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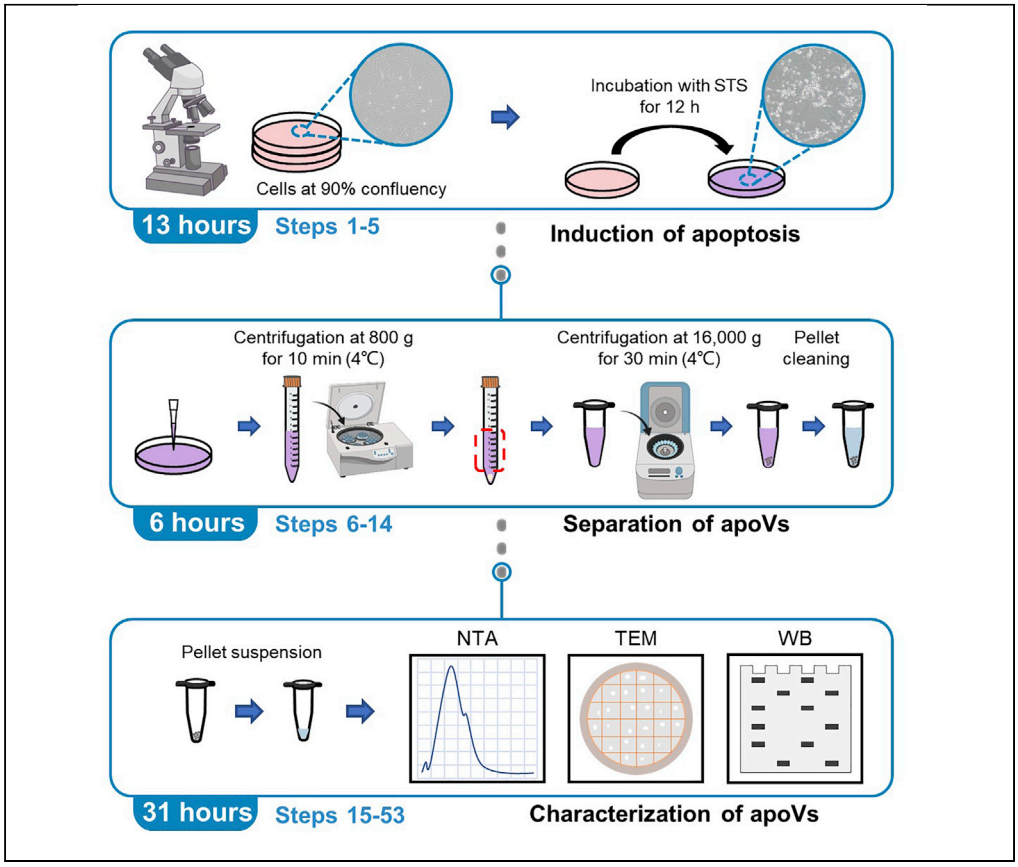
Protocol for differential centrifugation-based separation and characterization of apoptotic vesicles derived from human mesenchymal stem cells



Apoptotic vesicles (apoVs) are specific extracellular vesicles generated during apoptosis and play important roles in multiple physiological and pathophysiological settings. Here, we present a protocol using differential centrifugation to separate apoVs from human mesenchymal stem cells (MSCs) after induction of apoptosis. We describe how to characterize apoV size and morphology by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM), and determination of specific biomarker expression by immunoblotting. Our protocol will be useful for preparing apoVs for further functional analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Presentation of an approach for inducing MSC apoptosis *in vitro*

Description of apoV separation from apoptotic MSCs by differential centrifugation

Determination of apoV morphology and size distribution by NTA and TEM

Assessment of apoV yield and specific biomarkers by immunoblotting

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Protocol for differential centrifugation-based separation and characterization of apoptotic vesicles derived from human mesenchymal stem cells

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SUMMARY

Apoptotic vesicles (apoVs) are specific extracellular vesicles generated during apoptosis and play important roles in multiple physiological and pathophysiological settings. Here, we present a protocol using differential centrifugation to separate apoVs from human mesenchymal stem cells (MSCs) after induction of apoptosis. We describe how to characterize apoV size and morphology by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM), and determination of specific biomarker expression by immunoblotting. Our protocol will be useful for preparing apoVs for further functional analysis.

For complete details on the use and execution of this protocol, please refer to Zheng et al. (2021).

BEFORE YOU BEGIN

Each day, billions of cells undergo apoptosis within the body, constituting a crucial activity for homeostasis maintenance (Medina et al., 2020; Morioka et al., 2019). Of note, the generated apoptotic vesicles (apoVs) perform unique functions that canonical extracellular vesicles (EVs), typically released from viable cells, cannot achieve. One of these functions is the delivering of a variety of featured cargos, including DNA, proteins and RNA. For example, apoVs have been found to encapsulate proteins that can regulate apoptosis, such as GSN (Kothakota et al., 1997; Springer et al., 1999). It has also been confirmed that the danger signal protein called HMGB1, is exclusively within apoptotic cell-released EVs (Tucher et al., 2018). In addition, MPO and Arg1, which are contained within apoptotic neutrophil-EVs, may modulate T-cell responses (Shen et al., 2017). Notably, apoVs are becoming a research hotspot for cell-free therapies in a wide spectrum of pathological conditions to restore tissue homeostasis and promote damage repair (Caruso and Poon, 2018; Grant et al., 2019; Kakarla et al., 2020). Here we demonstrate apoV separation protocol from mesenchymal stem cells (MSCs) that emerge as one of the most promising sources for cell therapy (Zheng et al., 2021).

Institutional permissions

The use of human umbilical cord MSCs (hUCMSCs) are approved by the Ethics Committee of the Fourth Military Medical University with informed consent of the donors.

Cell culture

Timing: 1 h for plating cells; 48 h of culturing cells prior to the induction of apoptosis

Note: This is an optimized separation protocol for apoVs from adherent cells. We have cultured primary hUCMSCs and used the third passage of hUCMSCs as an example. This protocol can also be applied to suspension cells with relevant modification (Dou et al., 2020).

1. Plate cells in the 100-mm tissue culture dishes and incubate the cells in a humidified 37°C, 5% CO_2 incubator, which will be 80%–90% confluent in 48 h. In the case of hUCMSCs, 1.0 × 10^6 cells are plated in each of the dishes with 10 mL culture medium containing 10% fetal bovine serum (FBS).

Note: We suggest using cells before the fifth passages. Here we use the third passage of hUCMSCs.

Note: From our experience, this approach typically yields about 30 μg apoVs/100-mm dish. To ensure the overall apoV yield is sufficient for subsequent analyses, it is recommended that at least four 100-mm dishes be used and the procedure start when cells reach 80%–90% confluency. However, the exact amount of apoVs that can be collected needs to be determined by preliminary experiments.

Alternatives: More apoVs can be separated at one time by using 150-mm dishes or even larger size.

Reagents and centrifuge preparation

Timing: 20 h, reagents can be prepared on the day or before the day of the procedure

2. Centrifuge 200 mL FBS in 38.6 mL ultracentrifuge tubes at 160,000 × *g* at 4°C for 16 h.
3. Inject the FBS through a 0.22 μm filter for sterilization with a 10 mL syringe and store at −20°C.

Note: The purpose of ultracentrifugation is to prepare sterilized EV-free FBS.

4. Prepare apoptosis-inducing solution.
   a. Preparation of 2 mM staurosporine (STS) (Belmokhtar et al., 2001): dissolve 250 μg STS (in powder form) in 276.5 μL dimethyl sulfoxide (DMSO) (calculated according to MW=466.5) by pipetting.
   b. Preparation of 250 μM STS: take 100 μL from 2 mM STS and mix thoroughly with 700 μL DMSO.
   c. Preparation of 500 nM STS: take 100 μL from 250 μM STS and mix thoroughly with 45 mL culture medium and 5 mL EV-free FBS.

Note: STS should be stored away from light. Solid STS can be stored at −20°C for up to 2 years, while liquid STS can be stored at 4°C for up to 1 month or −20°C for up to 6 months.

5. Place the rotors in centrifuges and cool them to 4°C.
6. Filtrate phosphate buffered saline (PBS) through a 0.22 μm filter and store at 4°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-β-Actin (1:1,000)	Cwbio	Cat#CW0096M; RRID: AB_2665433
Rabbit polyclonal anti-Caspase-3 (1:1,000)	Cell Signaling Technology	Cat#9662; RRID: AB_331439

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse monoclonal anti-Flotillin (1:1,000)	PTM BIO	Cat#PTM-5369
Rabbit monoclonal anti-Lamin B1 (1:1,000)	PTM BIO	Cat#PTM-5495
Mouse monoclonal anti-Caveolin-1 (1:1,000)	Santa Cruz Biotechnology	Cat#sc-53564; RRID: AB_628859
Peroxidase affiniPure goat anti-Mouse IgG (H+L) (1:500)	Jackson ImmunoResearch	Cat#115-035-003; RRID: AB_10015289
Peroxidase affiniPure goat anti-Rabbit IgG (H+L) (1:500)	Jackson ImmunoResearch	Cat#115-035-003; RRID: AB_10015289
Chemicals, peptides, and recombinant proteins		
Minimum essential medium α (MEM α)	Gibco	Cat#12571063
FBS	Corning	Cat#35-081-CV
Penicillin-Streptomycin (100×), Sterile	MCE	Cat#HY-K1006
PBS (1 ×)	Corning	Cat#21-040-CVC
Trypsin-EDTA (0.25%)	Gibco	Cat#25200056
DMSO	MP Biomedicals	Cat#196055; CAS: 67-68-5
STS	Enzo	Cat#ALX-380-014-M001; CAS: 62996-74-1
2% Uranyl acetate	Electron Microscopy China	Cat#GZ02625; CAS: 541-09-3
Cell lysis buffer for Western and IP	Beyotime	Cat#P0013
SDS-PAGE loading buffer (5 ×)	Cwbio	Cat#CW0027S
Bovine serum albumin (BSA)	MP Biomedicals	Cat#FC0077; CAS: 9048-46-8
Omni-PAGE™Hepes-Tris Gels (4%–20%, 10 wells)	Epizyme	Cat#LK206
Tris	Thermo Fisher Scientific	Cat#17926; CAS: 77-86-1
UltraPure glycine	Thermo Fisher Scientific	Cat#15527013; CAS: 56-40-6
SDS	Thermo Fisher Scientific	Cat#28312; CAS: 151-21-3
Methanol	Cell Signaling Technology	Cat#13604S; CAS: 67-56-1
Tween-20	MP Biomedicals	Cat#194724; CAS: 9005-64-5
Tris buffered saline with Tween-20 (TBST) (10 ×)	Cell Signaling Technology	Cat#9997
Amersham enhanced chemiluminescence (ECL) detection reagent	Cytiva	Cat#RPN2235
Critical commercial assays		
Pierce Bicinchoninic Acid (BCA) Protein Assay Kit	Thermo Fisher Scientific	Cat#23227
Biological samples		
Human umbilical cord (female, 25–35 years old)	This study	N/A
Experimental models: Cell lines		
Primary human umbilical cord MSCs	This study	N/A
Other		
Cell culture dish	Thermo Fisher Scientific	Cat#150466
Optima™XPN-100	Beckman Coulter	A94469
Centrifuge Tubes	Beckman Coulter	354263
SW 32 Ti Swinging-Bucket Rotors	Beckman Coulter	18U5478
Minisart® Syringe Filter	Sartorius Stedim	16541-K
Centrifuge 5702R	Eppendorf	Cat#5703000390
A-4-38 centrifuge rotor	Eppendorf	Cat#5702720003
Centrifuge 5430R	Eppendorf	Cat#5428000090
FA-45-30-11 centrifuge rotor	Eppendorf	Cat#5418707005
Transmission Electron Microscope (TEM)	Hitachi – Science & Technology	H-7650
Double loop qualitative filter paper	Whatman	99-102-090
ZetaView® BASIC NTA - Nanoparticle Tracking Video Microscope PMX-120	Particle Metrix	N/A
Gel imaging system	Tanon	4600

MATERIALS AND EQUIPMENT

10 × SDS electophoresis buffer		
Reagent	Final concentration	Amount
Tris	250 mM	30.3 g
Glycine	1,920 mM	144 g
SDS	1%	10 g
ddH_2O	N/A	To 1 L
Total	**N/A**	**1 L**

Note: 10 × SDS electophoresis buffer can be stored at room temperature (25°C) for up to 3 months.

Note: Before use, the 1 × SDS electophoresis buffer should be obtained by diluted with ddH_2O.

10 × transfer buffer		
Reagent	Final concentration	Amount
Tris	250 mM	30.3 g
Glycine	1,920 mM	144 g
ddH_2O	N/A	To 1 L
Total	**N/A**	**1 L**

Note: 10 × transfer buffer can be stored at room temperature (25°C) for up to 3 months.

1 × transfer buffer		
Reagent	Final concentration	Amount
10 × transfer buffer	10%	100 mL
ddH_2O	70%	700 mL
Methanol	20%	200 mL
Total	**N/A**	**1 L**

Note: 1 × transfer buffer can be stored at room temperature (25°C) for up to 2 weeks.

STEP-BY-STEP METHOD DETAILS

Induction of apoptosis

Timing: 30 min for exchanging the complete culture medium with apoptosis-inducing solution; 12 h for apoptosis induction

This major step describes how to induce apoptosis of adherent cells (Figure 1). Given the length of apoptosis induction, we recommend that this step be carried out at the night before conditioned media collection and apoV separation.

⚠ CRITICAL: This section should be performed under sterile conditions in a biosafety cabinet.

1. Put the apoptosis-inducing solution and PBS into a 37°C water bath for pre-heating.
2. Aspirate the complete culture medium.
3. Add 5 mL pre-warmed PBS to rinse the cells for 10 s, and then aspirate the PBS.

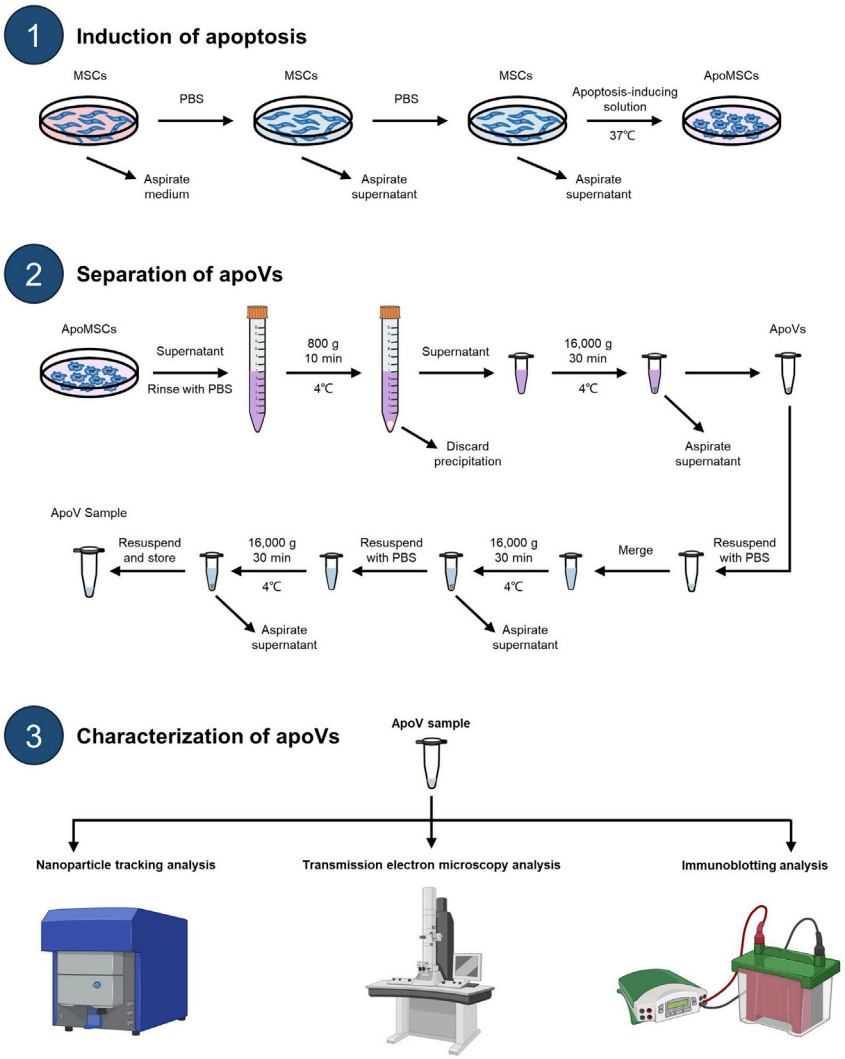

Figure 1. Schematic illustration of the protocol

The mesenchymal stem cells (MSCs) undergo apoptosis induction (1) and the conditioned media is collected for separation of apoVs *via* gradient centrifugation (2). After (1) and (2), apoVs are characterized by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and immunoblotting. Some figure elements were created with BioRender.com.

4. Repeat step 3 once.

   Note: All rinsing steps should be performed gently so as to avoid cell detachment.

5. Add 5 mL pre-warmed apoptosis-inducing solution to each dish and incubate the cells in a humidified 37°C, 5% CO_2 incubator for 12 h.

   Note: We recommend approximately 12 h of apoptosis induction for MSCs so that ~90% cells can undergo apoptosis and release apoVs for separation. Moreover, the maximum duration of apoptosis induction is recommended not to exceed 16 h.

   ⚠ CRITICAL: The duration of apoptosis induction depends on the sensitivity of the cells to the apoptosis-inducing solution. Pre-experiment is needed to determine the induction time length for a specific type of cells.

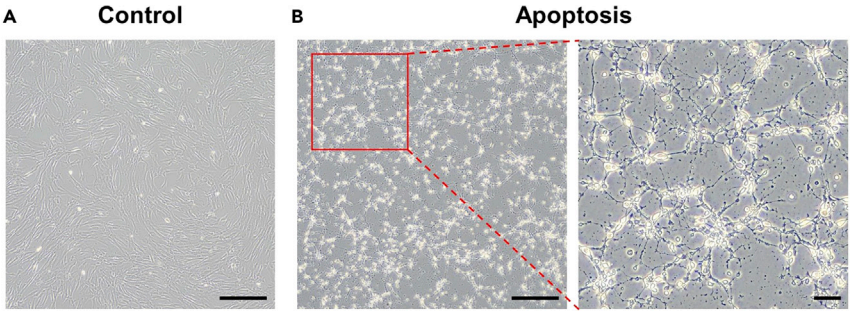

Figure 2. Microscopy images of the cells undergoing apoptosis

(A and B) Microscopy images comparing the morphology of the cells undergoing apoptosis (B) versus the control (A). The morphological characteristics of apoptosis include intercellular connection disappearance, cell shrinkage, membrane blebbing and fragmentation into membrane-bound vesicles. Scale bars, 100 μm (left and middle) and 20 μm (right).

Separation of apoVs

Timing: 1 h for collecting conditioned media; 5 h for separation of apoVs

This major step describes the isolation of apoVs from the conditioned media *via* differential centrifugation (Figure 1).

⚠ CRITICAL: This section should be performed in a sterile biosafety cabinet.

6. When ~90% cells undergo apoptosis (Figure 2), flush the cells thoroughly with pipetting the conditioned media to detach all apoptotic cells.

   Note: It is recommended that the cells be observed under an inverted microscope for evaluation of apoptosis. The morphological characteristics of apoptosis include intercellular connection disappearance, cell shrinkage, membrane blebbing and fragmentation into membrane-bound vesicles (Figure 2).

7. Transfer the supernatant, together with detached cells, to a sterile 15 mL centrifuge tube on ice.
8. Rinse each dish with 2 mL cold PBS, and transfer the PBS to the same centrifuge tube (Each tube contains 7 mL of liquid).
9. Place the centrifuge tubes in a centrifuge.
10. Centrifuge the samples for 10 min at 800 × *g* at 4°C to remove the cells and cell debris (Figure 3A).

    Note: All centrifugation steps should be performed at 4°C. The samples and reagents need to be kept on ice.

    ⚠ CRITICAL: Before centrifugation, centrifugal tubes with liquid in the same volume should be placed in a symmetrical position in the centrifuge.

11. Collect the sample supernatant with a pipette and discard the precipitation containing cells and cell debris (Figure 3A).
    a. Divide the supernatant into five 1.5 mL EP tubes.
    b. Place the EP tubes in a centrifuge.
    c. Centrifuge the tubes at 16,000 × *g* for 30 min at 4°C (Figure 3B).

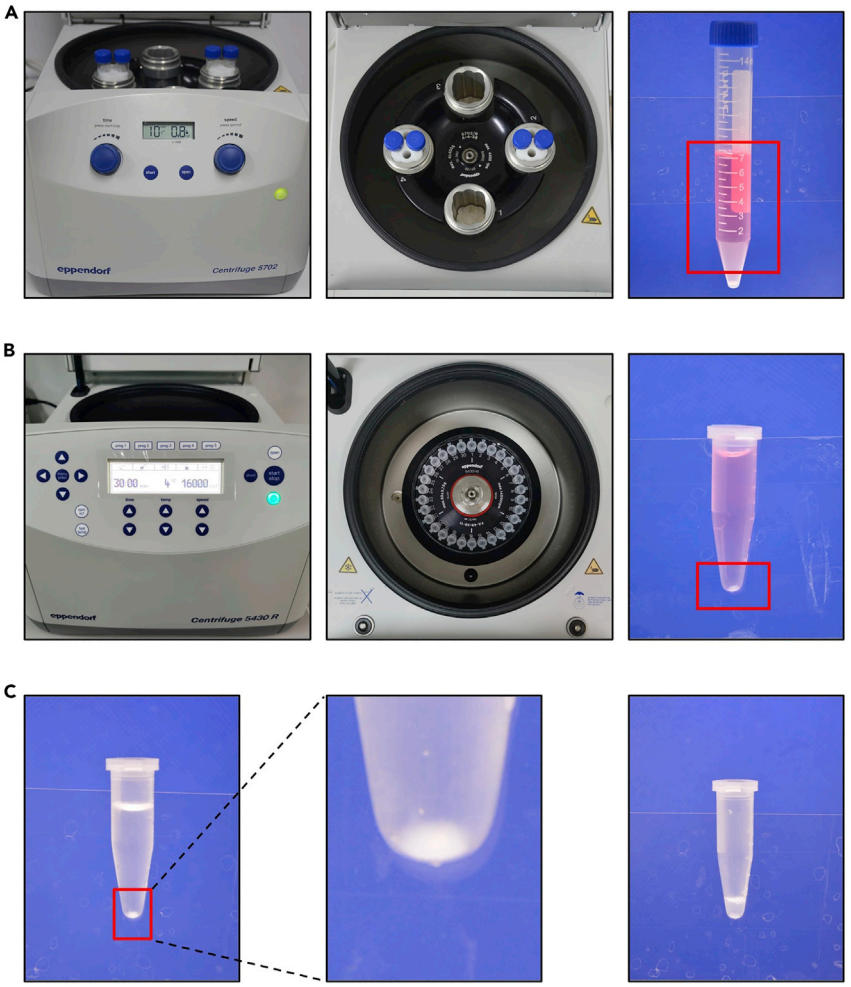

Figure 3. Representative images showing the steps of gradient centrifugation for separating apoVs

(A) Setting of the centrifuge, placement of the 15 mL centrifuge tubes in the rotor, and collection of the supernatant that contains apoVs.

(B) Setting of the centrifuge, placement of the 1.5 mL EP tubes in the rotor, and the appearance of the apoV pellets after centrifugation.

(C) The appearance of the apoV pellets after washing by PBS, which are re-suspended with the corresponding solvent.

Note: When collecting the supernatant, 5 mm supernatant in height above the precipitate can be retained to prevent precipitation from being sucked up.

⚠ CRITICAL: The EP tubes should be placed with the cap uniformly oriented towards the center of the rotor, so that the separated apoVs will remain at the bottom of a specific side of the EP tubes.

12. Aspirate the supernatant.
    a. Add 200 μL cold PBS to each tube and gently resuspend the pellet for sample cleaning by pipetting.
    b. Merge the suspension of every five EP tubes into one EP tube.
    c. Centrifuge the tubes at 16,000 × *g* for 30 min at 4°C (Figure 3C).

Note: When removing the supernatant, the EP tubes can be tilted to make the supernatant parallel to the precipitation so as to avoid unnecessary loss of apoVs (Methods video S1).

13. Aspirate the supernatant.
    a. Add 1 mL cold PBS to each tube and gently resuspend the pellet for sample cleaning by pipetting.
    b. Centrifuge the tubes at 16,000 × *g* for 30 min at 4°C.
14. Aspirate the supernatant, and re-suspend the precipitate with the corresponding solvent (Figure 3C). The precipitation is the apoVs. (If the yield of apoVs is low or unstable please read troubleshooting Problem 1–2).
    a. Re-suspend the precipitate separated from one dish with 50 μL cold PBS and store at 4°C for nanoparticle tracking analysis (NTA) analysis.
    b. Re-suspend the precipitate separated from one dish with 50 μL cold PBS and store at 4°C for TEM analysis.
    c. Re-suspend the precipitates separated from two dishes with 50 μL lysis buffer, incubate on the ice for 30 min and store at −80°C for Western blotting (WB) analysis.

Characterization of apoVs: Nanoparticle tracking analysis

Timing: 3 h

Note: The NTA protocol below is specifically for the microscope model PMX-120. NTA measurement using different machine will need different protocol.

NTA is used for measuring the amount and size of apoVs by capturing the movies of apoV particles that move under Brownian motion (Figure 1).

15. Take 10 μL of the 50 μL sample, and dilute to 10 mL with PBS.

Note: The samples should be maintained on ice during transportation.

16. Use a sterile 10 mL syringe to inject at least 5 mL ddH_2O to flush the ZetaView® BASIC NTA machine channel at a constant speed (0.5 mL/s to 1 mL/s), until the number of particles displayed on the detection interface is less than 10 (Methods video S2).
17. Prepare calibration solution (diluted at 1:250,000).
    a. Dilute 1 μL calibration solution provided with the NTA machine to 1 mL with ddH_2O.
    b. Take 100 μL and dilute to 25 mL with ddH_2O.

Note: The calibration solution needs to be freshly prepared immediately before use.

18. Use a sterile 10 mL syringe to inject at least 5 mL calibration solution to flush the machine channel (Methods video S3).

Note: Maintain a uniform and stable flow during the injection process so as to avoid the formation of bubbles.

19. When the number of particles displayed on the detection interface is between 50-400 (preferably around 200), run the calibration program (Methods video S4).
20. Repeat step 16 to clean the machine channel (Methods video S5).
21. Use a sterile 10 mL syringe to inject at least 5 mL buffer solution (PBS) to flush the machine channel at a constant speed (0.5 mL/s to 1 mL/s), until the number of particles displayed on the detection interface is less than 10 (Methods video S6).
22. Slowly inject the sample into the machine channel (at least 2 mL) using a sterile 1 mL syringe (Methods video S7).

Note: Before injection, hold the syringe upright and tap the side gently to drain the bubbles in the sample.

Note: For samples with an optimum particle concentration, the number of particles displayed on the detection interface should be between 50-400 (preferably around 200).

⚠ CRITICAL: If the reading is too high, repeat step 16 immediately to avoid particles retention at the channel wall. Use PBS to further dilute the sample, and when the number of particles displayed on the detection interface is less than 10, repeat step 21–22.

23. Conduct particle capture analysis and generate analysis report. (If contaminants are found in NTA please read troubleshooting Problem 3).
24. Repeat step 21 to clean the sample in the channel.
25. Repeat step 16 to clean the PBS in the channel.
26. Use a sterile 10 mL syringe to inject at least 10 mL air at a constant speed (0.5 mL/s to 1 mL/s) to remove the ddH_2O in the channel.

Note: If multiple samples need to be analyzed, carry out step 20–23 above in sequence.

Characterization of apoVs: Transmission electron microscope analysis

Timing: 4 h

TEM is used for analyzing the size and morphology of apoVs (Figure 1).

27. Drop 10 μL of the 50 μL sample onto a copper mesh and incubate for 10 min at 25°C. Remove excess liquid with filter paper.

Note: In order to ensure that single particles can be observed, the sample concentration should be adjusted and the suspension should be clear without obvious turbidity. The sample used here is diluted at 1:1,000. However, the dilution of different cells should be determined by the preliminary experiment.

Note: Before dropping, mix the suspension well and pipette from the middle of the liquid.

⚠ CRITICAL: Pay attention to dropping the sample to the front side of the copper mesh.

28. Rinse the copper mesh in 100 μL sterile ddH_2O and remove excess liquid with filter paper. Repeat this step for another two times.

Note: Use electron microscope tweezers to clamp the edge of the copper mesh, and immerse the front side downward into ddH_2O to rinse it.

29. Negatively stain the copper mesh by immersing the front side in 2% uranyl acetate solution for 1 min at 25°C, and remove the excess liquid with filter paper.
30. Repeat step 28.
31. Remove excess liquid with filter paper and put the copper mesh on a new filter paper to dry it under an incandescent lamp for 2 min or at room temperature (25°C) for 10 min.
32. Observe the copper mesh under a TEM operating at 100 kV and imaged with a digital camera. (If contaminants are found in TEM analysis please read troubleshooting Problem 3.) (If the morphology of apoVs is not typical please read troubleshooting Problem 4).

Characterization of apoVs: Immunoblotting analysis

Timing: 24 h

The protein concentration of apoVs is quantified *via* the BCA assay and the expression levels of specific markers for apoVs are detected *via* WB (Figure 1).

Note: Methods for determination of protein concentration can be selected according to laboratory conditions, such as using Qubit protein quantification assay (Hu et al., 2021). This protocol only provides a relatively simple and common determination method.

33. Centrifuge the lysates (the sample obtained at step 14c) at 13,000 × *g* for 5 min at 4°C.
34. Transfer the supernatant to a sterile 1.5 mL EP tube.

Note: Place all the samples on the ice during the operation.

35. Preparation of 0.5 mg/mL BSA standard solution.
36. Dilute 0.5 mg/mL BSA standard solution with normal saline to a serial concentration.
37. Add 20 μL of standard solution at different concentrations into each standard well of the 96-well plate, and add 2 μL sample and 18 μL normal saline into each sample well of the 96-well plate.

Note: Be careful when adding to the microplate so as to avoid the formation of bubbles.

38. Mix BCA reagent A and BCA reagent B in a 50 : 1 ratio to make the needed amount of working solution.

Note: It is recommended that the working solution be prepared right before use.

39. Add 200 μL working solution to each well, and incubate the 96-well plate in an incubator at 37°C for 30 min.
40. Put the 96-well plate into the microplate reader and measure the optical density (OD) value at the wavelength of 562 nm.
41. Establish the standard curve according to the OD data and calculate the protein concentration of each sample.
42. According to the results, dilute the samples to 1 μg/μL with normal saline and 5 × loading buffer.
43. Seal the tubes with parafilm and heat them at 95°C for 5 min.
44. Load equal protein mass per sample onto a precast 4%–20% (wt/vol) TGX stain-free gel.

Note: Choose the gel percentage according to the molecular weight of the proteins of interest.

45. Run the SDS-PAGE at 80 V for the concentrated gel and 120 V for the separation gel.

Note: The purpose of this step is to resolve the proteins based on their sizes.

Note: The running time may vary according to the equipment or percentage of gel.

46. Transfer the gel to a polyvinylidene difluoride (PVDF) membrane using a wet transfer system for 120 min at 200 mA.

Note: The PVDF membrane needs to be rinsed shortly (20 s) in methanol immediately before transferring.

47. Block the membrane in 1 × TBST containing 5% BSA for 2 h at 25°C with gentle shaking.

Note: The 1 × TBST is obtained by diluting the 10 × TBST with ddH_2O.

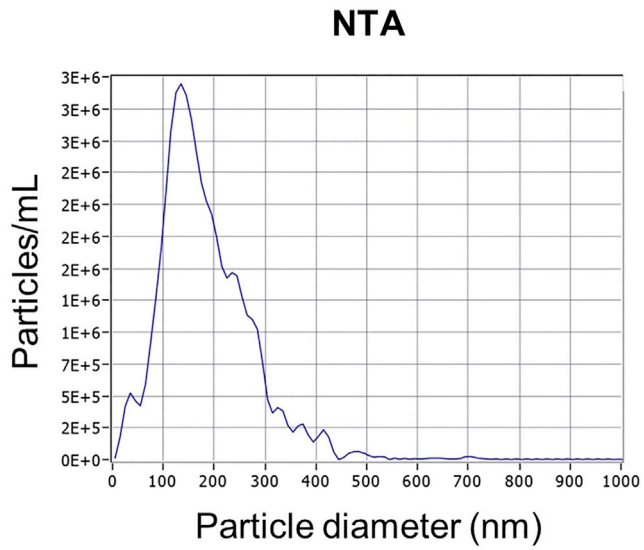

Figure 4. Representative NTA image showing the size distribution of apoVs

48. Incubate the membrane with the primary antibodies diluted in 1 × TBST at 4°C for 12–16 h with gentle shaking.
    a. The anti-Caspase-3 antibody that detects both full length Caspase-3 and Cleaved Caspase 3 is used at a 1:1,000 dilution as specific markers for apoptotic products (Nicholson et al., 1995).
49. Wash the membrane in 1 × TBST for 10 min at 25°C on a shaker. Repeat this step for another two times.
50. Incubate the membrane with the secondary antibodies diluted in 1 × TBST for 1 h at 25°C on a shaker.
51. Wash the membrane in 1 × TBST for 10 min at 25°C on a shaker. Repeat this step for another two times.
52. Incubate the membrane with the reagents of an ECL kit for 1 min away from light.
53. Detect the protein bands by a gel imaging system. (If low signal or no signal of Cleaved Caspase-3 is detected by immunoblotting please read troubleshooting problem 5).

EXPECTED OUTCOMES

Here, we provide a detailed protocol for the separation and characterization of apoVs produced by MSCs. From our experience, this approach typically yields about 30 μg apoVs/100-mm dish. The size distribution of apoVs analyzed by NTA shows that the diameter range of apoVs is 50–500 nm, with majority peak at 100–200 nm (Figure 4). Besides, the morphological characteristics of apoVs as cup-shaped membranous vesicles (Zheng et al., 2021) are verified by TEM observation (Figure 5). Moreover, immunoblotting analysis demonstrates that Cleaved Caspase-3, a typical apoptosis marker (Nagata, 2018), accumulates in apoVs, with expression of plasma membrane markers, Caveolin-1 and Flotillin (Durcin et al., 2017), and the cytoskeleton marker, β-actin, but without detection of the nuclear envelop protein, Lamin B1, as a negative control (Figure 6).

LIMITATIONS

The yield and quality of apoVs depends largely on the apoptosis inducing process. Since the sensitivity to apoptosis inducer varies among different cell types, the precise induction concentration and induction time need to be confirmed by pre-experiment. Moreover, during the apoptosis inducing process, the researcher needs to closely observe the morphological changes of cells in order to verify the appropriate timepoint for collecting conditioned media. It should be noted that excessive

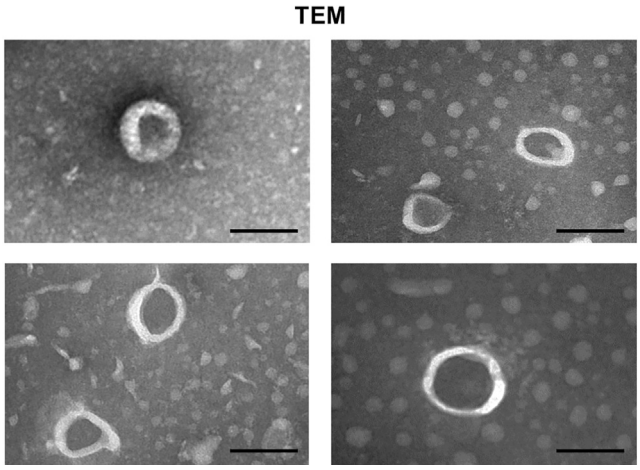

Figure 5. Representative TEM image showing the morphology of apoVs
Scale bar, 100 nm.

apoptosis can lead to secondary necrosis (Rogers et al., 2017; Silva, 2010), in which the cell membrane breaks down and the contents are released, which will contaminate the separated apoVs. The apoptosis versus secondary necrosis can be distinguished by Annexin V/PI or 7-AAD staining, if needed (Akiyama et al., 2012).

Centrifugation at 800 g for 10 min applied in this study removes most of the larger contaminants including cells and cell debris, but also results in loss of large apoVs. However, this protocol is advantageous on the separation of small apoVs (50–400 nm) from cultured cells without ultracentrifugation, which is thus simple, convenient and feasible for subsequent morphological, functional and therapeutic studies (Zheng et al., 2021). Notable, for apoVs separated from tissue or body fluid samples, the centrifugal speed or separation method must be adjusted according to the component complexity of these samples and the size of target apoVs in practical operation (Atkin-Smith and Poon, 2017; Atkin-Smith et al., 2017).

The purity of apoVs separated by this differential centrifugation approach is relatively low, due to the aggregation of EVs as well as contaminating proteins (Yang et al., 2020). Besides, the subpopulation of apoVs cannot be distinguished (Bordanaba-Florit et al., 2021). To further separate the apoVs subclasses, it is recommended that this approach be performed in combination with other methods such as immunoaffinity separation (Koliha et al., 2016) and high-sensitivity fluorescence-activated sorting (Kondratov et al., 2020).

The current protocol has been optimized for adherent cells. As for separating apoVs from suspension cells, when transferring the complete culture medium to apoptosis-inducing solution, the suspension cells need to be initially collected *via* extra-centrifugation step (Dou et al., 2020).

TROUBLESHOOTING

Problem 1

Low yield of apoVs (step 14).

Potential solution

Scaling up the cell number by preparing more plates will increase the overall yield of apoVs ("cell culture"). Besides, the apoptosis condition is a critical factor for apoV yield. Thus, the concentration of apoptosis-inducing solution and the length of induction time needs to be optimized *via* pre-experiment (steps 5 and 6).

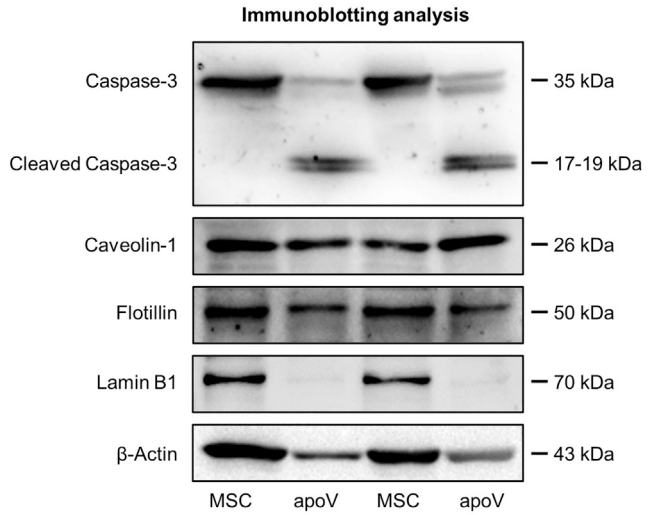

Figure 6. Immunoblotting analysis of the whole cell lysates and apoVs

An equal amount of protein was loaded for all the samples and analyzed for the presence of apoptosis marker, Caspase-3/Cleaved Caspase-3; the plasma membrane markers, Caveolin and Flotillin; the cytoskeleton marker, β-actin; and the nuclear envelop protein, Lamin B1, as a negative control.

Problem 2

The yield of apoVs is unstable (step 14).

Potential solution

The density and condition of cells when inducing apoptosis is important for the amount of released apoVs. We recommend cells at early passages be used and the procedure start when cells reach 80%–90% confluency ("cell culture").

Problem 3

Contaminants in TEM and NTA (steps 23 and 32).

Potential solution

In the characterization of apoVs, the cleanliness of PBS is also crucial. We suggest making it on the spot. Or use commercial PBS within 1 week of unsealing.

Problem 4

The morphology of apoVs is not typical when observed *via* TEM (step 32).

Potential solution

The concentration of apoV sample is crucial for the TEM analysis. Thus, adjust the sample concentration so as to make it within an appropriate range (step 27). In addition, since long-term storage will affect the morphology of EVs, it is recommended to perform TEM observation within a short time after apoV separation (step 14) (Gelibter et al., 2022).

Problem 5

Low signal or no signal of Cleaved Caspase-3 is detected by immunoblotting (step 53).

Potential solution

As has been stated above, the concentration of apoptosis-inducing solution and the length of induction time are critical for the efficiency of apoV secretion (step 5). Before the collection of conditioned media, the cells should be observed under an inverted microscope for evaluation of apoptosis (step

6). In addition, poor signal may result from problems during the WB process. We recommend using fresh primary and secondary antibodies, increasing the concentration of primary antibody and extending the incubation time (steps 48 and 50).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yan Jin (yanjin@fmmu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze any dataset or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101695.

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AUTHOR CONTRIBUTIONS

D.C., Z.Z., and K.Z. performed the experiments and wrote the manuscript. F.J. performed the experiments. C.Z. and Y.J. designed and supervised the study, and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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