Extraction and quality evaluation of exosomes from joint effusion

Anmin Ruan
Beijing University of Chinese Medicine
Yueshan Yin
Beijing University of Chinese Medicine
Pu Chen
Beijing University of Chinese Medicine
Jun Zhou
Beijing University of Chinese Medicine
Xiaozhe Zhang
Beijing University of Chinese Medicine
Yufeng Ma
Beijing University of Chinese Medicine
Qingfu Wang (qingpu-wang@sohu.com)

Research article

Keywords: Joint effusion, Exosomes, Osteoarthritis, Separation

Posted Date: October 23rd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-49025/v2

Abstract

Objective: The aim of this study was to compare the quality and efficiency of exosomes extracted from knee joint effusion by different methods, laying a foundation for further research on exosomes from knee joint effusion.

Methods: To separate and extract joint exosomes by 8% polyethylene glycol (PEG) precipitation method, ultracentrifugation method (UC) and ultrafiltration with exclusion chromatography (SECF). Transmission electron microscopy (TEM) and nanoparticle tracing technology (NTA) were used to detect particle morphology and particle size, and Western Blot (WB) was used to detect marker proteins of granule surface (CD9, CD63, Flotillin-1 and calnexin).

Results: Three methods separated vesicle-like round particles from joint effusion successfully. The results of TEM show that the particles obtained by the three extraction methods are round or oval vesicles, the plasma membrane is intact, the size is different, and the diameter is distributed between 30 and 150 nm. Compared with SECF group, PEG group had more background particle impurities. and the broken vesicle fragments can be seen in the UC group; The results of NTA show that the main peaks of the three groups of particles are between 100-120nm, and the particle concentration is greater than 1×10^{10}/ml; The results of WB show that the protein expressions of CD9, CD63 and Flotillin-1 in the suspension extracted by the three methods were higher, the expression of calnexin protein in the PEG group was higher than the UC group and SECF group.

Conclusion: The three extraction methods can extract the exosomes of joint effusion successfully. The quality of exosomes obtained by the SECF method is relatively high, while the PEG precipitation method contains a small amount of impurity particles. UC method does not guarantee the integrity of exosomes. In summary, we recommend the SECF method to extract and isolate joint effusion exudates-derived exosomes when further studying the role of exosomes in the pathogenesis of knee osteoarthritis.

1. Introduction

Knee Osteoarthritis (KOA) is a chronic degenerative musculoskeletal disease. Its pathological changes mainly include cartilage damage, subchondral bone hyperplasia, and inflammation of soft tissues such as the periosteum of the knee. Clinical manifestations include pain, stiffness, Swollen joints and restricted mobility etc., seriously affect the patient's quality of life.[1] The research report shows that the global incidence of KOA is close to 4%, and more than 35% of middle-aged people suffer from this disease. The high incidence of the disease brings a heavy economic burden to people. [2-3] We know that there is still no effective treatment that can fundamentally reverse the pathological process of KOA. The current main treatment method is symptomatic treatment, and there are certain hidden dangers, and only knee replacement can be performed in the late stage. [4-5] It is precisely because the pathogenesis of KOA is still unclear that it leads to unsatisfactory efficacy. At the same time, imaging technology alone cannot diagnose early lesions of KOA, which is not conducive to early detection and treatment. For many years, scholars generally believe that cartilage injury is the main pathological mechanism of KOA. With the deepening of research, some scholars have found that synovial inflammation changes may play a driving role in the process of KOA lesions. [6-7] Knee effusion is a common pathological product in KOA lesions. Under pain, KOA imaging examination can find that more than 55% of patients have moderate to large amounts of inflammatory exudation. [8] In a healthy state, joint synovial fluid is a living environment for tissue structures such as cartilage and synovium in the joint, and can provide nutritional support for cartilage. In the inflammatory state, it becomes the medium of intra-articular signal communication, which has an important influence on the pathological process of KOA. However, the transmission mechanism of these signals needs further study.

Exosomes are the smallest extracellular vesicles. They are commonly found in biological fluids and have a lipid bilayer structure with a size of about 30-150 nm. The formation process can be summarized as the formation of multivesicular bodies by endocytosis of the plasma membrane (MVB), the MVB plasma membrane buds inward to form a subset containing multiple exosomes, MVB fuse with the cell membrane and release the exosomes. [9] Studies have found that exosomes can carry a large amount of biologically active substances such as nucleic acids, proteins, lipids, etc. from maternal cells to complete signal transmission between cells. [10] It is widely involved in pathological and physiological processes such as immune regulation, tissue damage repair, and metastasis promotion. [11] According to reports, exosomes in joint effusions carry a large number of cytokines, which can recruit inflammatory cells and cause further damage to cartilage. [12] In vitro experiments also found that joint exudate-derived exosomes can significantly stimulate M1 macrophages to release a variety of inflammatory cytokines, chemokines and metalloproteases. [13] These findings provide a new perspective for understanding KOA lesions. However, there are only a few articles that mention the study of joint fluid

exosomes, and there is no comprehensive comparison of extraction methods of joint fluid exosomes. Therefore, this study focuses on the analysis of the quality and efficiency of extracting exosomes of knee joint effusion through different techniques such as transmission electron microscopy (TEM), nanoparticle tracer analysis (NTA) and Western Blot (WB). The research on the mechanism of action in KOA lesions laid the foundation.

2. Materials And Methods

2.1 Main instruments and reagents

Transmission electron microscope H-7650 was purchased from Hitachi (Japan); Nanoparticle tracking analyzer was purchased from PMX (Germany); 0.22um filter was purchased from Millipore Corporation (USA); Size exclusion chromatography and filtration kit from Echo Biotech (China) Purchase; BCA protein concentration detection kit, cryostat centrifuge were purchased from Thermo Scientific (USA); anti-CD9, CD63, Flotillin-1 and calnexin primary antibodies were purchased from CST (Cambridge, Massachusetts, USA); Hyaluronidase and polyethylene glycol (PEG6000) were purchased from Sigma (USA).

2.2 Acquisition of joint effusion

According to the diagnostic criteria developed by the American College of Rheumatology, [14] 24 patients with primary KOA were collected from the outpatient clinic of the The Third Affiliated Hospital of Beijing University of Chinese Medicine from August 2019 to December 2019. All patients were randomly divided into 3 groups, PEG group, UC group and SECF group, 8 cases in each group. This study was approved by the Ethics Committee of the The Third Affiliated Hospital of Beijing University of Chinese Medicine, and all patients signed an informed consent form.

After sterilization, the knee joint was punctured along the lower patellar edge with a puncture needle in the outpatient operating room to extract joint effusion. The joint effusion sample was centrifuged at 3000 rpm and 4°C for 10 minutes to remove cell debris. In order to clarify the sample, the sample was treated with 2ug/ml hyaluronidase for 30min, then centrifuged at 10000×g for 20min, the supernatant was taken through a 0.22um filter, and then stored at -80°C.

2.3 Exosome isolation

2.3.1 PEG group The first step is to configure a concentration of 16% PEG6000 solution (16g PEG6000, 5.844g NaCl dissolved in 100mL ultrapure water), after filtering through a 0.22um filter, to be used. Mix the above solution with the thawed sample at a 1:1 ratio (to make the final PEG concentration 8%), and incubate overnight at 4°C. The next day at 12 000×g, centrifuge at 4°C for 30 min, discard the supernatant, add 100ul PBS to resuspend, and store at -80°C.

2.3.2 UC group, the samples were thawed at room temperature, vortexed and mixed, balanced, and placed in a 100 000×g centrifuge. After ultracentrifugation at 4° C. for 2 h, the supernatant was discarded, resuspended in PBS and placed in 100 000×g again. In a centrifuge, ultracentrifuge at 4℃ for 2h, discard the supernatant, add 100ul PBS to resuspend, and store at -80℃ until use.

2.3.3 SECF group The procedures used strictly follow the kit instructions. The resulting exosomes were resuspended in 100ul PBS, and then placed at −80°C and kept for use.

2.4 Characterization of exosomes

2.4.1 Transmission electron microscopy (TEM)

Take 10μL of the sample re-suspension solution on the sample-loaded copper mesh and let it stand at room temperature for 5 min. Use a filter paper to suck the liquid from the side. Add 10ul of 4% phosphotungstic acid solution onto the copper mesh. The filter paper absorbs the negative dye solution, after baking under the incandescent lamp, observe under the transmission electron microscope and image under 80KV.

2.4.2 Nanoparticle tracer analysis (NTA)

Nanometer tracing technology was used to detect the size and concentration distribution of vesicles. According to the corresponding operation process, set the parameters, the measurement time is 60s, the 500ul resuspended sample is appropriately diluted, and the

temperature is monitored throughout the measurement process. The samples were measured three times and analyzed using NanoSight NS300 NTA software.

2.5 Western Blot(WB)

After quantification of BCA protein, protein immunoblotting (WB) was used to detect the expression of the corresponding protein. The protein was separated by 12% SDS-PAGE gel electrophoresis. The electrophoresis environment was (80V, 30min; 120V, 60min); The protein was transferred to the PVDF membrane at 250mA constant flow ,5% non-fat milk was blocked at room temperature for 2 hours, and then added to the primary antibody and incubated at 4°C overnight, and TBST was washed 4 times for 5 min each time,added with corresponding secondary antibodies and incubated for 1 h at room temperature, and TBST was washed 4 times for 5 min each time. The ECL luminescence kit was developed, and the Bio-Rad fluorescence scanner scanned the image.

2.6 Statistical analyses

The data of this study are expressed as mean ± standard deviation (SD). GraphPad Prism 7.0 software was used for statistical analysis, and t-test or one-way analysis of variance was used for comparison between groups. $P<0.05$ is considered significant.

3. Results

3.1 Morphological observation and particle size analysis of exosomes

The TEM results show that all three separation methods can successfully extract exosomes from joint fluid. The circular or elliptical membranous microvesicle structure with a particle diameter in the range of 30-150nm contains a dense cloud-like substance, which is Exosome contents. From the background of TEM, the PEG group electron microscope is more blurred and there are more impurities, while the background of UC group and SECF group is cleaner and the impurities are least; from the perspective of the integrity of exosomes, PEG group and SECF group exocrine the body is relatively complete, and there are some fragments of exosome rupture in the UC group. As shown in Figure 1.

NTA results show that the main peaks of exosomes obtained by the three separation methods are all distributed between 100-120nm, as shown in Figure 2; the particle concentration of the three groups of exosomes is higher than 1×10^{10}/ml, the particle size distribution and accumulation the percentages are shown in Table 1. The cumulative percentages of the three groups of exosomes at 25-155 nm are 70.42% in the PEG group, 75.65% in the UC group, and 82.52% in the SECF group.

3.2 Determination of exosome protein concentration

The exosome protein concentration obtained by the three separation methods was determined according to the operating instructions of the BCA kit. As shown in Figure 3, the PEG histone concentration was the highest, and the SECF group was significantly different from the PEG and UC group ($P<0.05$).

3.3 Western blot verification of exosome markers

WB method was used to detect exosome biomarkers CD9, CD63, Flotillin-1 and Calnexin. The results showed that the expression of CD9, CD63 and Flotillin-1 were higher in the sample suspensions extracted by the three methods, and lower in the joint effusion. However, Calnexin was significantly expressed in the joint effusion and PEG groups, but was low in the UC and SECF groups (Figure 4).

4. Discussion

Extracellular vesicles (EVs) can be classified according to size and formation. They include multiple subgroups, mainly microvesicles (MVs) and exosomes. MVs are membranous extracellular vesicles with diameters ranging from 100 to 1000 nm, while the range of exosomes is only 30-150 nm. [15] Since winning the Nobel Prize in Physiology or Medicine for the study of cellular vesicle transport regulation in 2013, exosomes have become research hotspots in many fields. With the deepening of research, many functions of exosomes have been gradually discovered. In recent years, exosomes have been functionally studied in tumor growth and migration, immune regulation, disease diagnosis, repair of tissue damage, and drug transport. Significant progress. [16] The biological

composition of exosomes is gradually becoming clear. Because exosomes are derived from the endocytosis of cellular MVB, they contain heat shock proteins (Hsp70 and Hsp90), membrane transport and fusion proteins (GTPases), annexin and flotillin) And tetrapeptides (CD9, CD63, CD81 and CD82). [15] Among these proteins, heat shock proteins, annexin and Rab family proteins are often used as body surface markers of exosomes. With the development of research, the separation and identification of exosomes is an important step in determining the success of the experiment. The current separation methods of exosomes include ultracentrifugation, density gradient centrifugation, immunomagnetic bead method, precipitation method and various Commercial kits, etc. [17] As for exosome detection technology, currently recognized transmission electron microscopy technology, nanoparticle tracking analysis, Western blot, dynamic light scattering, mass spectrometry, etc. [18] In 2018, the guidelines for vesicle research published by the International Extracellular Vesicle Society pointed out that the main characteristics of current exosomes obtained by separation technology are high recovery and low specificity,intermediate recovery and intermediate specificity, and low recovery and high specificity. [19] However, there are only a few literatures that mention the research on exosomes derived from joint effusions. There is no uniform standard for the isolation and identification of joint effusion exosomes. The guidance of our comprehensive guide selects three representative separation methods, And identify the quality and efficiency of exosomes obtained by the three techniques.

Previously, it was generally believed that the main lesion of KOA was cartilage injury. In fact, the inflammation of the synovium played a huge role in the pathological process of KOA, and joint effusion was the pathological product of synovial inflammation. Wang [6] believed that synovial inflammation may affect articular cartilage damage through the innate immune system and aggravate the course of OA. Joint effusion as an intra-articular environment, in which exosomes carry a large number of inflammatory factors and participate in the signal transmission of pathological mechanisms. [12] Therefore, how to isolate and extract exosomes from joint effusion is of great significance for further study of the pathological mechanism of KOA.

Current research generally believes that the role of exosomes in knee joints can be divided into two categories: one is to promote the development of inflammation and accelerate joint degradation; the other is to relieve inflammation and repair damaged cartilage. Kato [20] stimulated chondrocytes by exosomes secreted by normal synovial fibroblasts and IL-1β-stimulated synovial fibroblasts, and the results showed that the latter could significantly up-regulate the expressions of MMP-3, MMP-13, IL-1, adamTS-5 and vascular endothelial growth factor in articular chondrocytes, and significantly down-regulate the expressions of COL2A1 and ACAN.Gao[13] have found that the exosomes of joint fluid contain a large amount of inflammatory factors and chemokines, and the content of inflammatory factors in the synovial exosomes of patients with different grades of KOA (Kellgren-Lawrence) is also different. Moreover, exosomes derived from KL3-4 can significantly inhibit the proliferation of chondrocytes compared to exosomes derived from KL1-2. In vitro experiments also found that joint exudate-derived exosomes can significantly stimulate M1 macrophages to release a variety of inflammatory cytokines, chemokines and metalloproteases [13]. Wang [21] intervene in damaged cartilage tissue by extracting exosomes secreted by mesenchymal stem cells (ESC-MSCs), which can significantly increase type II collagen and promote the repair of damaged cartilage. Tofiño-Vian[22] found that exosomes secreted by adipose-derived mesenchymal stem cells down-regulate the expression of aging factors in OA osteoblasts induced by IL-1β, which indicates that exosomes can play a role in OA osteoblasts Anti-inflammatory response and anti-oxidative stress. However, the mechanism of synovial fluid exosomes and exosomes involved in KOA lesions is still unclear, and we still need to further study them.

In this study, we selected PEG precipitation method, UC method and SECF method to isolate joint fluid exosomes, using TEM and NTA to detect the morphology and particle size of the obtained exosomes, and WB method to detect three Positive exosome surface marker proteins CD9, CD63 and Flotillin-1 and negative marker protein Calnexin. The results showed that the positive protein was highly expressed in the three groups of exosomes, and the negative protein was significantly expressed in the joint effusion group and the PEG group, indicating that the exosomes extracted from the PEG group were mixed with more impurities, while the UC group and the impurities in the SECF group are relatively small, which is consistent with the observation of the background impurity particles in the PEG group under TEM. However, under the TEM, the UC group clearly observed broken fragments, which is consistent with the results reported in the previous literature. NTA results showed that the enrichment of the vesicle diameter within 25-155nm was highest in SECF group (82.52%), while that of PEG group and UC group (70.42% and 75.65%) was relatively low. BCA results show that the concentration of SECF histones is relatively low. Comprehensive experimental results SECF method is better than PEG group and UC group in separating exosomes.

In summary, we believe that the SECF method is more suitable for the separation of joint fluid exosomes. This result lays a foundation for the study of the role of joint fluid exosomes in the pathogenesis of KOA.

Abbreviations

NTA=Nanoparticle tracer analysis; KOA= Knee Osteoarthritis; PEG=Polyethylene glycol;SECF=ultrafiltration with exclusion chromatography;TEM=Transmission electron microscopy; UC=Ultracentrifugation; WB= Western Blot; MVB= multi-vesicular body biogenesis.

Declarations

Acknowledgements

The authors thank all participants for their contributions in the study.

Authors' contributions

Research design:Anmin Ruan,Pu Chen,Qingfu Wang;experiment implementation: Anmin Ruan,Pu Chen, Jun Zhou, Yueshan Yin;Methodology: Anmin Ruan,Pu Chen, Jun Zhou,Qingfu Wang;Data curation: Anmin Ruan, Yufeng Ma;

Writing-original draft: Anmin Ruan;Writing – review & editing: Anmin Ruan, Qingfu Wang.

Funding

This study was funded by the General Program of National Natural Science Foundation of China (no.81373662).

Availability of data and materials

All data are fully available without restriction

Ethics approval and consent to participate

This article was approved by the Ethics Committee of the Third Affiliated Hospital of Beijing University of Chinese Medicine.

Consent for publication

Yes.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] School of Graduates, Beijing University of Chinese Medicine,Beijing,China

[2]Department of Orthopaedic Surgery, Beijing University of Chinese Medicine Third Affiliated Hospital, No. 51, Xiaoguan Street, Anding Gate, ChaoyangDistrict, Beijing, China.

References

1. Onuora S. Osteoarthritis. Molecular imaging detects activated macrophages. Nat Rev Rheumatol. 2016;12(6):313.
2. Cross M, Smith E, Hoy D,et al. The global burden of hip and knee osteoarthritis: estimates from the global burden of disease 2010 study. Ann Rheum Dis. 2014;73(7):1323–30.
3. Neogi T. The epidemiology and impact of pain in osteoarthritis. Osteoarthritis cartilage. 2013;21(9):1145–53.

4. Mendel OI, Luchihina LV, Mendel W. Aging and osteoarthritis; Chronic nonspecific inflammation as a link between againg and osteoarthritis. Adv Gerontol. 2015;28(2):274–83.
5. Deyle GD, Allen CS, Allison SC, et al. Physical Therapy versus Glucocorticoid Injection for Osteoarthritis of the Knee. N Engl J Med. 2020;382(15):1420–9.
6. Wang H, Wang Q, Yang M, et al. Histomorphology and innate immunity during the progression of osteoarthritis: Does synovitis affect cartilage degradation? Journal of cellular physiology. 2018;233(2):1342–58.
7. .Ozeki N, Muneta T, Koga H, et al. Not single but periodic injections of synovial mesenchymal stem cells maintain viable cells in knees and inhibit osteoarthritis progression in rats. Osteoarthritis cartilage. 2016;24(6):1061–70.
8. Hill CL, Gale DG, Chaisson CE, et al. Knee effusions, popliteal cysts, and synovial thickening: association with knee pain in osteoarthritis. J Rhuematol. 2001;28(6):1330–7.
9. Milane L, Singh A, Mattheolabakis G, et al. Exosome mediated communication within the tumor microenvironment. Journal of controlled release: official journal of the Controlled Release Society. 2015;219:278–94.
10. Pérez-González R, Gauthier SA, Kumar A, et al. A Method for Isolation of Extracellular Vesicles and Characterization of Exosomes from Brain Extracellular Space. Methods in molecular biology. (Clifton NJ). 2017;1545:139–51.
11. De Gregorio C, Díaz P, López-Leal R, et al. Purification of Exosomes from Primary Schwann Cells, RNA Extraction, and Next-Generation Sequencing of Exosomal RNAs. Methods in molecular biology (Clifton, NJ) 2018;1739:299–315.
12. Miyaki S, Lotz MK. Extracellular vesicles in cartilage homeostasis and osteoarthritis. Curr Opin Rheumatol. 2018;30(1):129–35.
13. Gao K, Zhu W, Li H, et al. Association between cytokines and exosomes in synovial fluid of individuals with knee osteoarthritis. Modern rheumatology. 2020;30(4):758–64.
14. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis rheumatism. 1988;31(3):315–24.
15. Vlassov AV, Magdaleno S, Setterquist R, et al. Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. Biochim Biophys Acta. 2012;1820(7):940–8.
16. Hurwitz SN, Rider MA, Bundy JL, et al. Proteomic profiling of NCI-60 extracellular vesicles uncovers common protein cargo and cancer type-specific biomarkers. Oncotarget. 2016;7(52):86999–7015.
17. Ha D, Yang N, Nadithe V. Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges. Acta pharmaceutica Sinica B. 2016;6(4):287–96.
18. Batrakova EV, Kim MS. Using exosomes, naturally-equipped nanocarriers, for drug delivery. Journal of controlled release: official journal of the Controlled Release Society. 2015;219:396–405.
19. 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. Journal of extracellular vesicles. 2018;7(1):1535750.
20. Kato T, Miyaki S, Ishitobi H, et al. Exosomes from IL-1β stimulated synovial fibroblasts induce osteoarthritic changes in articular chondrocytes. Arthritis research therapy. 2014;16(4):R163.
21. Wang Y, Yu D, Liu Z,et al. Exosomes from embryonic mesenchymal stem cells alleviate osteoarthritis through balancing synthesis and degradation of cartilage extracellular matrix. Stem Cell Res Ther. 2017;8(1):189.
22. Tofiño-Vian M, Guillén MI, Pérez D, Caz MD, et al. Extracellular Vesicles from Adipose-Derived Mesenchymal Stem Cells Downregulate Senescence Features in Osteoarthritic Osteoblasts. Oxidative Med Cell Longev. 2017;2017:7197598.

Tables

Table 1 The particle size distribution and cumulative percentage of exosomes

Size (nm)	PEG			UC			SECF		
	Number (10^6/ml)	Percent(%)	Cumulative percent(%)	Number (10^6/ml)	Percent(%)	Cumulative percent(%)	Number (10^6/ml)	Percent(%)	Cumulative percent(%)
5	0	0.00	0.00	1	0.10	0.10	0	0.00	0.00
15	5	0.32	0.32	1	0.10	0.20	3	0.25	0.25
25	5	0.32	0.64	6	0.61	0.81	10	0.83	1.08
35	15	0.96	1.59	3	0.30	1.11	7	0.58	1.67
45	19	1.21	2.80	11	1.11	2.22	11	0.92	2.58
55	31	1.98	4.78	13	1.31	3.54	18	1.50	4.08
65	63	4.02	8.80	30	3.03	6.57	63	5.25	9.33
75	97	6.18	14.98	46	4.65	11.21	80	6.66	15.99
85	122	7.78	22.75	72	7.27	18.48	115	9.58	25.56
95	130	8.29	31.04	112	11.31	29.80	118	9.83	35.39
105	155	9.88	40.92	108	10.91	40.71	137	11.41	46.79
115	132	8.41	49.33	98	9.90	50.61	129	10.74	57.54
125	112	7.14	56.47	88	8.89	59.49	110	9.16	66.69
135	99	6.31	62.78	76	7.68	67.17	89	7.41	74.10
145	66	4.21	66.99	52	5.25	72.42	78	6.49	80.60
155	64	4.08	71.06	40	4.04	76.46	36	3.00	83.60
165	54	3.44	74.51	33	3.33	79.80	30	2.50	86.09
175	45	2.87	77.37	25	2.53	82.32	28	2.33	88.43
185	43	2.74	80.11	26	2.63	84.95	20	1.67	90.09
195	46	2.93	83.05	18	1.82	86.77	19	1.58	91.67
205	50	3.19	86.23	19	1.92	88.69	10	0.83	92.51
215	34	2.17	88.40	16	1.62	90.30	12	1.00	93.51
225	29	1.85	90.25	20	2.02	92.32	16	1.33	94.84
235	28	1.78	92.03	14	1.41	93.74	8	0.67	95.50
245	18	1.15	93.18	14	1.41	95.15	8	0.67	96.17
255	11	0.70	93.88	9	0.91	96.06	9	0.75	96.92
265	15	0.96	94.84	7	0.71	96.77	7	0.58	97.50
275	13	0.83	95.67	3	0.30	97.07	5	0.42	97.92
285	14	0.89	96.56	6	0.61	97.68	0	0.00	97.92
295	9	0.57	97.13	2	0.20	97.88	5	0.42	98.33
305	14	0.89	98.02	1	0.10	97.98	2	0.17	98.50
315	5	0.32	98.34	2	0.20	98.18	3	0.25	98.75
325	3	0.19	98.53	2	0.20	98.38	3	0.25	99.00
335	4	0.25	98.79	2	0.20	98.59	1	0.08	99.08

345	4	0.25	99.04	3	0.30	98.89	4	0.33	99.42
355	1	0.06	99.11	2	0.20	99.09	1	0.08	99.50
365	1	0.06	99.17	0	0.00	99.09	1	0.08	99.58
375	1	0.06	99.24	3	0.30	99.39	0	0.00	99.58
385	2	0.13	99.36	1	0.10	99.49	1	0.08	99.67
395	2	0.13	99.49	1	0.10	99.60	0	0.00	99.67
405	1	0.06	99.55	1	0.10	99.70	0	0.00	99.67
415	4	0.25	99.81	0	0.00	99.70	2	0.17	99.83
425	2	0.13	99.94	1	0.10	99.80	1	0.08	99.92
435	0	0.00	99.94	0	0.00	99.80	1	0.08	100.00
445	1	0.06	100.00	0	0.00	99.80	0	0.00	100.00
455	0	0.00	100.00	1	0.10	99.90	0	0.00	100.00
465	0	0.00	100.00	0	0.00	99.90	0	0.00	100.00
475	0	0.00	100.00	1	0.10	100.00	0	0.00	100.00

Figures

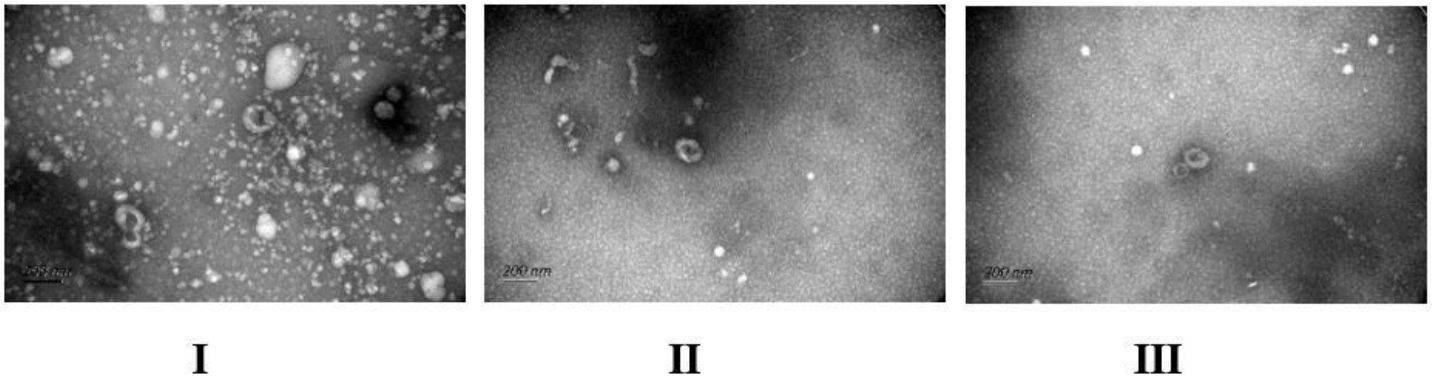

Figure 1

Observed the morphology and size of exosomes separated by three separation techniques under electron microscope. the PEG group, the UC group, and the SECF group.

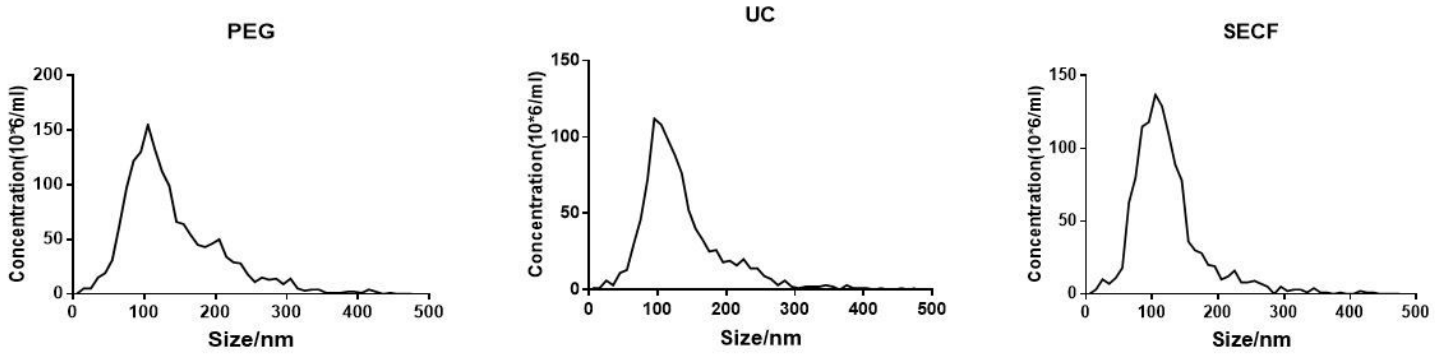

Figure 2

Nano-tracer technology detects the size distribution of exosomes obtained by three separation techniques.

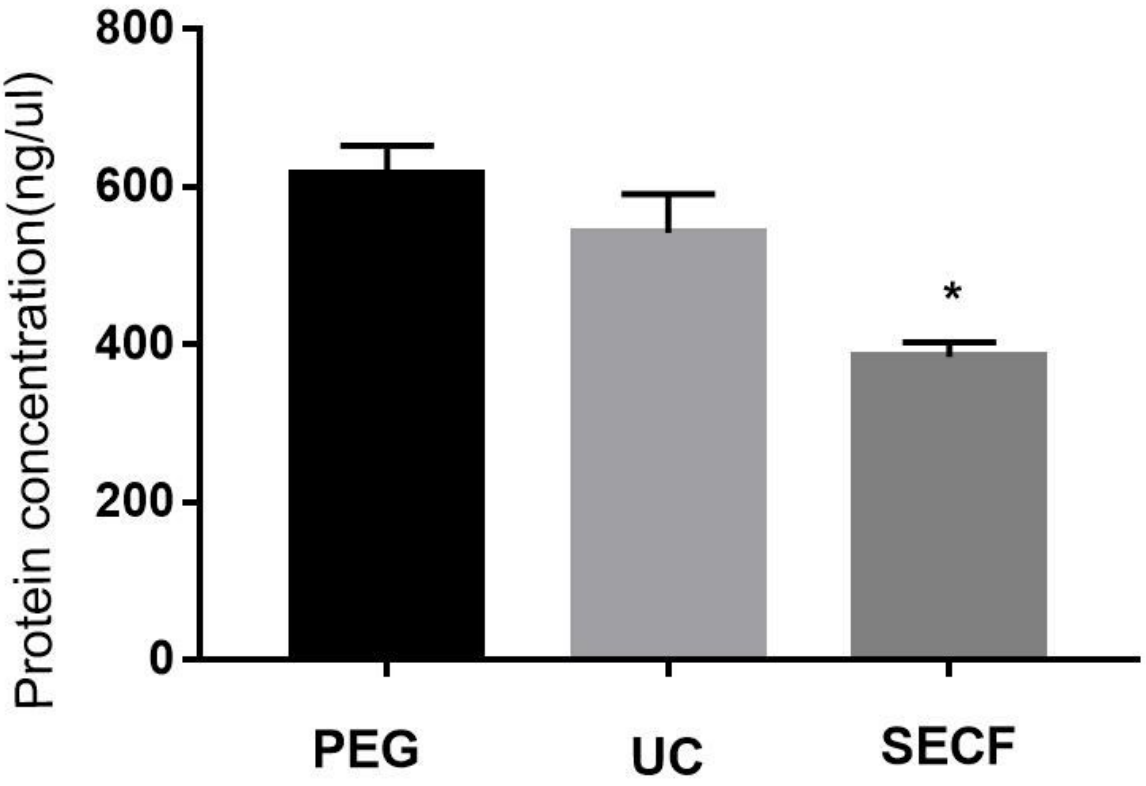

Figure 3

The protein concentration of exosomes obtained by three separation methods. *Compared with PEG group and UC group, P<0.05

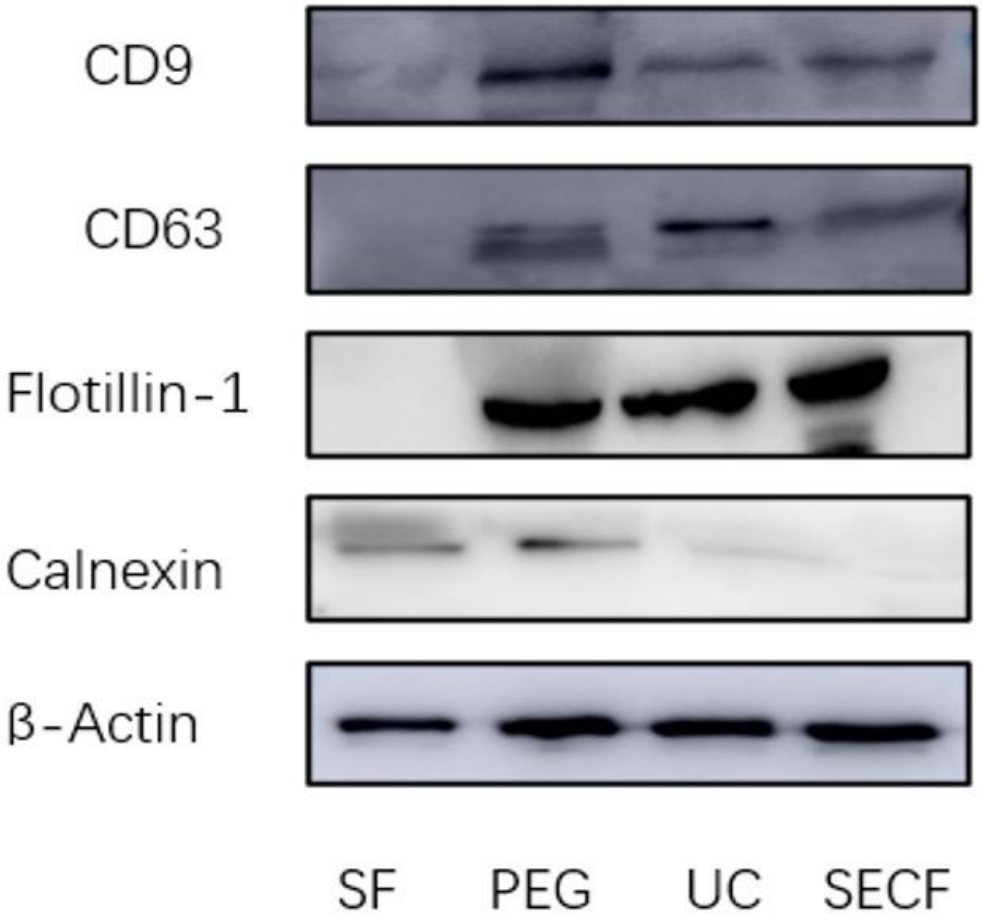

Figure 4

The expression of exosome body surface marker protein obtained by three separation methods