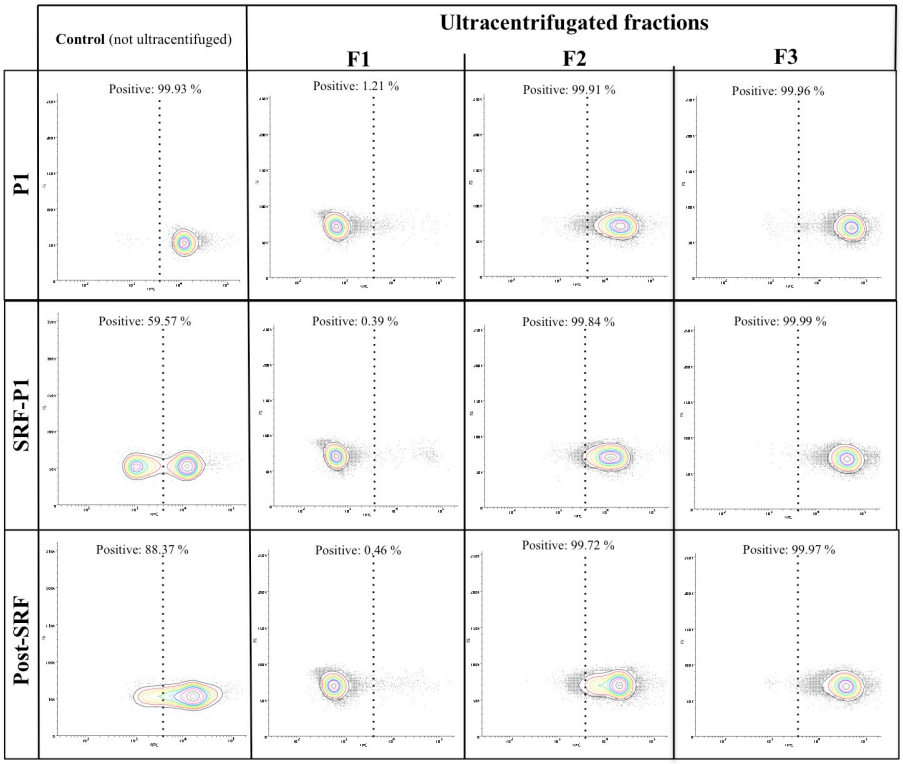

Figure 3.

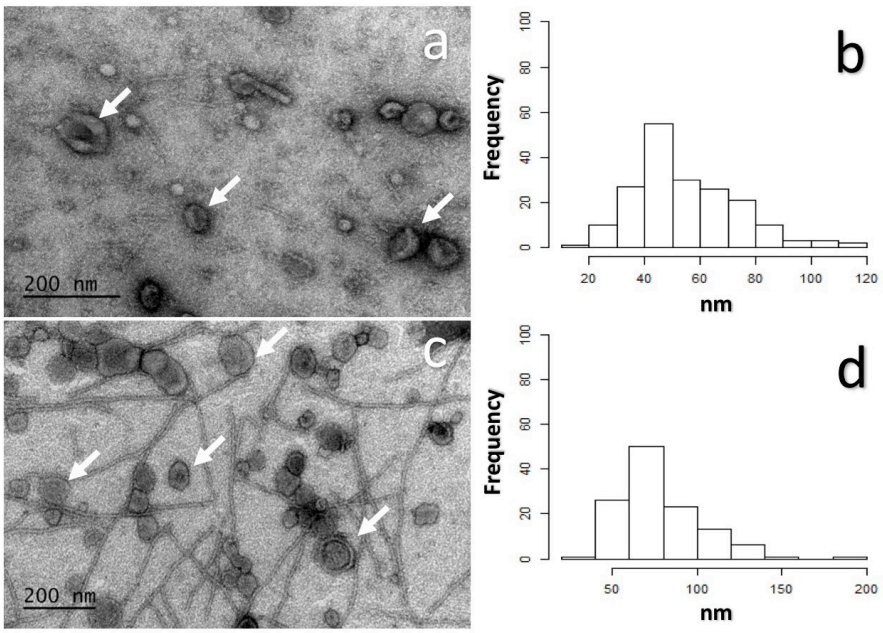

Figure 4.