

Isolation and Identification of Exosomes from Feline Plasma, Urine and Adipose-Derived Mesenchymal Stem Cells

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Abstract

Background: Exosomes, internal proteins, lipids, and nucleic acids coated by phospholipid bilayer membranes, are one type of small extracellular vesicles, which can mediate cell-cell communication. In recent years, exosomes have gained considerable scientific interest due to their widely applied prospect in the diagnosis and therapeutics of human and animal diseases. In this study, we describe for the first time a feasible method designed to isolate and characterize exosomes from feline plasma, urine and adipose-derived mesenchymal stem cells.

Results: Exosomes from feline plasma, urine and adipose-derived mesenchymal stem cells were successfully isolated by differential centrifugation. Quantification and sizing of exosomes were assessed by transmission electron microscopy, flow nano analysis and western blotting. Detected particles showed the normal size (30-100 nm) and morphology described for exosomes, as well as presence of the transmembrane protein (TSG101, CD9, CD63, and CD81) known as exosomal marker.

Conclusions: The results suggest that differential centrifugation is a feasible method for isolation of exosomes from different types of feline samples. Moreover, these exosomes can be used to further diagnosis and therapeutics in veterinary pre-clinical and clinical studies.

Background

Exosomes, widely existing in various biological fluids such as blood[1], saliva[2], cerebrospinal fluid[3], urine[4], milk[5], semen[6] and synovial fluid[7], are membrane structures of 30 to 100 nm in size produced by cells and one of carriers of cell-cell communication[8, 9]. The functional materials carried by exosomes, such as nucleic acids, proteins and lipids, are considered as biomarkers for animal physiological diagnosis and as communication carriers for animal physiological states regulation or diseases treatment[10]. Exosomes have been extensively studied as their great development potential.

Exosomes isolated from biological fluids such as plasma and urine hold diagnostic potential[11]. Plasma-derived exosomes (Plasma-exo), which are easily collected and does not usually cause discomfort to the patient, are considered potential biomarkers for diagnosis and prognosis of several diseases such as cancer[12], hematonosis[13], angiocardopathy[14] or cerebrovascular disease[15]. The study of their protein or nucleic acid content is also promising for disease. Urine-derived exosomes (Urine-exo) are secreted by various cells in the urinary system and released into the urine. The changes of urinary exosome-derived miRNAs and proteins can be used as biomarkers in kidney diseases for monitoring the changes of diseases and judging prognosis, and also have important value in the disease treatment[16].

Mesenchymal stem cells (MSCs) have emerged as a promising therapeutic strategy for several diseases. There is accumulating evidence suggesting their therapeutic effects are largely mediated by paracrine factors including cytokines, growth factors, and exosomes[17, 18]. Numerous studies have revealed that

MSC-derived exosomes (MSC-exo) might represent a novel cell-free therapy with compelling advantages over MSCs such as lower immunogenicity and no tumorigenicity[19–21].

The establishment of mature, stable, convenient and fast exosome isolation methods is a prerequisite for ensuring the smooth progress of exosome related research[22]. The isolation and identification methods of different tissues-derived exosomes from dogs[23], horses[24] and cattle[25] samples have been established, but the exosomes from feline samples have been rarely reported. The objective of this study was to develop an efficient and robust method for MSC-exo, Plasma-exo and Urine-exo from feline samples (Fig. 1). This study provides comprehensive techniques such as transmission electron microscopy, flow nano analysis and western blotting to identify and characterize exosomes, allowing them to be quantified and sized, as well as characterized through specific morphology and a distinct protein expression.

Results

Identification of adipose-derived mesenchymal stem cells (AD-MSCs)

Differentiation of AD-MSCs. After induction with adipogenic medium for 14 days, AD-MSCs gradually changed from fibroblast-like cells to flattened cells, and many different sizes lipid droplets appeared in the cytoplasm. Cellular staining was positive and the multiple lipid droplets in differentiated cells were stained red by staining with Oil red-O. After incubation with osteogenic medium for 5 days, MSCs exhibited obvious morphological alterations. Calcium nodules appeared on the 10th day of induced differentiation and tightly packed colonies forming nodule-like structures were observed and deposition of calcium in these cells was observed by staining with alizarin red (Fig. 2-A).

Flow cytometry analysis of AD-MSCs.

AD-MSCs were highly-expressed mesenchymal stem cell surface markers CD44, CD90 and CD105, while for the lowly-expressed haematopoietic stem cells surface markers CD34, leukocyte common antigen CD45 and major histocompatibility complex class II HLA-DR (Fig. 2-B). That is, the isolated and cultured cells conformed the characteristics and identification criteria of mesenchymal stem cells.

Transmission electron microscopy (TEM)

TEM confirmed 3 different sources-derived exosomes showed the cup-shaped spherical morphology with of exosomal vesicles that are concave in the middle (Fig. 3). The vesicles observed ranged in size from 30 to 100 nm.

Flow nano analyzer

The exosomes from MSCs cell culture medium, plasma, and urine exhibited an ideal mean diameter of 74.76 nm, 66.62 nm, and 72.88 nm, a concentration of 2.62×10^{10} /ml, 6.42×10^{10} /ml, and 8.49×10^{11} /ml, as detected by Flow NanoAnalyzer (Fig. 4).

Western blotting

We analysis revealed detection of four EVs-surface-marker proteins (TSG101, CD9, CD63, and CD81), with results showing all samples isolated by our ultrafiltration technique were positive for TSG101, CD9, CD63, and CD81, indicating the presence of EV proteins (Fig. 5).

Discussion

Exosomes are released by virtually every cell type in the body cells into biological fluids in vivo and cell culture conditioned media in vitro[26]. Exosomes have been shown to be key mediators of cell to cell communication, delivering a distinct cargo of lipids, proteins and nucleic acids that reflects their cell of origin[27]. As a new biomarker, exosomes have been widely used in the diagnosis and therapeutics of human diseases, but there are few researches in related fields of pet medical. The research interest in exosomes is continuously increasing however the lack of standard methods for isolation and quantification, limits the reliability and reproducibility of exosome use[28, 29].

This study provided a method based on differential centrifugation of exosome isolation for 3 different biofluids from feline samples, laying a foundation for the application of exosomes in disease diagnosis and treatment of pet cats in the future. The differential centrifugation method is based on the difference in size and density between the exosome sample and other substances, through a series of centrifuges with different centrifugal forces and different centrifugal time lengths, non-exosomes are gradually removed after precipitation, and then exosomes are precipitate by ultracentrifugation and re-suspended finally[30]. Ultracentrifugation is the most widely used method for exosome isolation and was once called the “gold standard” for exosome preparation[31]. Due to its simple operation and stable separation effect, about more than half of exosomes related researcher used this method to extract exosomes.

In this study, the ultrastructure, particle size and surface markers of exosomes were identified by transmission electron microscopy, flow nano analyzer and western blot. The results showed that the three exosomes were round or elliptic vesicles with membranous structures around the vesicles, similar in shape to those previously described in mammals. The particle size of Urine-exo detected by flow nano analyzer is the largest of the three exosome samples, while Plasma-exo is the smallest, but all within the range of 30–100 nm. Compared to plasma and urine samples, the number of exosomes found in MSCs cell culture medium was significantly lower. This may be because the volume of 50ml cell culture medium is too small, and a larger volume of medium is needed to obtain higher production of exosomes. Tetraspanins (including CD81, CD63 and CD9 protein) are common exosomal specific markers for extracellular vesicles such as exosomes and were suggested by the International Society of Extracellular Vesicles (ISEV) for the identification of exosomes[24]. As a cytosolic protein, Tumor Susceptibility Gene

101 (Tsg101) is involved in multivesicular body formation of exosome, is considered to be another important exosome marker[32]. Our western blotting result showed that the marker proteins were detected to be all positive in exosomes from 3 different biofluids. But all proteins signal strengths of MSC-exo are weaker than those in the serum and urine, probably because number of exosomes are fewer of them. Therefore, combined with the above results, it is demonstrated that the methods of exosome isolation we established is feasible and effective, allowing nanoparticles to be analysed in downstream applications.

Conclusions

Overall, our results evidence the feasibility to easily isolate exosome from the supernatants of feline adipose derived mesenchymal stem cells, as well as from plasma and urine of feline. This method for isolating exosomes from feline samples can be used to further diagnosis and therapeutics in veterinary pre-clinical and clinical studies.

Methods

Isolation, culture and identification of adipose-derived mesenchymal stem cells

Abdominal subcutaneous adipose tissues were collected aseptically at Affiliated Animal Hospital, Department of Veterinary Medicine of Foshan University. The tissues were cut into tissue blocks about 1 mm² in size and were digested with 1 mg/mL collagenase type I at 37°C for 2 ~ 3 h. The digestive juices were filtered with 200-mesh cell strain and centrifuged at 800×g for 5min to collect AD-MSCs. Approximately 5000 isolated suspended cells per cm² were transferred to cell culture flask (Corning, USA) in Dulbecco's Modified Eagle's Medium supplemented with 10% exosome-free Fetal Bovine Serum (FBS, Biological Industries, Israel), 1% Pen-Strep (Gibco, USA), and 1% L-glutamine (Gibco, USA) and placed into the incubator at 37°C in a humidified incubator containing 5% CO₂. After 24 h, the medium was replaced for the first time to remove most of the blood cells and replaced every 3 d thereafter. AD-MSCs were digested with 0.25% trypsin and passaged routinely when 80 ~ 90% confluence was reached.

The AD-MSCs were characterized by multipotential differentiation and flow cytometry analysis. In vitro adipogenic and osteogenic differentiation were examined using MSCs Adipogenic Differentiation Kit (Cyanogen, China) and MSCs Osteogenic Differentiation Kit (Cyanogen, China) following the manufacturer's protocol for each kit. Cells were stained with Oil Red O solution to assess adipogenic differentiation and alizarin red solution to assess osteogenic differentiation.

Flow cytometry analysis was performed using a CytoFLEX flow cytometry instrument (Beckman, USA). Data acquisition and analysis was performed with CytExpert (Beckman, USA). Briefly, AD-MSCs of passage 2 were stained for 30 min with FITC-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies at 37°C. The following monoclonal antibodies were used: anti-CD34-PE (cat.no.ab23830; Abcam), anti-CD44-FITC (cat.no.MA1-10229; Invitrogen), anti-CD45-FITC (cat.no.ab27287; Abcam), anti-

CD90-PE (cat.no.11-0900-81; Invitrogen), anti-CD105-FITC(cat.no.ab11415; Abcam) ,and anti-HLA-DR-FITC (cat.no.L243-347400; BD Biosciences). Chilled PBS was used to wash and remove unbound antibodies, and then a total of 2×10^5 cells from each sample tube were acquired for analysis using Flow Cytometer.

Preparation of cell culture medium samples

50–80% confluent AD-MSCs at passage 2–5 were washed twice in PBS and further cultured in an exosome-free medium as described above. Briefly, cell culture medium was harvested after 48 h of incubation with exosome-free medium and stored at $-80\text{ }^{\circ}\text{C}$ for subsequent experiments.

Preparation of plasma samples

Samples were mixed from 3 female and 2 male felines presented at Affiliated Animal Hospital, Department of Veterinary Medicine of Foshan University. Blood samples are collected into a collections tubes containing anticoagulant and the cell components were removed by centrifugation ($800 \times g$, $4\text{ }^{\circ}\text{C}$, 15 min). The supernatant was diluted with phosphate buffered saline of the same volume (1:1) and stored at $-80\text{ }^{\circ}\text{C}$ for subsequent experiments.

Preparation of Urine samples

Urine samples were mixed from 1 female and 2 male felines presented at Affiliated Animal Hospital, Department of Veterinary Medicine of Foshan University. Samples are collected into tubes and stored at $-80\text{ }^{\circ}\text{C}$ for subsequent experiments.

Isolation of exosomes

Exosomes were isolated by differential centrifugation. Briefly, Cell culture medium (50 mL) were centrifuged at $4\text{ }^{\circ}\text{C}$, $300 \times g$ for 10 min to remove dead cells, followed by centrifuging at $12,000 \times g$ for 30 min at $4\text{ }^{\circ}\text{C}$ to remove cell debris. Supernatant was collected and filtered through 0.22 mm filters (Merck Millipore, USA) to remove contaminating microvesicles. Following this, supernatants were transferred to polycarbonate tubes for ultracentrifugation in ultra-speed centrifuge (Beckman Coulter XL-90, SW28Ti rotor; Beckman Coulter; USA) at $100,000 \times g$ for 70 min at $4\text{ }^{\circ}\text{C}$. Diluted plasma samples (5 ml) were centrifuged at $12,000 \times g$ for 30 min at $4\text{ }^{\circ}\text{C}$ to remove cell debris. Clarified supernatant was then ultracentrifuged at $50,000 \times g$ for 70 min at $4\text{ }^{\circ}\text{C}$ to remove large proteins and microvesicles. Following this, supernatants were transferred to polycarbonate tubes for ultracentrifugation at $100,000 \times g$ for 70 min at $4\text{ }^{\circ}\text{C}$. Urine samples (25 ml) were centrifuged at $4\text{ }^{\circ}\text{C}$ $300 \times g$ for 10 min to remove dead cells, followed by centrifuging at $12,000 \times g$ for 30 min at $4\text{ }^{\circ}\text{C}$ to remove cell debris. Clarified supernatant was then ultracentrifuged at $50,000 \times g$ for 70 min at $4\text{ }^{\circ}\text{C}$ in ultra-speed centrifuge remove large proteins and microvesicles. Following this, supernatants were transferred to polycarbonate tubes for ultracentrifugation at $100,000 \times g$ for 70 min at $4\text{ }^{\circ}\text{C}$. The all final pellets were resuspended in $100\text{ }\mu\text{l}$ PBS and then stored at $-80\text{ }^{\circ}\text{C}$ until further analysis.

Transmission electron microscopy (TEM)

Exosome samples were diluted in PBS, dropped on a carbon-coated copper grid, and then stained with 1% uranyl acetate for 1 min. Grids were imaged under a Hitachi H-7650 transmission electron microscope.

Flow nano analyzer

Exosome samples were diluted 1:100 and analyzed using the Flow Nano Analyzer (NanoFCM Inc.) according to manufacturer's protocol. Briefly, the lasers were calibrated using 200 nm control beads (NanoFCM Inc.), which were then analyzed as a reference for particle concentration. Additionally, a mixture of different sized beads (NanoFCM Inc.) was analyzed to set reference for size distribution.

Western blotting

Exosome samples were denatured in protein loading buffer (10% sodium dodecyl sulfate (SDS), 250 mM Tris-HCl (pH 6.8), 0.5% Bromophenol blue, 50% glycerin, 5% β -Mercaptoethanol) at 95°C for 10 min. Proteins were separated by 10% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), and were then transferred to a polyvinylidene fluoride (PVDF) membranes (Merck Millipore, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 hour at room temperature and afterwards incubated at room temperature for 1 hour with antibodies against TSG101 (Santa Cruz, sc-7964, 1:1000), CD81 (Affinity, DF2306, 1:1000), CD63 (Santa Cruz, sc-5275, 1:1000) and CD9 (Affinity, AF5139, 1:1000), followed by incubation with horseradish peroxidase conjugated secondary antibodies at room temperature for 1 hour. Luminescent visualization was done using an ECL kit (Tanon, China) to identify immunoreactive protein bands.

Abbreviations

AD-MSCs adipose-derived mesenchymal stem cells

FBS fetal bovine serum

ISEV international society of extracellular vesicles

MSCs mesenchymal stem cells

MSC-exo mesenchymal stem cells derived exosomes

Plasma-exo plasma derived exosomes

PVDF polyvinylidene fluoride

SDS sodium dodecyl sulfate

Tsg101 tumor susceptibility gene 101

Urine-exo urine derived exosomes

Declarations

Ethics approval and consent to participate

All procedures in the present study were approved by the Animal Ethics Committee of Foshan University and written informed consent was obtained from all donors.

Consent for publication

Written informed consent for publication was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Funding

Not applicable.

Authors' contributions

BYW and YX designed the study. DSL and HNL performed the experiments and analysed the data. ZSC and SFC collected the samples. All authors read and approved the final manuscript.

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Figures

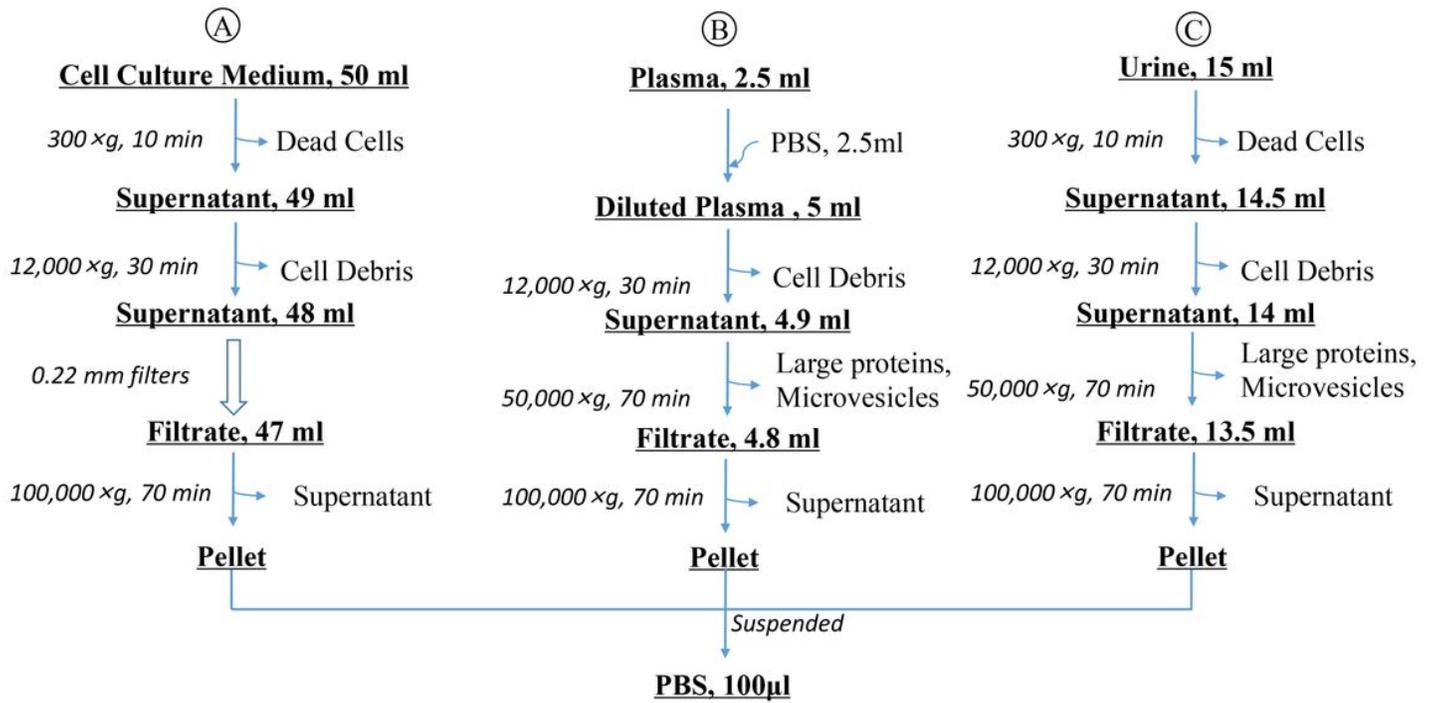
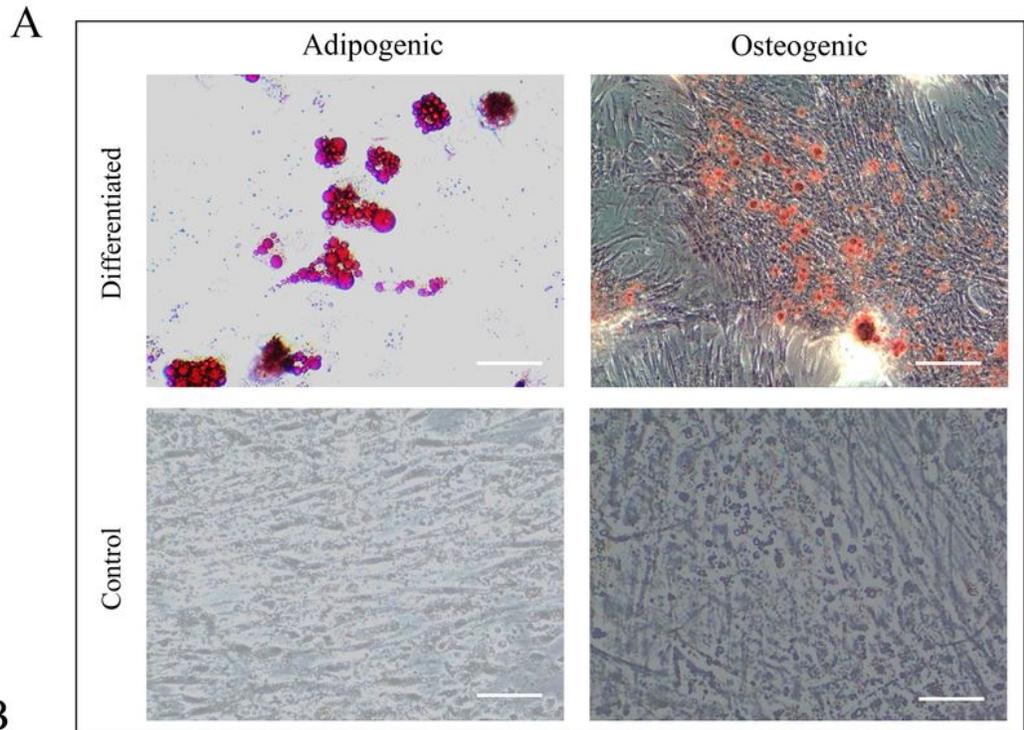


Figure 1

Procedures of the methods used for the isolation of exosomes from feline samples by differential ultracentrifugation. (A) The method for isolating exosomes from feline adipose-derived mesenchymal stem cell culture medium, (B) the method for isolating exosomes from feline plasma, and (C) the method for isolating exosomes from feline urine.



B

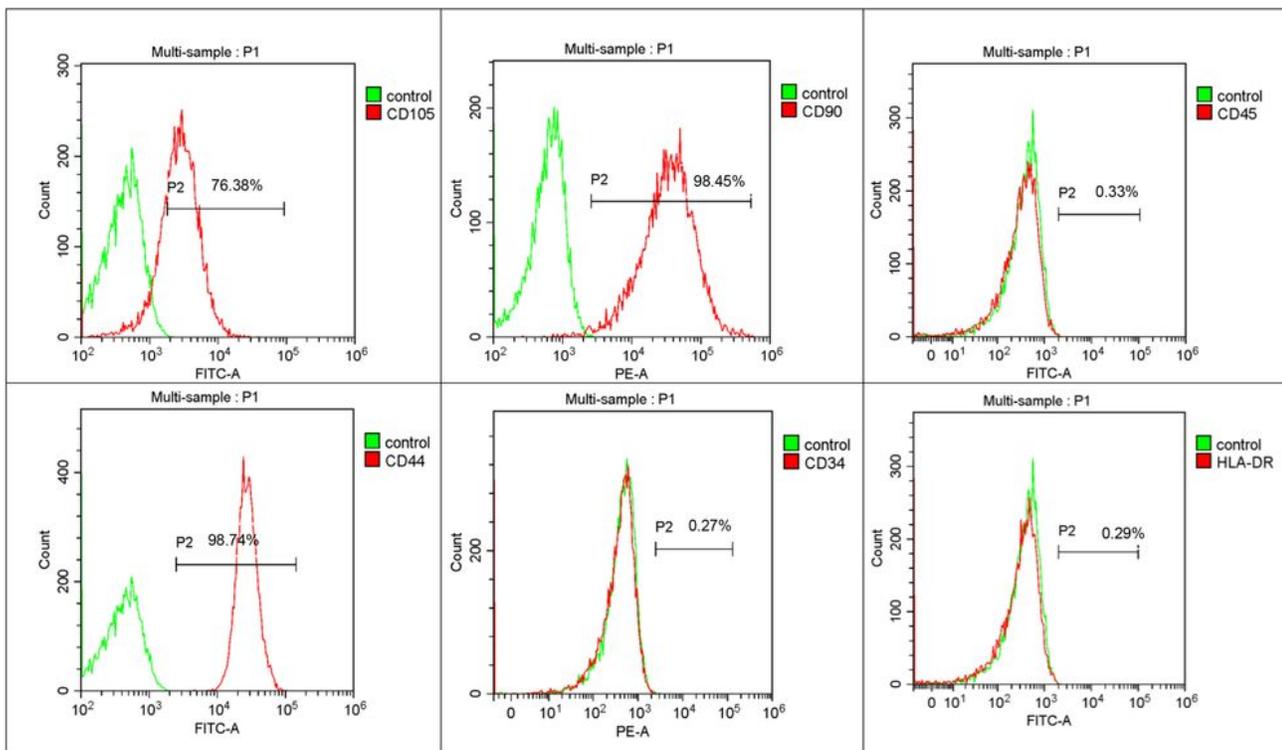


Figure 2

Identification of feline adipose-derived mesenchymal stem cells. (A) Adipogenic and osteogenic differentiation of feline AD-MSCs. AD-MSCs were positive for Oil red-O staining and alizarin red staining. Scale bars, 50 μ m. (B) Surface markers of feline AD-MSCs. Based on flow cytometric analysis, surface molecule markers CD44, CD90, and CD105 were highly expressed on feline AD-MSCs, whereas the

expression of hematopoietic stem cell markers CD34, leukocyte common antigen CD45, and major histocompatibility complex HLA-DR were rarely expressed.

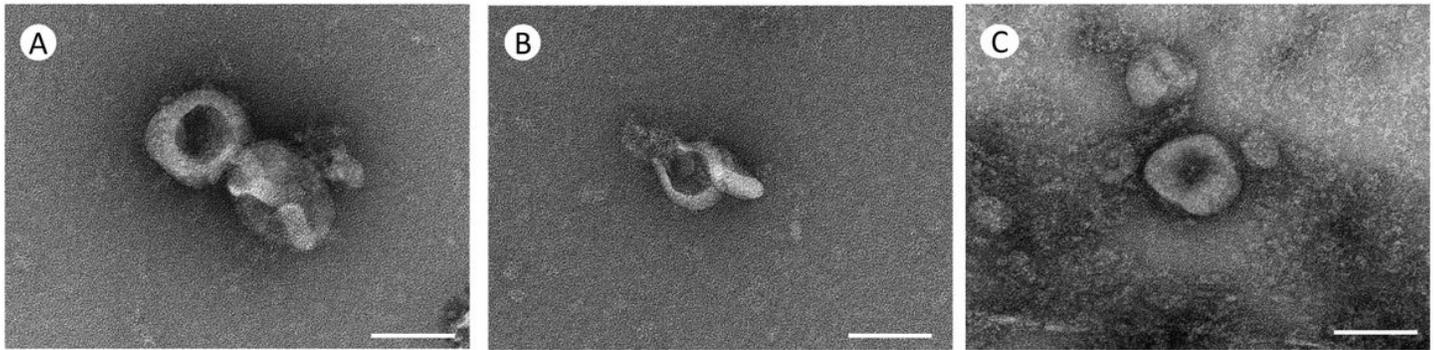


Figure 3

Transmission electron microscopy of feline samples-derived exosomes. (A) Exosomes isolated from feline adipose-derived mesenchymal stem cell culture medium (MSC-exo). (B) Exosomes isolated from feline plasma (Plasma-exo). (C) Exosomes isolated from feline urine (Urine-exo). Scale bars, 100 nm.

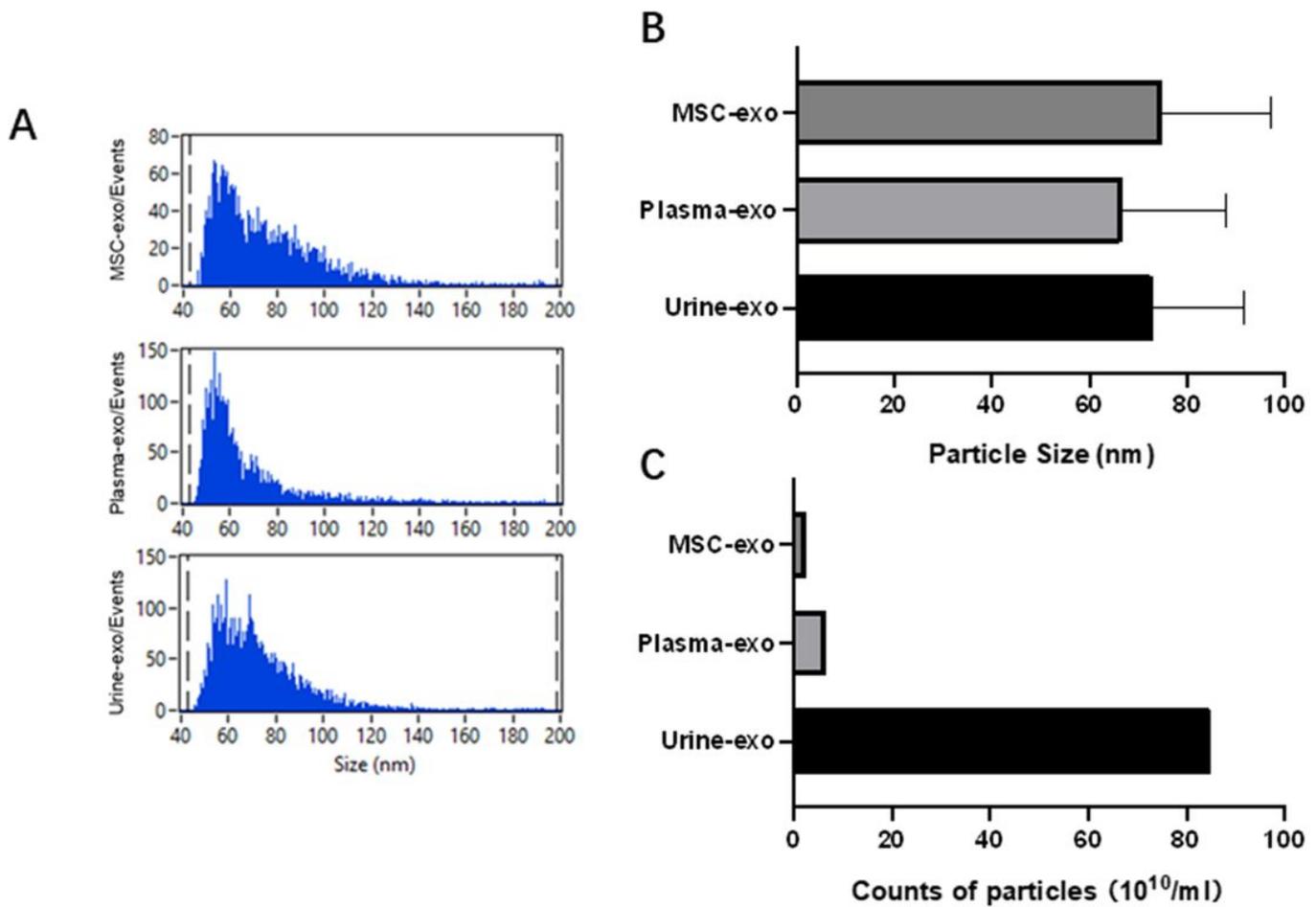


Figure 4

The size and concentration of feline samples-derived exosomes measured by flow nano analyzer. (A) Nano track analysis size distribution of exosomes isolated from feline adipose-derived mesenchymal stem cell culture medium (MSC-exo), feline plasma (Plasma-exo), and feline urine (Urine-exo). (B) Diameter of isolated particles (exosomes) from feline plasma, urine and adipose-derived mesenchymal stem cells. (C) Counts of particles (exosomes) from feline plasma, urine and adipose-derived mesenchymal stem cells.

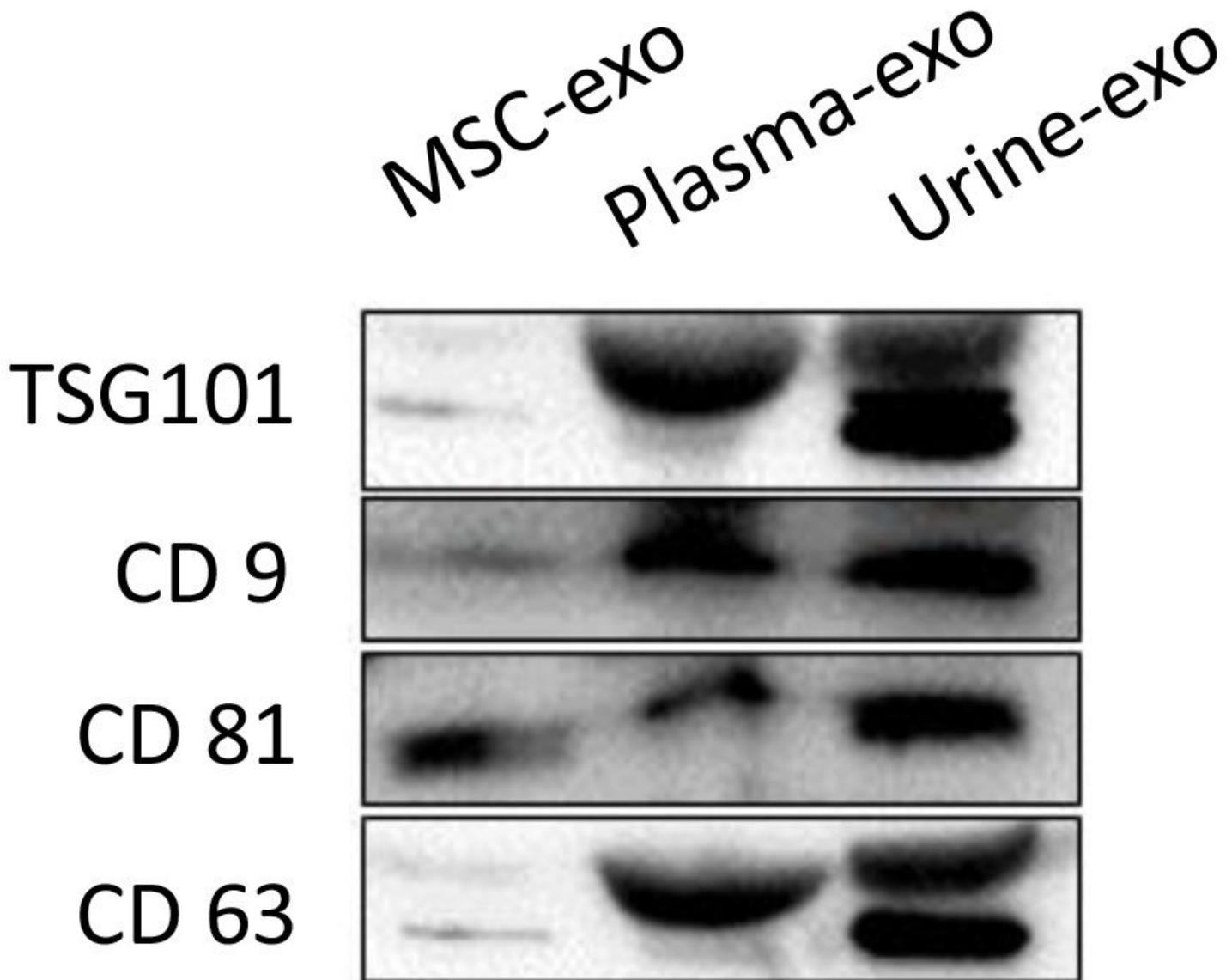


Figure 5

Western blotting analysis of feline samples-derived exosomes. Markers TSG101, CD9, CD63, and CD81 were positive expressed by all exosomes.

Supplementary Files

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