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Chicken seminal fluid lacks CD9- and CD44-bearing extracellular vesicles

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Abstract

The avian seminal fluid (SF) is a protein-rich fluid, derived from the testis, the rudimentary epididymis and, finally, from the cloacal gland. The SF interacts with spermatozoa and the inner cell lining of the female genital tract, to modulate sperm functions and female immune responsiveness. Its complex proteome might either be free or linked to extracellular vesicles (EVs) as it is the case in mammals, where EVs depict the tetraspanin CD9; and where those EVs derived from the epididymis (epididymosomes) also present the receptor CD44. In the present study, sperm-free SF from Red Jungle Fowl, White Leghorn and an advanced intercross (AIL, 12th generation) were studied using flow cytometry of the membrane marker tetraspanin CD9, Western blotting of the membrane receptor CD44 and electron microscopy in non-enriched (whole SF) or enriched fractions obtained by precipitation using a commercial kit (Total Exosome Precipitation Solution). Neither CD9- nor CD44 could be detected, and the ultrastructure confirmed the relative absence of EVs, raising the possibility that avian SF interacts differently with the female genitalia as compared to the seminal plasma of mammals.

KEYWORDS

CD44, CD9, electron microscopy, exosome precipitation, rooster, seminal fluid, tetraspanins, western blot

1 | INTRODUCTION

Seminal fluid (SF), the extracellular medium which spermatozoa are suspended in, is derived from the testis, epididymis and accessory sexual glands. In the case of the latter, these do not exist in all species, but note these are present and involved in SF production in mammals (Rodríguez-Martínez et al., 2009; Samanta, Parida, Dias, & Agarwal, 2018). With the absence of counterpart accessory sexual glands in avian species, such as the chicken, a complex SF derives from the testis, the rudimentary epididymis and the cloacal glands. Avian SF contains hundreds of proteins and peptides (including antioxidative enzymes and cytokines), many of them conserved over taxa (Atikuzzaman, Alvarez-Rodriguez, et al., 2017). The chicken SF plays a crucial role in several important functions, from modulating

sperm transport, to sperm motility and function and to the induction of genetic regulation of the immune system of the female increasing towards tolerance of the foreign paternal spermatozoa and accompanying proteins (Atikuzzaman, Alvarez-Rodriguez, et al., 2017; Atikuzzaman, Sanz, et al., 2017) in a manner similar to that which has been reported in mammals (Alvarez-Rodriguez, Atikuzzaman, Venhoranta, Wright, & Rodriguez-Martinez, 2019; Barranco et al., 2015; Rodríguez-Martínez et al., 2009). The chicken SF is, via this conserved interaction with the female immune function, capable of influencing fertility (Atikuzzaman, Alvarez-Rodriguez, et al., 2017; Atikuzzaman, Sanz, et al., 2017).

The mammalian seminal plasma (SP) not only contains a complex proteome, with proteins evidently related to modulate fertility (Pérez-Patiño et al., 2018) but also contains extracellular vesicles (EVs), spherical membrane-coated structures released as part of the genital cell secretome. In the mammalian SP, the EVs are classified by size, from those as small (30–100 nm) as exosomes to those as large (100–1,000 nm) as microvesicles (Aalberts, Stout, & Stoorvogel, 2014; Machtinger, Laurent, & Baccarelli, 2015). The exosomes are also classified by point of origin, such as epididymosomes, prostasomes or even vesiculosomes (Bai et al., 2018; Höög & Lötvall, 2015; Sullivan & Saez, 2013), and they interact with both spermatozoa and the internal epithelial lining of the female genital tract (Bai et al., 2018; Du et al., 2016). Sperm modifications after spermatogenesis are caused by, amongst other factors, by EVs trafficking components to the spermatozoa (Girouard, Frenette, & Sullivan, 2009).

In the chicken, however, exosome-like vesicles carrying CD9 have been found delivered to the oviductal lumen in hens by Huang, Isobe, and Yoshimura (2017) who speculated they would eventually interact with spermatozoa. On the other hand, in the specific case of avian SF, studies of the SF proteome of the polyandrous Red Jungle Fowl (RJF), including detection of the CD9 antigen (Borziak, Álvarez-Fernández, L Karr, Pizzari, & Dorus, 2016), suggested the existence of an abundant cargo of exosomes in the SF (Atikuzzaman, Sanz, et al., 2017). However, neither of these studies provided any firm evidence of their presence in chicken SF, a critical oversight.

The tetraspanin CD9 plays a role in cell-to-cell communication by building fusion-competent sites together with integrins (Andreu & Yáñez-Mó, 2014), having a wide tissue distribution. The CD9 is therefore included in most commercial kits to isolate and identify EVs (Keerthikumar et al., 2016). Mapping for this marker in chicken SF has, to the best of our knowledge, yet to be performed. Another marker to be considered is the hyaluronan-receptor CD44, present in exosomes of the pig seminal plasma particularly in the vanguard ejaculate fraction which mainly contains epididymosomes (Alvarez-Rodriguez, Ljunggren, Karlsson, & Rodriguez-Martinez, 2019). Since the rooster has an epididymis (albeit less developed than in mammals), this marker is also ripe for exploration in this context.

The present study therefore aimed to detect EVs in rooster seminal fluid using flow cytometry (FC) of an EV-marker (tetraspanin CD9), the receptor CD44 via Western blotting (WB) and electron microscopy. Three varieties of chicken were included, the ancestor RJF, the highly selected domestic White Leghorn (WL) and specimens resulting of an advanced intercross (12th generation AIL) between the RJF and WL.

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

Sexually mature (25-weeks old) RJF, WL and advanced intercross (AIL) cocks (n = 4 per breed) kept at the facilities of Linköping University were used (58°24′39.1″N, 15°37′17.65″E). Feed, water and perches were available ad libitum and chicken were held under controlled temperature and light regimes (12:12 hr light/dark cycle) as per the geographical location, in 1–2 m^2 pens depending on age for their first 7 weeks. Throughout all experiments, birds were handled carefully and in such a way to avoid any unnecessary stress.

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

Semen was collected from pre-trained roosters, avoiding the presence of a transparent fluid, via gentle dorso-abdominal massage until cloacal eversion was obtained, followed by pressure on the phallus (Burrows & Quinn, 1935). The semen was collected with a plastic Pasteur pipette and transferred to a 1 ml Eppendorf tube. A drop of semen was extended with warmed 37°C Dulbecco's medium and assessed for sperm concentration and motility (velocity and forward progressive motility) using a light microscope (Axio Scope; Carl Zeiss) equipped with a thermal plate (Temp Controller 2000-2; Pecon GmbH) kept at 41°C for chicken semen, positive phase contrast optics (10× objective), a charge-coupled device (CCD) camera (UI-1540LE-M-HQ; Ueye, IDS Imaging Development Systems GmbH) and the QUALISPERM® Software (Biophos SA); a high-throughput system (usually 4 fields per min), analysing >2,000 spermatozoa/field. Ejaculates with >70% motile spermatozoa were used for the experiments; original ejaculates being centrifuged at 21,000 g at 4°C for 10 min (Centrifuge 5424R; Eppendorf AG). The supernatants of avian SF were harvested, microscopically checked for absence of spermatozoa, pooled per type of bird and stored at −80°C prior to analysis.

2.4 | Experimental design

Samples of pooled seminal fluid (using seminal plasma from boars as positive control) (1 ml) were centrifuged again (2,000 *g* at 5°C for 30 min) and harvested supernatants. Then, 200 µl of Total Exosome Isolation Reagent (Invitrogen™; Product code: 13355394) was 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: (a) Ultrastructural examination of the SF by transmission electron microscopy (TEM), (b) exosome identification and analysis using the tetraspanin CD9-detection and (c) extraction of exosome proteins for CD44 identification by WB.

2.4.1 | Ultrastructure of SF samples

Aliquots (5 µl) of the samples obtained by Total Exosome Precipitation Reagent and original SF-supernatant pools were fixed at room temperature in a 0.1% (w/v) paraformaldehyde solution (50 µl) for at least 18 hr. A 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 min at room temperature. The grids were thereafter transferred to 2% uranyl acetate (w/v) drops for negative staining (Cizmar & Yuana, 2017). Electron micrographs were obtained using a transmission electron microscope (EM JEM 1230; JEOL Ltd.), operated at 100 kV. Two-dimensional data were collected, and images were processed for assessing the size of the EVs using IMAGEJ (https://imagej.nih.gov/ij/) and RSTUDIO (https://www.rstudio.com) for illustration of the collected summarized data.

2.4.2 | Exosome identification

Exosome presence was analysed using the Exo-FLOW™ Exosome purification beads (System Biosciences [SBI]) linked to a biotinylated capture antibody against the tetraspanin CD9 (EXOFLOW15OA-1) following the protocols of the manufacturer. In brief, 40 µl streptavidin magnetic bead slurry was 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. The biotinylated capture antibody (CD9) was added, kept on ice for 2 hr, 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 hr. 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 FC using a GalliosTM (Beckman Coulter) instrument equipped with standards optics, violet laser (405 nm) two colours, argon laser (488 nm) five colours and HeNe-laser (633 nm) three colours. Filter configuration: Blue: FL1 550SP 525BP (FITC). The instrument is controlled with NAVIOS software (Beckman Coulter). Analyses of acquired data were performed using the KALUZA software (Beckman Coulter) 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 capture antibody (CD9) 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) versus Forward Scatter (Average-linear).

2.4.3 | Western blot for CD44

Seminal fluid samples eluted by Total Exosome Isolation Reagent were used for protein isolation following our previous protocol (Álvarez-Rodriguez, Vicente-Carrillo, & Rodriguez-Martinez, 2018) with slight modifications. In brief, 200 µl of RIPA buffer (Sigma-Aldrich) were added to the eluted samples prior to sonication (Amplitude 60 W, 10 s, 2 cycles). Then, samples were kept at 4°C for 40 min. After centrifugation of 13,000 *g* for 10 min, proteins were quantified using a DC Protein assay kit (Bio Rad), following manufacturer's instructions. Protein suspensions (0.625 µg protein/µl) were denatured by heating at 70°C for 10 min and an aliquot (10 µl) of each protein suspension were loaded into 4%–20% Mini-PROTEAN® TGX™ Precast Protein Gels (BIORAD). After electrophoresis (150 V for 1 hr) and transfer of the proteins to nitrocellulose membranes (LI-COR Biosciences; 100 V for 1 hr), the membranes were blocked at room temperature for 1 hr with blocking solution (LI-COR Biosciences) and washed in phosphate-buffered saline (PBS) (Thermo Fisher Scientific) containing 0.1% Tween-20 (Sigma-Aldrich) (PBST). After three washes in PBST for 10 min, one membrane was incubated at 4°C overnight with the primary monoclonal anti-CD44 antibody 60224-1-Ig (mouse monoclonal antibody to CD44; Nordic BioSite, Proteintech Europe) at 1:500 dilution rate. The day after, the membrane was washed three times in PBST and incubated for 1 hr with a dilution 1:15,000 of the secondary antibody (goat anti-mouse IRDye 800 CW [925-32210; LI-COR Biosciences]) followed by extensive washing in PBST. The membranes were scanned using the Odyssey CLx (LI-COR Biosciences), and images of the blots were obtained using the IMAGE STUDIO 4.0 software (LI-COR Biosciences).

3 | RESULTS

The electron microscopy of the samples depicted scattered presence of round particles (Figure 1a–c) whose ultrastructural aspect was mostly compatible with lipid droplets rather than with EVs (Figure 1a–c), compared to findings in mammals (Alvarez-Rodriguez,

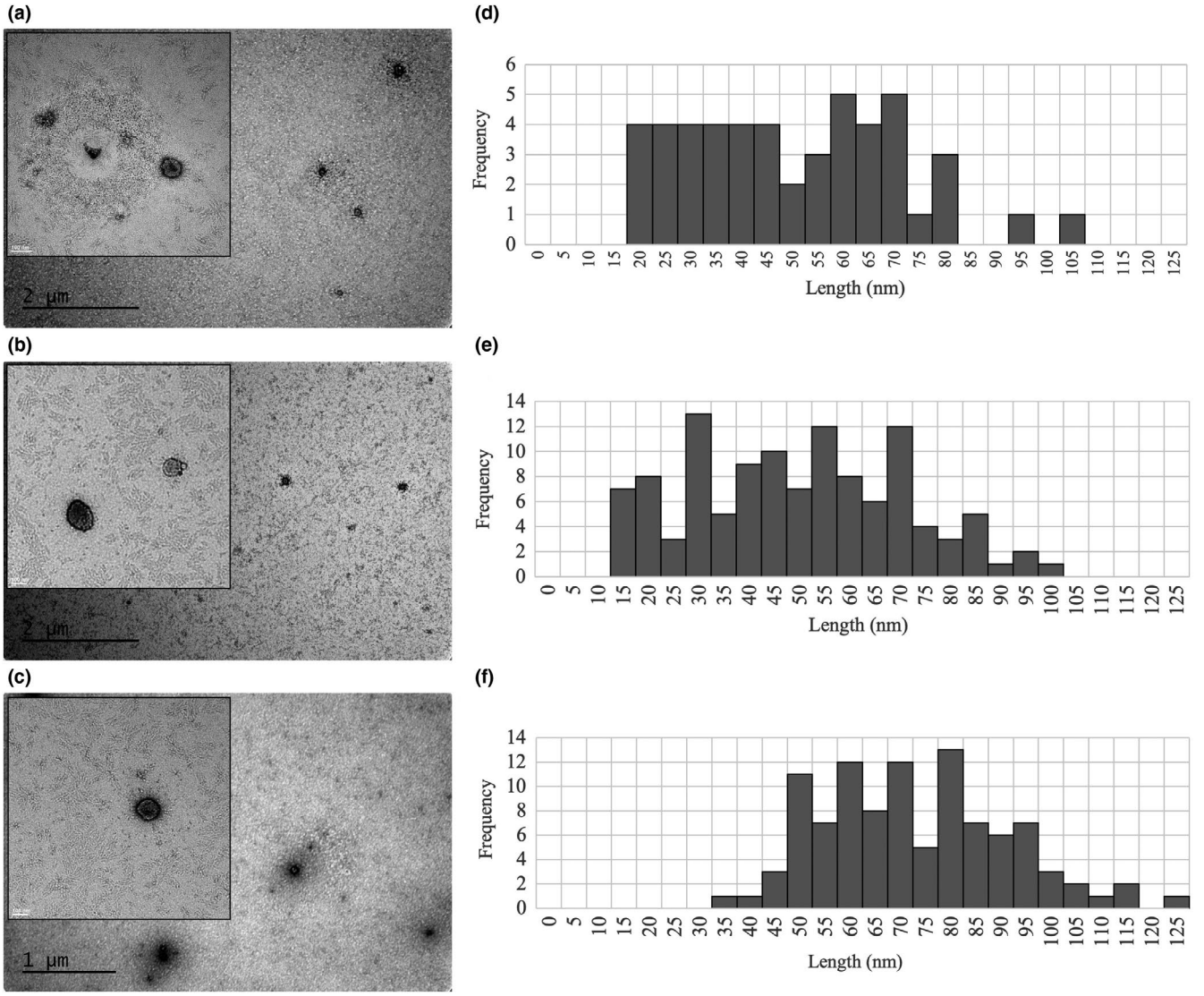

FIGURE 1 Transmission electron micrographs of rooster seminal fluid processed by Total Exosome Isolation Reagent from (a) Red Jungle Fowl, (b) advanced intercross and (c) White Leghorn and cocks. The insets depict higher magnifications of the very few extracellular vesicles (EVs) detected. The accompanying histograms in (d–f) depict frequencies of size intervals, indicating the EVs were virtually absent/present in very low concentration in the seminal fluid of the respective breeds

Ljunggren, et al., 2019; Barranco et al., 2019). A further striking finding was their scarcity and low size (Figure 1d–f), irrespective of the samples assessed, either from original SF or following isolation/enrichment by use of the Total Exosome Precipitation Reagent.

The flow cytometric sorting procedure was able to clearly separate the distinct particular surface markers for the EVs in the positive control (porcine exosomes). The uncentrifuged pooled chicken SF appeared consistently negative to the CD9 antibody staining in all replicates tested, suggesting that the avian SF lacks EVs with this particular tetraspanin (Figure 2, Table 1), thus reinforcing the findings of the electron microscopy.

The WB using the specific anti-CD44 monoclonal antibody (60224-1-Ig) detected the receptor CD44 in pig seminal plasma, used as positive control (Figure 3a; L1: P1) but not in pooled (n = 4) rooster seminal fluid. The antibody identified expected bands at 85 kDa in controls, indicative of a full-size CD44 (Lane 1, Figure 3a). The pooled rooster seminal fluid appeared consistently negative (RJF, AIL respectively WL; L2-L4) in all replicates assayed.

4 | DISCUSSION

In the present study, sperm-free rooster seminal fluid was studied in both bulk rooster SF and SF that was further centrifuged, using FC of EXO-FLOW™ Exosome purification beads. Samples were explored for presence, aspect and distribution frequency using electron microscopy (EM), FC (incubated with an antibody against CD9) and WB (using an antibody against the membrane receptor CD44). Boar seminal plasma was used as comparative positive control (Alvarez-Rodriguez, Ljunggren, et al., 2019).

Among the many methods applied in different fluids and species (reviewed by Konoshenko, Lekchnov, Vlassov, & Laktionov, 2018) to

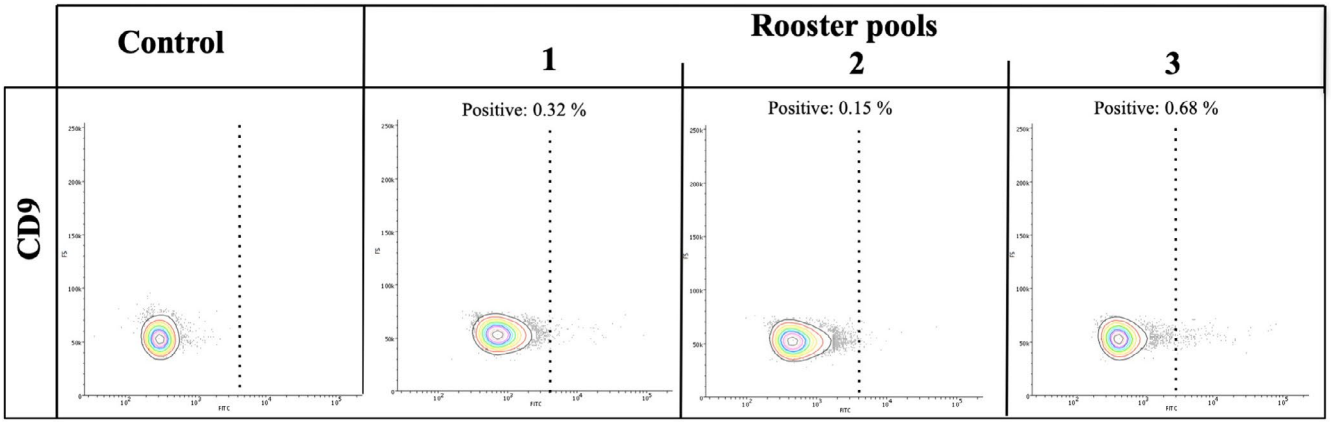

FIGURE 2 Density plots (flow cytometry/EXO-FLOW) of the tetraspanin CD9 in pooled seminal fluid (SF) from pure White Leghorn roosters. The dotted line marks the limit between negative (left hand side) and positive (right hand side, %) plot allocation. Control: SF excluded

TABLE 1 Proportions of flow cytometric FITC staining (EXO-FLOW) for the extracellular vesicle (EV)-marker tetraspanin CD9 in pooled rooster seminal fluid

EV-surface marker	Sample type	SF/SP tested	FITC– (%)	FITC+ (%)	EXO-FLOW result[a]
CD9	Negative control	No sample	99.99	0.12	–
CD9	Positive control	Boar	0.4	99.6	+
CD9	Bulk RJF SF	Pool 1	99.68	0.32	–
	Bulk AIL SF	Pool 2	99.85	0.15	–
	Bulk WL SF	Pool 3	99.32	0.68	–

Note: Negative control: sample absent. Positive control: boar seminal plasma exosomes.

Abbreviations: AIL, advanced inter-cross; RJF, Red Jungle Fowl; SF, seminal fluid; WL, White Leghorn.

[a]EXO-FLOW Result: exosome presence +/Absence –.

FIGURE 3 Western blot (WB) detection of the receptor CD44 in control pig seminal plasma (three ejaculate fractions; L1), and pooled (n = 4) rooster seminal fluid (L2: red jungle fowl [RJF], L3: advanced intercross [AIL] and L4: pure White Leghorn [WL]). The anti-CD44 monoclonal antibody (60224-1-Ig) identified expected bands at 85 kDa for CD44 in seminal plasma boar exosomes (L1), but not in rooster seminal fluid (L2–L4)

isolate EVs (microvesicles and/or exosomes), immunoaffinity precipitation and capture is the method most often commercially offered (Exo-Quick-TC; System Biosciences) as being easy to perform and quick (Bai et al., 2018). However, due to the low density and small size of the exosomes, purity is low-to-poor and demands further screening of other exosome surface markers, such as CD44 for example (Alvarez-Rodriguez, Ljunggren, et al., 2019). In the present study, we studied SF samples harvested by strong centrifugation to separate the spermatozoa and also SF samples further centrifuged and where precipitation of all the EVs was intended using the Total Exosome Isolation Reagent (Invitrogen™; Product code: 13355394). The findings appeared initially disappointing, since the rooster SF (of three different lines of birds) appeared negative to the presence of CD9, of CD44, and at EM examination. Despite such protein markers, as the highly conserved transmembrane-4 superfamily tetraspanins, associated to the use of ultrafiltration or ultracentrifugation have been successfully used to determine the presence and even type of EVs (Alvarez-Rodriguez, Ljunggren, et al., 2019; Barranco et al., 2019). The tetraspanin CD9, which plays a role in cell-to-cell communication by building fusion-competent sites together with integrins (Andreu & Yáñez-Mó, 2014), is due to its broad tissue distribution (Keerthikumar et al., 2016), included in most commercial kits to isolate and identify EVs.

At least two publications from 2016 have indirectly suggested exosomes ought to be present in rooster SF (particularly of the ancestor RJF), owing to indirect findings in their SF proteome (Atikuzzaman, Sanz, et al., 2017; Borziak et al., 2016). Our current findings indicate this is in fact not the case. EVs were not detected in sufficient amounts, neither via EM nor via FC or WB of specific markers. Further studies are warranted to attempt detection via other ways, or a revision of the proteomics in avian SF, since the origin of the CD9 assumed as present might have another origin than from SF-exosomes.

The cell-surface glycoprotein receptor for hyaluronan CD44 depicts a diverse biological activity related to numerous functions

and pathologies (Jordan, Racine, Hennig, & Lokeshwar, 2015). CD44 is expressed, in relation to the local production/presence of the ligand hyaluronan, by numerous cell types including pig spermatozoa (Alvarez-Rodriguez, Ljunggren, et al., 2019) but also in the human epididymis and prostate (Alam et al., 2004).

The rooster SF samples were also consistently negative to CD44. The seminal fluid of a rooster contains mainly material from testicular origin and of the rudimentary epididymal duct, plus some secretion for the cloacal gland. We obviously speculated that epididymosomes ought to be present in the fluid, therefore we tested not only for the tetraspanin CD9 but also for CD44, since this is considered to be a suitable marker for epidydimosomes in pig (Alvarez-Rodriguez, Ljunggren, et al., 2019).

The CD44 is present in the female and male genital tract of most mammalian species, 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 Rodriguez-Martinez et al., 2015). The presence of CD44 in the EVs of the sperm-free seminal plasma play a crucial role in most mammals, 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 (Aalberts et al., 2014). In addition, it is known that rooster spermatozoa do not complete maturation in the epididymis (Froman & Kirby, 2005), thus we hypothesized about a role for CD44 in this process, as documented in mammals (Rodriguez-Martinez et al., 2015). However, no CD44 binding by Western blotting was found in any of the SF samples from the three chicken breeds studied.

Tetraspanin proteins are present in the oviduct of the female chicken, particularly in the sperm-storage tubules that act as sperm reservoirs in hens. Here, exosomes depicting CD63 appeared to be delivered to the lumen to contact the stored spermatozoa, presumably providing them with a cargo meant to keep the cells functional (Huang et al., 2017). However, not only do the EVs carry ncRNAs, and specific proteins; lipoproteins are also carriers with similar delivery capacity for miRNAs, both low-density (LDL) and particularly high-density lipoproteins (HDL) (Vickers, Palmisano, Shoucri, Shamburek, & Remaley, 2011). Since exosomes are comprised to a large extent by membrane lipids, further studies are needed to aid in the more accurate isolation and classification of the different EVs (Piehl et al., 2006; Skotland, Hessvik, Sandvig, & Llorente, 2019) and to determine their eventual protective role on chicken spermatozoa (Atikuzzaman, Alvarez-Rodriguez, et al., 2017) and its signalling for already documented changes in gene expression by the female genital tract, including immune process genes (Alvarez-Rodriguez, Atikuzzaman, et al., 2019; Bai et al., 2018).

The electron microscopy results further corroborated the overall findings. Very few round particles were present in corresponding SF samples. The findings were mostly compatible with lipid droplets and not with EVs, owing to their ultrastructural aspect, size distribution and relative frequency.

Overall, the findings of the present study failed in both finding EVs in rooster SF as confirmed by the lack of signal identifying the presence of CD9 in EVs (MVs/exosomes) in the FC-based FC/EXO-FLOW platform; a method which has been demonstrated to work in boar seminal plasma exosomes (Alvarez-Rodriguez, Ljunggren, et al., 2019). This lack of signal was also determined via Western blotting using a monoclonal antibody to CD44 (Álvarez-Rodriguez et al., 2018) where controls were clearly positive. Lastly, the electron microscopy examinations confirmed the absence of clear-cut exosomes. How avian SF is able to stimulate and convey proteomic and genomic changes in the oviduct of the hen after semen deposition is most likely not mediated via EVs but by proteins, peptides and/or other free components of the SF as demonstrated previously (Atikuzzaman et al., 2015; Atikuzzaman, Alvarez-Rodriguez, et al., 2017; Atikuzzaman, Sanz, et al., 2017).

5 | CONCLUSION

Extracellular vesicles (EVs) were absent or extremely rare in rooster seminal fluid, as confirmed by electron microscopy, explaining why neither total precipitation solution reagent failed in isolating EVs, and yielding negative results when specific markers for tetraspanin CD9 and CD44 were tested. Further studies are warranted in order to establish how chicken SF elicits transcriptomic changes in the female.

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CONFLICTS OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

M.A-R., K.I.K., D.W., M.L-B., C.A.M. and H.R-M. designed the experiments. M.A-R. 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.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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