Received: 2 May 2022 | Revised: 20 June 2022 | Accepted: 6 September 2022

DOI: 10.1111/vco.12858

Veterinary and Comparative Oncology WILEY

ORIGINAL ARTICLE

Characterization and function of extracellular vesicles in a canine mammary tumour cell line: Ultracentrifugation versus size exclusion chromatography

Valentina Moccia[1] | Alessandro Sammarco[1,2] | Silvia Ferro[1] | Laura Cavicchioli[1] | Valentina Zappulli[1]

[1]Department of Comparative Biomedicine and Food Science, University of Padua, Padua, Italy

[2]Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, Los Angeles, California, USA

Correspondence
Valentina Moccia, Department of Comparative Biomedicine and Food Science, University of Padua, Legnaro, 35020, Padua, Italy.
Email: valentina.moccia@phd.unipd.it;

Funding information
American-Italian Cancer Foundation

Abstract

Extracellular vesicles (EVs) are cell-derived membrane-bound vesicles involved in many biological processes such as tumour progression. For years, ultracentrifugation (UC) has been considered the gold standard for EV isolation but limited purity and integrity allowed the diffusion of alternative techniques. In this study, EVs were isolated from a canine mammary tumour cell line using UC and size exclusion chromatography (SEC) and analysed for size and concentration by nanoparticle tracking analysis (NTA) and for protein expression by western blot (WB). EV autocrine effect on cell proliferation, migration and invasiveness was then evaluated in vitro. In all samples, particles were in the EV size range (50–1000 nm), with a higher concentration in UC than in SEC samples (10^{11} and 10^{10} particles/ml respectively), and expressed EV markers (Alix, CD9). Functional assays did not show statistically significant difference among conditions, but EV treatment slightly increased cell proliferation and invasiveness and treatment with SEC-isolated EVs slightly enhanced cell migration compared to UC-isolated EVs. In conclusion, the main differences between the two isolation techniques are the quantity of the final EV-product and slight differences on EV functionality, which should be further explored to better highlight the real autocrine effect of tumoral EVs.

KEYWORDS
CIPp, dog, extracellular vesicles, mammary tumour cell line, size exclusion chromatography, ultracentrifugation

1 | INTRODUCTION

Extracellular vesicles (EVs) are a heterogeneous family of membrane bound vesicles originating from endosomes or cellular plasmatic membrane.[1] EVs are involved in physiological and pathological processes, being used by cells to communicate and to modify the behaviour of target cells through autocrine or paracrine interactions, exchanging molecules such as proteins, lipids, sugars and nucleic acids.[1–4] EVs have been classified according to their size and biogenesis as small "exosomes" (30–100 nm), originating within endosomes, and larger plasma membrane-derived "ectosomes" (microparticles/microvesicles) (100–1000 nm).[2,3] Since biogenesis is not easily definable, the use of size ranges has been recommended, identifying small (<100 nm exosomes and 100–200 nm microvesicles) and medium-large EVs (>200 nm).[5,6] More recently, larger (1–10 μm) tumour-derived EVs–named oncosomes–and new specific EV subtypes have also been described.[7]

The role of EVs in tumorigenesis, tumour prognosis and therapy has been the focus of many studies in human medicine in the last years.[2,8,9] Tumour-derived EVs can target and transfer their cargo to different cells within and outside the tumour.[3,10] The uptake of tumoral EVs by neoplastic cells has consequences on many tumour-associated pathways, stimulating angiogenesis, regulating immune response and transferring drug resistant phenotypes.[3,8,11] EV cargo can also promote cancer cell migration, invasiveness, and metastasis and EVs isolated from malignant cells and transferred to less malignant cells were demonstrated to increase the migration of the recipients in vitro.[12]

When studying EVs, the isolation procedure represents a critical step. EVs can be isolated through different methods. In the past years, ultracentrifugation (UC) was considered the gold standard for EV purification and concentration, but this technique presents major limitations,[5,13] such as the co-deposition of non-EV components, the formation of EV/protein aggregates and the damage of EVs during centrifugation.[14] For these reasons, the popularity of other isolation techniques has increased, and different methods can be combined to gain higher specificity.[5] An alternative isolation method to UC is size exclusion chromatography (SEC). SEC should reduce the co-precipitation of contaminants, preserve EV integrity and avoid EV-aggregation more efficiently than UC.[10,14]

In veterinary medicine, few studies investigated EVs isolated from different species, mainly focusing on identification, characterization and preliminary cargo description.[15–18] However, tumour-derived EVs have not been widely explored yet in veterinary medicine. Studies on circulating EVs demonstrated the higher concentration of EVs in the blood of tumour-bearing dogs compared to healthy dogs[19,20] and investigated EV-related RNAs to find possible diagnostic and prognostic biomarkers.[18,21,22] In vitro research focused on preliminary isolation and characterization of EVs from tumoral cell culture medium (CCM) and on the analysis of their RNA content to elucidate tumour biological behaviour.[22–25] To our knowledge, no study has explored the role of canine mammary tumour-derived EVs in tumour progression and aggressiveness performing in vitro functional assays.

In this study, we compared the isolation of EVs with UC and SEC from a canine mammary tumour cell line (CIPp) and evaluated EV autocrine effects in vitro on cell proliferation, migration and invasiveness. A comparison between the presence or absence of fetal bovine serum (FBS) in CCM was included and assessed for particle concentration and effects on cell proliferation. Our findings showed that UC allows the isolation of more particles consistent with EVs than SEC; however, SEC-isolated EVs manifested a slightly higher autocrine effect.

2 | MATERIALS AND METHODS

2.1 | Cell line validation statement and culture conditions

Canine primary mammary carcinoma CIPp cell line was established by Uyama et al.[26] and kindly provided by Prof. R. De Maria (University of Turin, Italy). The cell line has been regularly tested and confirmed to be mycoplasma-free. Cells were cultured in Rosewell Park Memorial Institute (RPMI 1640) (Thermo Fisher Scientific) supplemented with 10% FBS (PANTM BIOTECH) and 1% penicillin/streptomycin (Corning).

2.2 | Isolation of EVs with UC and SEC

To isolate EVs, two p150 petri dishes were seeded with 1.6×10^6 cells each. CCM was replaced 24 or 48 h before EV isolation with FBS-free (FBSf) medium or with 5% EV-depleted FBS (EV-dFBS) medium respectively, in a volume of 25 ml for UC and 16 ml for SEC. Growth medium from plates processed in the same way but without cells, unconditioned medium (UCM), was included in all experiments as negative control.

EV-dFBS was prepared by overnight (16 h) ultracentrifugation at 100 000 g at 4°C. The pellet was discarded and the supernatant (EV-dFBS) was sterile filtered using a 0.2 μm filter (Sartorius Stedim Biotech).

EVs were isolated by UC and SEC (50 ml and 32 ml of medium respectively) from two plates with 90% confluent cells. The medium was first centrifuged at 300 g for 10 min at 4°C to remove any cell/cell debris. The supernatant was then centrifuged at 2000 g for 10 min at 4°C to remove additional debris. For EV isolation through UC (herein UC EV) the supernatant was transferred to a clean ultracentrifuge tube (Beckman Coulter) and ultracentrifuged at 100 000 g for 90 min at 4°C. The supernatant was discarded and the EV-enriched pellet resuspended in 100 μl of double filtered (0.2 μm) PBS (dfPBS). For EV isolation with SEC (herein SEC EV), the supernatant was transferred into a 100 kDa ultrafiltration tube (Merck Millipore) and centrifuged at 5000 g for 30 min at 4°C. All the material that did not pass through the filter was loaded onto qEVoriginal columns (Izon Science) and SEC was performed according to manufacturer's instructions. Yielded fractions #7, #8, and #9 were pooled and centrifuged with a 100 kDa ultrafiltration tube (Merck Millipore) at 4000 g for 20 min at 4°C. The remaining material that did not passed through the filter was collected and resuspended in 100 μl of dfPBS.

All functional studies were performed in biological triplicates. For western blot (WB) and nanoparticle tracking analysis (NTA), also SEC EV fractions from #10 to #18 were collected. UC and SEC were similarly performed on UCM, so that the herein called UC UCM and SEC UCM (pooled SEC UCM fractions #7, #8, and #9) were used as negative control in each experiment.

2.3 | Nanoparticle tracking analysis

After EV purification, samples of UC EV, UC UCM, SEC EV and SEC UCM from fFBS and 5% EV-dFBS media were quantified and evaluated for concentration and size distribution using NanoSight NS300 (Malvern). For SEC EV fractions from FBSf medium, fractions from #7 to #18 were pooled and analysed. Resuspended (100 μl dfPBS) samples were kept on ice for 1 h and then progressively diluted in dfPBS until reliable measurements were obtained by NTA. To assess background particles in the original media, unprocessed samples (herein unprocessed media) of FBSf RPMI and 5% EV-dFBS RPMI without

cells were also measured by NTA. Three movies of 60 s each were recorded for each sample and analysed using the 3.4 NTA software with camera level set at 12. For particle quantification, reliable values were those within instrument optimal working ranges: particles per frame from 20 to 120; particles concentration between 10^6 and 10^9 per ml; total particles to valid particles ratio higher or equal to 1/5. In addition, for size measurements we also reported D90, D50 and D10, which represent the size point below which 90%, 50% and 10% of the particles, respectively, is included.

2.4 | Protein extraction and western blotting analysis

Cell proteins were extracted from 90% confluent cells on a 15-cm plate using 2 ml of RIPA buffer (Thermo Fisher Scientific) supplemented with protease inhibitor according to manufacturer's protocol. Proteins from UC EV and SEC EV from FBSf media and from UC UCM and SEC UCM from both FBSf and 5% EV-dFBS media were extracted resuspending them in 60 or 20 μl of RIPA buffer supplemented with protease inhibitor after UC and SEC, respectively.

Cells and EV-derived protein concentrations were calculated using Pierce BCA protein Assay Kit (Thermo Fisher Scientific), according to manufacturer's protocol.

For WB, 20 μg of proteins from cells/EVs were used for samples, which were in BCA assay quantification range. Instead, 21 μl were used for samples with protein concentration below detection range. Samples were first denaturated at 70°C for 10 min or at 95°C for 5 min, then were resolved using NuPAGE 4%–12% Bis-Tris gel (Thermo Fisher Scientific) and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked for 90 min in 5% non-fat dry milk in TBS-T (TBS containing 0.05% Tween-20) at room temperature. Blots were then incubated at 4°C overnight with rabbit or mouse primary antibodies against human Alix (1:200; Santa Cruz sc-5358), CD9 (1:200; Bio-Rad MCA694GT) and Calnexin (1:1000; Cell Signalling #2679). Then, membranes were incubated with a peroxidase-conjugate secondary antibody (1:3000; anti-Rabbit #32260 or anti-Mouse #32230, Thermo Fisher Scientific) for 1 h at room temperature. All antibodies were diluted in TBS-T containing 1% non-fat dry milk. Reactive bands were visualized using a chemoluminescent detection kit (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific) with the iBright instrument (Thermo Fisher Scientific).

2.5 | Cell proliferation assay

A 5000 cells per well were seeded in 100 μl of FBSf medium in a 96 well plate. Within 1 h 10 μl of dfPBS resuspended samples (UC EV, SEC EV, UC UCM and SEC UCM from both FBSf and 5% EV-dFBS media) were added to each well. After 24 or 48 h, 20 μl of CellTiter 96® Aqueous One Solution cell proliferation assay (MTS, Promega) was added to each well and after 1 h of incubation at 37°C, absorbance was measured with a spectrophotometer (Packard Instrument, Meriden) at 490 nm.

2.6 | Cell migration assays

Cell migration was studied using wound healing assays and transwell migration assays. For the wound healing assay, 2×10^5 cells per well were seeded on a 6-well plate in 10% FBS medium. When cells reached confluency, cells were washed with PBS and the medium replaced with FBSf medium and 100 μl of dfPBS resuspended samples (UC EV, SEC EV, UC UCM or SEC UCM from FBSf medium) were added to each well. After overnight incubation (14 h), the cell monolayer in each well was scratched twice vertically using a 1 ml sterile pipette tip with a distance of approx. 1 cm between the two scratches. Cells were photographed at 10x using an inverted microscope (Olympus IX50) after 0, 4 and 8 h in three fixed points per scratch (6 fixed point per well). The width of the gap was calculated in each fixed point using ImageJ (https://imagej.nih.gov/ij/), considering 10 measurements per fixed point. To measure migration over time, the mean of measurements after 4 and 8 h were subtracted to measurements at time 0.

In the transwell migration assay, 1.5×10^4 cells were seeded in 100 μl of FBSf medium on a 8-μm pore membrane insert of a transwell 24-well plate (Corning) and 15 μl of dfPBS resuspended samples (UC EV, SEC EV, UC UCM or SEC UCM from FBSf medium) were added to each well. In the lower chamber 400 μl of RPMI with 10% FBS were added. After 6 h, the migrated cells on the lower surface of the transwell membrane were stained with 0.5% crystal violet and visualized using a digital microscope (DMD108, Leica). Pictures were taken at 5× on 5 different fields per condition and ImageJ was used to count the migrated cells.

2.7 | Transwell invasion assay

Cell invasion ability after EV treatment was detected using a 8-μm pore membrane transwell 24-well plate (Corning). Each transwell membrane was pre-coated with 50 μl of 1% Matrigel (BD Biosciences) diluted in FBSf medium and dried at 37°C overnight. A 1.8×10^4 cells were seeded in the upper chamber of each well with 100 μl of FBSf medium and 18 μl of dfPBS resuspended samples (UC EV, SEC EV, UC UCM or SEC UCM from FBSf medium) were added to each well. In the lower chamber, 600 μl of RPMI with 10% FBS were added. After 24 h, membranes were stained with 0.5% crystal violet and cells on the lower surface were visualized using a digital microscope (DMD108, Leica). Pictures were taken at 5× on 5 different fields per condition and ImageJ was used to count the migrated cells.

2.8 | Statistical analysis

Statistical analyses were performed with GraphPad Prism 8 software. Differences between two groups were tested with the two-tailed unpaired Student's t-test when data were normally distributed or the Mann–Whitney test when data were not normally distributed. Differences between more than two groups were tested with ANOVA

TABLE 1 Particle size recorded at nanoparticle tracking analysis

Sample type			Size mean (nm) ± *SD*	Size mode (nm) ± *SD*	D90 (nm) ± *SD*	D50 (nm) ± *SD*	D10 (nm) ± *SD*
Unprocessed medium		FBSf	Unreliable	–	–	–	–
		EV-dFBS	129.7 ± 4.9	97.9 ± 7.9	198.8 ± 9.6	115.4 ± 4.7	83.8 ± 1.3
UCM	UC	FBSf	Unreliable	–	–	–	–
		EV-dFBS	142.1 ± 4.5	118.1 ± 8.1	222.1 ± 6.0	121.8 ± 0.6	87.6 ± 2.7
	SEC 7/8/9	FBSf	Unreliable	–	–	–	–
		EV-dFBS	141.5 ± 4.6	105.1 ± 2.6	211.1 ± 8.5	126.9 ± 4.8	88.5 ± 2.9
EV	UC	FBSf	180.0 ± 2.0	138.2 ± 2.3	283.4 ± 11.8	154.9 ± 3.2	109.0 ± 1.5
		EV-dFBS	188.2 ± 4.8	141.7 ± 7.5	295.2 ± 10.9	162.4 ± 6.3	119.1 ± 3.3
	SEC 7/8/9	FBSf	139.1 ± 1.0	101.2 ± 2.5	201.4 ± 4.7	127.7 ± 4.4	97.1 ± 1.7
		EV-dFBS	169.1 ± 1.6	117.6 ± 4.7	254.1 ± 10.5	150.3 ± 1.5	109.9 ± 3.0
	SEC 10/11/12	FBSf	140.0 ± 3.6	99.0 ± 3.3	207.9 ± 17.5	121.6 ± 1.3	90.5 ± 1.7
	SEC 13/14/15	FBSf	Unreliable	–	–	–	–
	SEC 16/17/18	FBSf	Unreliable	–	–	–	–

Note: D90, D50, D10 represent the size point below which 90%, 50% and 10% of the particles is included.
Abbreviations: EV, extracellular vesicles; EV-dFBS, 5% EV-depleted FBS medium; FBSf, FBS-free medium; SD, standard deviation; SEC #/#/#, fractions of size exclusion chromatography; UC, ultracentrifugation; UCM, unconditioned medium.

when data were normally distributed and Kruskal-Wallis when data were not normally distributed. Level of significance was set at $p < .05$.

3 | RESULTS

3.1 | EV size and concentration

After EV purification with SEC and UC, concentration and size of isolated particles were measured by NTA. Results are shown in Table 1 and Figure 1.

Measurements were not reliable in UCM samples from FBSf medium and SEC EV fractions 13/14/15 and 16/17/18 because of the low particle concentration (Table 1). The size distribution of particles from all other conditions showed instead size ranges within the size range considered for EVs (mode and mean of measurements ranging from 94 to 187 nm) (see Table 1). D90, D50 and D10 of the evaluated samples ranged from 195–289 nm, 115–164 and 84–116 nm, respectively, indicating the purification of mainly small EVs (Table 1).

In UC UCM and in SEC UCM fractions 7/8/9 from 5% EV-dFBS a concentration of 10^{10} and of 10^{9} particles/ml respectively, was observed (Figure 1).

Nanoparticle tracking analysis (NTA) showed similar concentration of particles in UC EV from FBSf medium and from 5% EV-dFBS medium ($7.7 \times 10^{11} \pm 1.8 \times 10^{10}$ and $4.2 \times 10^{11} \pm 6.2 \times 10^{10}$ particles/ml respectively). Particle concentration of UC EV samples from both medium was higher of one order of magnitude than particle concentration in SEC EV fractions 7/8/9 from 5% EV-dFBS or FBSf medium which resulted similar ($7.9 \times 10^{10} \pm 4.1 \times 10^{9}$ and $6.2 \times 10^{10} \pm 3.2 \times 10^{9}$ respectively) (Figure 1). These two latter samples had more particles than SEC EV fractions 10/11/12 from FBSf ($9.1 \times 10^{9} \pm 5.1 \times 10^{8}$ particles/ml) (Figure 1).

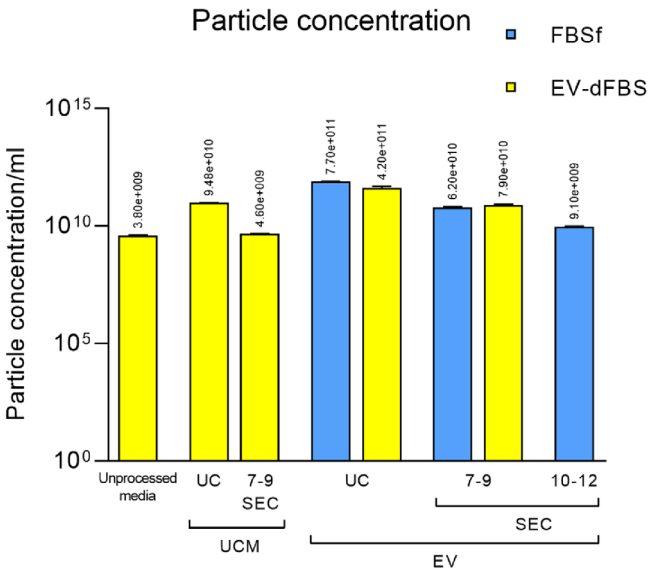

FIGURE 1 Particle concentration/ml recorded at nanoparticle tracking analysis (NTA); UCM, unconditioned medium; EV, extracellular vesicles; UC, ultracentrifugation; #-# SEC, fractions of size exclusion chromatography; FBSf, FBS-free medium; EV-dFBS, 5% EV-depleted FBS medium. Samples with unreliable measurements are not included.

3.2 | EV characterization

WB was performed to characterize EVs isolated by UC and SEC.

Alix, a cytosolic marker of EVs was detected in UC EV and in SEC EV fractions 7/8/9, where the majority of EVs should elute according to manufacturer. Alix was also detected in SEC EV fractions 10/11/12, suggesting EV presence in later fractions (Figure 2). One of

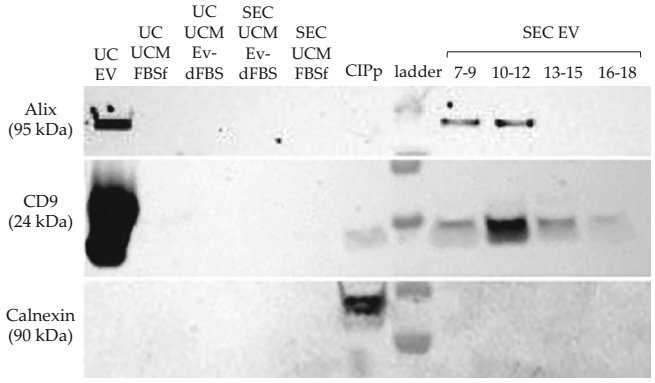

FIGURE 2 Western Blot analysis for EV markers on cells and on EV/UCM samples. UC EV, extracellular vesicles purified with ultracentrifugation; UC UCM, unconditioned medium from ultracentrifugation; SEC UCM, unconditioned medium from size exclusion chromatography; SEC EV #-#, pooled fractions of extracellular vesicles purified with size exclusion chromatography; FBSf, FBS-free medium; EV-dFBS, 5% EV-depleted FBS medium.

the most widely accepted tetraspanin marker for EVs, CD9, was detected in UC EV and in SEC EV fractions from 7 to 18, also suggesting EV presence in late SEC fractions (Figure 2). Calnexin, a marker of the endoplasmic reticulum commonly used as negative control for EVs and positive control for cells, was not detected in EVs but as expected, was detected in cells (Figure 2).

In negative controls (UC UCM and SEC UCM from both FBSf and 5% EV-dFBS media) no EV markers were detected.

3.3 | EV effect on proliferation, migration and invasion

To evaluate EV function, we performed cell proliferation, migration and invasion assays.

First, we evaluated the proliferation of CIPp treated for 24 or 48 h with SEC EV, UC EV, SEC UCM and UC UCM from both FBSf medium and 5% EV-dFBS medium. We did not evidence any

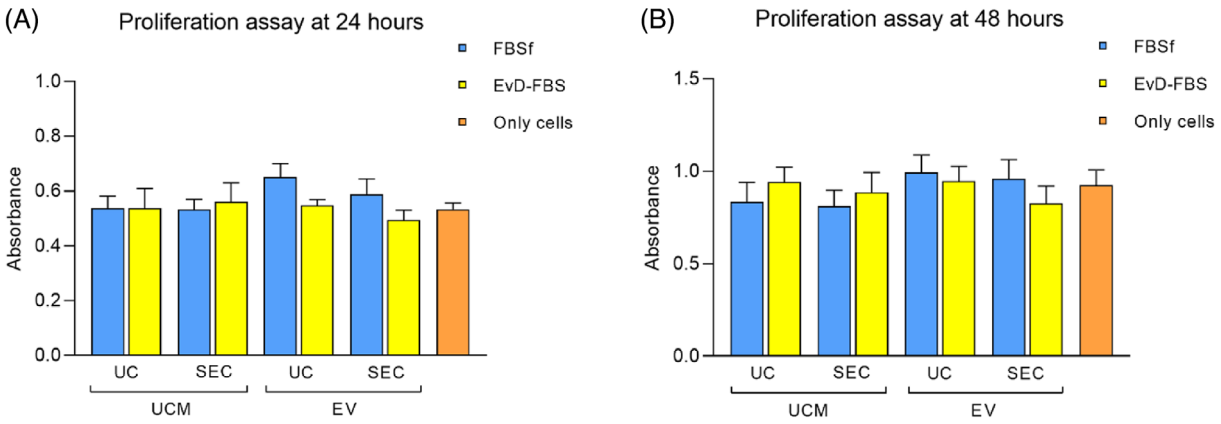

FIGURE 3 (**A**) Proliferation assay performed with MTS assay showing cells absorbance level at 24 h after EV treatment and (**B**) at 48 h after EV treatment. On the ordinate axis, absorbance value is proportional to the number of living cells. UCM, unconditioned medium; EV, extracellular vesicles; UC, ultracentrifugation; SEC, fractions 7/8/9 of size exclusion chromatography; FBSf, FBS-free medium; EV-dFBS, 5% EV-depleted FBS medium. The error bar represents the SE of the mean.

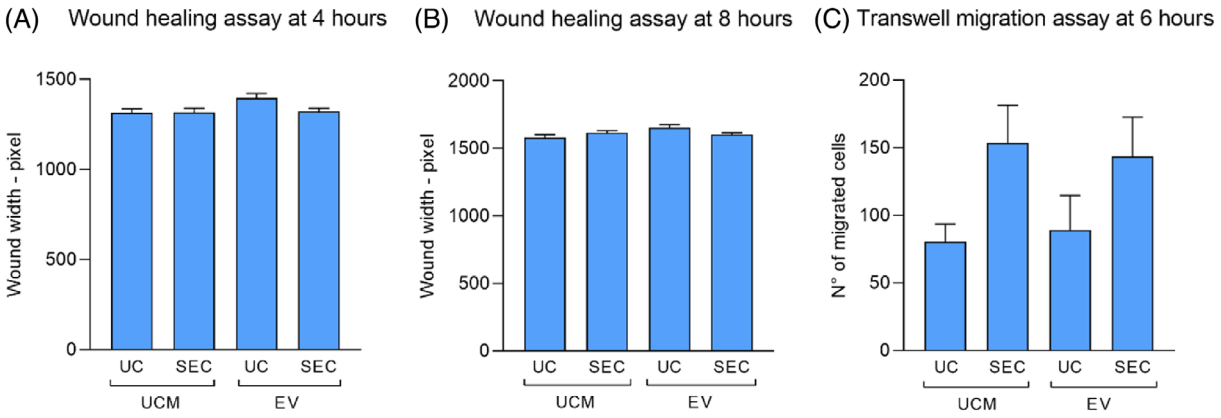

FIGURE 4 (**A**) Wound healing assay showing the reduction of wound width in pixels after 4 h from EV treatment; (**B**) and after 8 h from EV treatment; (**C**) Transwell migration assay showing the number of migrated cells after 6 h from EV treatment. UCM, unconditioned medium; EV, extracellular vesicles; UC, ultracentrifugation; SEC, fractions 7/8/9 of size exclusion chromatography. The error bar represents the SE of the mean.

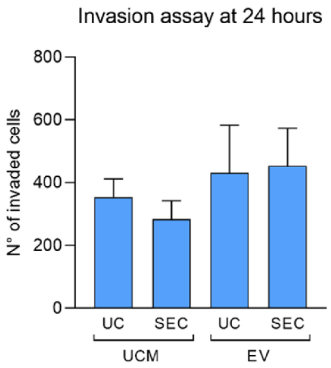

FIGURE 5 Transwell invasion assay results showing the number of invaded cells after 24 h from EV treatment; UCM, unconditioned medium; EV, extracellular vesicles; UC, ultracentrifugation; SEC, fractions 7/8/9 of size exclusion chromatography. The error bar represents the SE of the mean.

significant difference in cell proliferation among conditions after 24 and 48 h, except for a slight increase of cell proliferation of CIPp treated with UC EV and with SEC EV compared to their corresponding controls (UC UCM and SEC UCM, respectively) (Figure 3A,B). This increase was only evident when treating with EVs from FBSf medium and not from 5% EV-dFBS medium.

Secondly, to evaluate the ability of EVs to influence cell migration, we performed wound healing and transwell migration assays considering only FBSf medium samples. In the wound healing assay, cell migration was similar among conditions, both 4 and 8 h after scratch (Figure 4A,B). In the transwell migration assay, 6 h after EV treatment, we evidenced an increase in cell migration of cells treated with SEC EV compared to those treated with UC EV (Figure 4C) but with no statistical significance. However, EV treatments did not differ significantly from their relative controls (SEC UCM and UC UCM respectively).

Finally, to evaluate the effect of EVs on cell invasiveness, we performed a transwell invasion assay considering only FBSf medium samples. We did not find any statistically significant difference in cell invasiveness 24 h after treatment (Figure 5). However, cells treated with SEC EV and UC EV migrated more compared to their corresponding controls (SEC UCM and UC UCM).

4 | DISCUSSION

The aim of this study was to compare two EV isolation techniques, SEC and UC, on a canine mammary tumour cell line to evaluate the autocrine effect of EVs on cell proliferation, migration and invasiveness, including an evaluation of the presence or absence of FBS within the CCM.

We were able to isolate EVs from CIPp cell line using both UC and SEC.

Considering the technical aspects, the main difference between the two EV purification methods was the quantity of the isolated particles measured by NTA, that was higher with UC compared to SEC. It is now recognized that UC allows to collect a greater quantity of EVs compared to SEC, although EVs tend to aggregates and to co-precipitate with extra-EV proteins.[5,14] The presence of co-precipitated contaminants and aggregates can influence NTA measurements, with the impossibility to precisely discern EVs from non-EV particles or EV complexes.[5] Therefore, NTA concentration measurements can be influenced by particles present in FBS and in culture media before cell growth.[27]

In our study, while in unprocessed media and in UCM from FBSf medium, particle concentration was low (unreliable), in unprocessed media and UCM from 5% EV-dFBS medium, particles were within detection range (10^8/ml in unprocessed media, 10^9/ml in SEC UCM and 10^{10}/ml in UC UCM), meaning that the addition of 5% EV-dFBS increased particle concentration. However, since no EV marker was detected in UCM samples at WB, these particles could be FBS-derived EVs with a quantity of proteins below WB detection threshold, proteins/protein aggregates or other media/FBS components (e.g., amino acids, vitamins, salts, minerals), detected as particles at NTA. Additionally, FBS-related particles were no longer detectable after cell growth, since NTA revealed the same order of magnitude of particle concentration in SEC and UC EV from FBSf or 5% EV-dFBS medium. This may indicate that FBS-related particles were consumed by cells, being media components or that in EV samples from 5% EV-dFBS medium, EV concentration was lower compared to EV samples from FBSf medium, where there were no FBS-derived particles. A previous study performed on N2a neuroblastoma cells, showed trough NTA that cells cultured in serum free conditions shed more EVs than cells cultured with EV-depleted serum.[28] In our study, the absence of difference in particle concentration between EV from FBSf and 5% EV-dFBS medium may also be due to different cellular behaviour (N2a vs. CIPp), different culture conditions or by the impossibility to distinguish EV from non-EV particles.[5,29] Anyhow, based on this data and as already mentioned in other studies,[5,27] removal of FBS from CCM is strongly suggested to avoid any biases when working with CCM-derived EVs.

According to WB results, we found EVs distributed in SEC fractions from 7 to 18. The SEC columns producer company indicates SEC fractions #7/8/9 as those where the majority of EVs elute with the least contaminant proteins. EVs elute also in later fractions but with more contaminant proteins. Even if mainly performed on plasma, other studies have evidenced the presence of EVs through WB and NTA in SEC fractions beyond those suggested to collect when isolating EVs by manufacturers.[30–32]

Considering the functional studies, we did not detect any statistically significant biological effect of EVs nor any remarkable functional difference between UC EV and SEC EV. A slight increase in proliferation of cells treated with UC or SEC EV compared to controls, in cell migration when treating with SEC EV compared to UC EV, and in cell invasiveness when treating with SEC EV compared to controls were found.

The poor measured effect of EVs may be related or to technical aspects, or to the lack of a real biological autocrine effect of the studied EVs.

Considering the technical aspects, UC EV might be damaged and less functional,[14,33] and this might have contributed to the slightly higher effect of SEC EV, despite the lower concentration, in the transwell migration assay, as already demonstrated in other studies.[34–36] UC EV might also be associated with non-EV proteins,[5,14] which could influence cell growth. In a study performed on EVs isolated from starved Mesenchymal Stromal Cells, the authors found a deleterious effect of UC EVs on treated cells, which, after a comparison with EVs and proteins isolated with SEC, was referred to the presence of UC co-isolated particles and not to EVs themselves.[37]

Co-isolated particles might have also influenced the proliferation assays, where we evidenced a slight increase in cell proliferation only when treating cells with EVs isolated from FBSf medium compared to controls, but not when treating cells with EVs from 5% EV-dFBS. This latter lack of effect may be related to FBS co-isolated proteins, which could have interfered with EVs, or to starving which might have changed EV composition or quantity.[5,28,29]

Another technical issue in EV functional studies is the EV/recipient cells treatment ratio, for which standardization and overlapping with in vivo situation is arduous.[38] In our assays, EV concentration might have been too low or different from physiological concentration. In functional studies, EVs have been demonstrated to have dose-dependent effects on cell proliferation and migration, especially in regenerative medicine.[39–41] Hence, in further studies, EV dose could be changed to define dose-dependent effects in vitro and enhance broader qualitative differences. In stem cell research, EV effect on cell proliferation has been reported to be also time dependent, with incubation being usually longer than 24 h.[40,42] Unfortunately, in our study longer incubation was not possible because of the fast doubling time of CIPp cell line (24.6 h).[26]

Generally, considering the investigated biological effects of EVs, the literature suggests an enhancing effect of tumour EVs on cell proliferation, migration and invasiveness.[4] However, most studies deal with heterologous EVs, treating recipient cells with EVs isolated from different donor cells.[43–45] Studies on the functional effects of autologous EVs are fewer and some of them report a lack of clear effects. Menck and co-authors reported that the administration of autologous microvesicles to breast cancer cell lines enhanced cell invasion but had no effects on cell proliferation.[46] Moreover, in the same study, microvesicles enhanced cell invasiveness more than exosomes, suggesting the different properties of different EV subpopulations.[46] In our study, using UC at 100 000 × g, we mainly isolated small EVs, apparently mainly microvesicles (100–200 nm size range), and fewer large vesicles (>200 nm).[14] In another study, exosomes isolated from glioma associated-human mesenchymal stem cells, enhanced glioma stem-like cells (GSC) proliferation, but the administration of autologous exosomes to GSC had no effect on cell proliferation.[44] An additional work highlighted the ability of tumoral EVs to enhance cell migration in recipient cells according to the metastatic potential of the donor cells.[47] The authors compared two human breast cancer cell lines, the metastatic/invasive MDA-MB-231 and the non-metastatic/non-invasive MCF7, treated with autologous or heterologous EVs isolated from the same cell lines. EVs isolated from MDA-MB-231 cells had greater autocrine and paracrine migratory potential compared to MCF7-derived EVs.[47] In our study, the lack of significant pro-proliferative, migratory and invasive effects of CIPp-derived EVs may also be related to a low metastatic/invasive profile of the donor cells.[26]

To conclude, we purified EVs from a canine mammary tumour cell line with two different techniques: UC and SEC. UC allowed the isolation of a greater number of particles compared to SEC but SEC EV apparently showed a slightly higher effect on proliferation, migration and invasion. Further analyses are needed to better elucidate the role of EVs and their autocrine effect, taking into consideration their quantity, heterogeneity, purity, integrity and the cells of origin.

ACKNOWLEDGEMENTS

Alessandro Sammarco was supported by an American-Italian Cancer Foundation Postdoctoral Research Fellowship. Open Access Funding provided by Universita degli Studi di Padova within the CRUI-CARE Agreement.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

ORCID

Valentina Moccia https://orcid.org/0000-0001-8843-0395
Alessandro Sammarco https://orcid.org/0000-0003-1977-3292

REFERENCES

1. Abels ER, Breakefield XO. Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release and uptake. *Cell Mol Neurobiol*. 2016;36(3):301-312. doi:10.1007/s10571-016-0366-z
2. Bebelman MP, Smit MJ, Pegtel DM, Baglio SR. Biogenesis and function of extracellular vesicles in cancer. *Pharmacol Ther*. 2018;188:1-11. doi:10.1016/j.pharmthera.2018.02.013
3. Minciacchi VR, Freeman MR, Di Vizio D. Extracellular vesicles in cancer: exosomes, microvesicles and the emerging role of large oncosomes. *Semin Cell Dev Biol*. 2015;40:41-51. doi:10.1016/j.semcdb.2015.02.010
4. Maacha S, Bhat AA, Jimenez L, et al. Extracellular vesicles-mediated intercellular communication: roles in the tumour microenvironment and anti-cancer drug resistance. *Mol Cancer*. 2019;18(1):55. doi:10.1186/s12943-019-0965-7
5. Théry C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles*. 2018;7(1):1535750. doi:10.1080/20013078.2018.1535750
6. Jeppesen DK, Fenix AM, Franklin JL, et al. Reassessment of exosome composition. *Cell*. 2019;177(2):428-445.e18. doi:10.1016/j.cell.2019.02.029
7. Zijlstra A, Di Vizio D. Size matters in nanoscale communication. *Nat Cell Biol*. 2018;20(3):228-230. doi:10.1038/s41556-018-0049-8
8. Becker A, Kumar Thakur B, Weiss JM, Kim HS, Peinado H, Lyden D. Extracellular vesicles in cancer: cell-to-cell mediators of metastasis. *Cancer Cell*. 2016;30(6):836-848. doi:10.1016/j.ccell.2016.10.009

9. Urabe F, Kosaka N, Ito K, Kimura T, Egawa S, Ochiya T. Extracellular vesicles as biomarkers and therapeutic targets for cancer. *Am J Physiol - Cell Physiol*. 2020;318(1):C29-C39. doi:10.1152/ajpcell.00280.2019
10. Yamamoto T, Kosaka N, Ochiya T. Latest advances in extracellular vesicles: from bench to bedside. *Sci Technol Adv Mater*. 2019;20(1):746-757. doi:10.1080/14686996.2019.1629835
11. El Andaloussi S, Mäger I, Breakefield XO, Wood MJA. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov*. 2013;12(5):347-357. doi:10.1038/nrd3978
12. Maas SLN, Breakefield XO, Weaver AM. Extracellular vesicles: unique intercellular delivery vehicles. *Trends Cell Biol*. 2017;27(3):172-188. doi:10.1016/j.tcb.2016.11.003
13. Shao H, Im H, Castro CM, Breakefield X, Weissleder R, Lee H. New technologies for analysis of extracellular vesicles. *Chem Rev*. 2018;118(4):1917-1950. doi:10.1021/acs.chemrev.7b00534
14. Coumans FAW, Brisson AR, Buzas EI, et al. Methodological guidelines to study extracellular vesicles. *Circ Res*. 2017;120(10):1632-1648. doi:10.1161/CIRCRESAHA.117.309417
15. Zhou D, Xue J, He S, et al. Reticuloendotheliosis virus and avian leukosis virus subgroup J synergistically increase the accumulation of exosomal miRNAs. *Retrovirology*. 2018;15(1):1-11. doi:10.1186/s12977-018-0427-0
16. Gatien J, Mermillod P, Tsikis G, et al. Metabolomic profile of oviductal extracellular vesicles across the estrous cycle in cattle. *Int J Mol Sci*. 2019;20(24):6339. doi:10.3390/ijms20246339
17. Almiñana C, Vegas AR, Tekin M, et al. Isolation and characterization of equine uterine extracellular vesicles: a comparative methodological study. *Int J Mol Sci*. 2021;22(2):1-31. doi:10.3390/ijms22020979
18. Garnica TK, Lesbon JCC, Ávila ACFCM, et al. Liquid biopsy based on small extracellular vesicles predicts chemotherapy response of canine multicentric lymphomas. *Sci Rep*. 2020;10(1):1-11. doi:10.1038/s41598-020-77366-7
19. Šimundić M, Švara T, Štukelj R, et al. Concentration of extracellular vesicles isolated from blood relative to the clinical pathological status of dogs with mast cell tumours. *Vet Comp Oncol*. 2019;17(4):456-464. doi:10.1111/vco.12489
20. Żmigrodzka M, Witkowska-Piłaszewicz O, Rzepecka A, Cywińska A, Jagielski D, Winnicka A. Extracellular vesicles in the blood of dogs with cancer—a preliminary study. *Animals*. 2019;9(8):1-13. doi:10.3390/ani9080575
21. Narita M, Nishida H, Asahina R, et al. Expression of micrornas in plasma and in extracellular vesicles derived from plasma for dogs with glioma and dogs with other brain diseases. *Am J Vet Res*. 2020;81(4):355-360. doi:10.2460/ajvr.81.4.355
22. Andriessen A, Bongiovanni L, Driedonks TAP, et al. CDC6: a novel canine tumour biomarker detected in circulating extracellular vesicles. *Vet Comp Oncol*. 2021;20(2):381-392. doi:10.1111/vco.12781
23. Sammarco A, Finesso G, Cavicchioli L, et al. Preliminary investigation of extracellular vesicles in mammary cancer of dogs and cats: identification and characterization. *Vet Comp Oncol*. 2018;16(4):489-496. doi:10.1111/vco.12405
24. Fish EJ, Irizarry KJ, Deinnocentes P, et al. Malignant canine mammary epithelial cells shed exosomes containing differentially expressed microRNA that regulate oncogenic networks. *BMC Cancer*. 2018;18(1):832.
25. Asada H, Tomiyasu H, Uchikai T, et al. Comprehensive analysis of miRNA and protein profiles within exosomes derived from canine lymphoid tumour cell lines. *PLoS One*. 2019;14(4):1-15. doi:10.1371/journal.pone.0208567
26. Uyama R, Nakagawa T, Hong SH, Mochizuki M, Nishimura R, Sasaki N. Establishment of four pairs of canine mammary tumour cell lines derived from primary and metastatic origin and their E-cadherin expression. *Vet Comp Oncol*. 2006;4(2):104-113. doi:10.1111/j.1476-5810.2006.00098.x
27. Lehrich BM, Liang Y, Khosravi P, Federoff HJ, Fiandaca MS. Fetal bovine serum-derived extracellular vesicles persist within vesicle-depleted culture media. *Int J Mol Sci*. 2018;19(11):3538. doi:10.3390/ijms19113538
28. Li J, Lee Y, Johansson HJ, et al. Serum-free culture alters the quantity and protein composition of neuroblastoma-derived extracellular vesicles. *J Extracell Vesicles*. 2015;4(2015):1-12. doi:10.3402/jev.v4.26883
29. Martinez-Pacheco S, O'driscoll L. Evidence for the need to evaluate more than one source of extracellular vesicles, rather than single or pooled samples only, when comparing extracellular vesicles separation methods. *Cancers (Basel)*. 2021;13(16):4021. doi:10.3390/cancers13164021
30. Baranyai T, Herczeg K, Onódi Z, et al. Isolation of exosomes from blood plasma: qualitative and quantitative comparison of ultracentrifugation and size exclusion chromatography methods. *PLoS One*. 2015;10(12):1-13. doi:10.1371/journal.pone.0145686
31. Karimi N, Cvjetkovic A, Jang SC, et al. Detailed analysis of the plasma extracellular vesicle proteome after separation from lipoproteins. *Cell Mol Life Sci*. 2018;75(15):2873-2886. doi:10.1007/s00018-018-2773-4
32. Takov K, Yellon DM, Davidson SM. Comparison of small extracellular vesicles isolated from plasma by ultracentrifugation or size-exclusion chromatography: yield, purity and functional potential. *J Extracell Vesicles*. 2019;8(1):1-18. doi:10.1080/20013078.2018.1560809
33. Sidhom K, Obi PO, Saleem A. A review of exosomal isolation methods: is size exclusion chromatography the best option? *Int J Mol Sci*. 2020;21(18):1-19. doi:10.3390/ijms21186466
34. Nordin JZ, Lee Y, Vader P, et al. Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties. *Nanomed Nanotechnol Biol Med*. 2015;11(4):879-883. doi:10.1016/j.nano.2015.01.003
35. Benedikter BJ, Bouwman FG, Vajen T, et al. Ultrafiltration combined with size exclusion chromatography efficiently isolates extracellular vesicles from cell culture media for compositional and functional studies. *Sci Rep*. 2017;7(1):1-13. doi:10.1038/s41598-017-15717-7
36. Mol EA, Goumans MJ, Doevendans PA, Sluijter JPG, Vader P. Higher functionality of extracellular vesicles isolated using size-exclusion chromatography compared to ultracentrifugation. *Nanomed Nanotechnol Biol Med*. 2017;13(6):2061-2065. doi:10.1016/j.nano.2017.03.011
37. Forteza-Genestra MA, Antich-Rossellò M, Calvo J, Gayà A, Monjo M, Ramis JM. Purity determines the effect of extracellular vesicles derived from mesenchymal stromal cells. *Cell*. 2020;9(2):422.
38. Ha DH, Kim HK, Lee J, et al. Mesenchymal stem/stromal cell-derived exosomes for immunomodulatory therapeutics and skin regeneration. *Cell*. 2020;9(1157):289-313. doi:10.1007/978-981-10-2293-7_10
39. Nojima H, Freeman CM, Schuster RM, et al. Hepatocyte exosomes mediate liver repair and regeneration via sphingosine-1-phosphate. *J Hepatol*. 2016;64(1):60-68. doi:10.1016/j.jhep.2015.07.030
40. Haertinger M, Weiss T, Mann A, Tabi A, Brandel V, Radtke C. Adipose Stem Cell-Derived Extracellular Vesicles Induce Proliferation of Schwann Cells via Internalization. *Cell*. 2020;9(1):163. doi:10.3390/cells9010163
41. Gissi C, Radeghieri A, Lamorgese Passeri CA, et al. Extracellular vesicles from rat-bone-marrow mesenchymal stromal/stem cells improve tendon repair in rat Achilles tendon injury model in dose-dependent manner: A pilot study. *PLoS One*. 2020;15(3):1-17. doi:10.1371/journal.pone.0229914
42. Zhang W, Bai X, Zhao B, et al. Cell-free therapy based on adipose tissue stem cell-derived exosomes promotes wound healing via the PI3K/Akt signalling pathway. *Exp Cell Res*. 2018;370(2):333-342. doi:10.1016/j.yexcr.2018.06.035
43. Grange C, Tapparo M, Collino F, et al. Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche. *Cancer Res*. 2011;71(15):5346-5356. doi:10.1158/0008-5472.CAN-11-0241

44. Figueroa J, Phillips LM, Shahar T, et al. Transfer of miR-1587 exosomes from glioma-associated mesenchymal stem cells increase the tumorigenicity of glioma stem-like cells via transfer of miR-1587. *Cancer Res*. 2017;77(21):5808-5819. doi:10.1158/0008-5472.CAN-16-2524
45. Li X, Tang M, Zhu Q, Wang X, Lin Y, Wang X. The exosomal integrin α5β1/AEP complex derived from epithelial ovarian cancer cells promotes peritoneal metastasis through regulating mesothelial cell proliferation and migration. *Cell Oncol*. 2020;43(2):263-277. doi:10.1007/s13402-019-00486-4
46. Menck K, Scharf C, Bleckmann A, et al. Tumor-derived microvesicles mediate human breast cancer invasion through differentially glycosylated EMMPRIN. *J Mol Cell Biol*. 2015;7(2):143-153. doi:10.1093/jmcb/mju047
47. Harris DA, Patel SH, Gucek M, Hendrix A, Westbroek W, Taraska JW. Exosomes released from breast cancer carcinomas stimulate cell movement. *PLoS One*. 2015;10(3):1-18. doi:10.1371/journal.pone.0117495

How to cite this article: Moccia V, Sammarco A, Ferro S, Cavicchioli L, Zappulli V. Characterization and function of extracellular vesicles in a canine mammary tumour cell line: Ultracentrifugation versus size exclusion chromatography. *Vet Comp Oncol*. 2023;21(1):36-44. doi:10.1111/vco.12858