Scaled Isolation of Mesenchymal Stem/Stromal Cell-Derived Extracellular Vesicles

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Mesenchymal stem/stromal cells (MSCs) provide therapeutic effects in many diseases. Contrary to initial hypotheses, they act in a paracrine rather than a cellular manner. To this end, extracellular vesicles (EVs) have been found to mediate the therapeutic effects, even when harvested from MSC-conditioned cell culture supernatants. Lacking self-replicating activity and being so small that MSC-EV preparations can be sterilized by filtration, EVs provide several advantages as therapeutic agents over cellular therapeutics. At present, methods allowing EV preparation from larger volumes are scarce and regularly require special equipment. We have developed a polyethylene glycol−based precipitation protocol allowing extraction of EVs from several liters of conditioned medium. MSC-EVs prepared with this method have been successfully applied to a human graft-versus-host disease patient and to several animal models. Although the method comes with its own limitations, it is extremely helpful for the initial evaluation of EV-based therapeutic approaches. Here, we introduce the technique in detail and discuss all critical steps. © 2020 The Authors.

Basic Protocol 1: Preparation of MSC-conditioned medium for scaled MSC-EV production
Basic Protocol 2: PEG precipitation OF MSC-EV from MSC-conditioned medium

Keywords: EVs • exosomes • large-scale preparation • MSCs

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INTRODUCTION

At the turn of the millennium, mesenchymal stem/stromal cells (MSCs) were reported as multipotent cells (Pittenger et al., 1999). Considering them as an allogenic stem cell source for cell replacement strategies, several groups studied their interaction with immune cells. Quickly, it was demonstrated that MSCs were able to suppress pro-inflammatory immune responses (Bartholomew et al., 2002; Di Nicola et al., 2002). Due to their beneficial effects in various preclinical animal models, they were quickly translated into the clinic, either intended as cellular replacement or as immunomodulating

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cells. Up to now, MSCs have been applied in more than 1000 different clinical trials for the treatment of numerous different diseases (Heldring, Mager, Wood, Le Blanc, & Andaloussi, 2015). Despite positive effects in various settings, the MSCs were barely detected in affected tissues, resulting in the hypothesis that they mainly act via their secretome rather than in a direct cellular manner (Caplan & Correa, 2011; Caplan & Dennis, 2006). Indeed, it was quickly demonstrated using the examples of an acute kidney injury model and a myocardial infarction model that MSCs exert their therapeutic effects via small extracellular vesicles (EVs), such as exosomes and microvesicles, having diameters of up to 200 nm. Indeed, an array of different head-to-head studies confirmed that preparations that are highly enriched for such vesicles exert comparable therapeutic activities as their parental MSCs (Bruno et al., 2009; Doeppner et al., 2015; He et al., 2012).

Compared to cellular transplants, EV products provide some significant advantages. Due to their small sizes, MSC-EV preparations can be processed by filtration through filters with 0.22-μm pores, which is considered as sterilization. In contrast to cells, EVs are not self-replicating and thus lack any endogenous tumor-formation potential. Furthermore, their overall handling is much easier than that of cellular products. All of these features are essential requirements for an off-the-shelf product (Lener et al., 2015).

Although we are not aware of the exact mode of action, it appears MSC-EV preparations act multimodally. Among other activities, they can modulate pro-inflammatory into regulatory immune responses, presumably an essential requirement for regenerative processes (Börger et al., 2017; Giebel & Hermann, 2019).

A bottleneck in preparing MSC-EVs for clinical applications is the fact that liters of conditioned medium (CM) need to be prepared for the treatment of an individual patient. Classically, EVs have been prepared by differential centrifugation procedures, in which the initial volume is reduced during ultracentrifugation (see Current Protocols article: Thery, Amigorena, Raposo, & Clayton, 2006). Since even the largest rotors cannot process more than 400 ml in a single, typically 2-hr run, a challenge in the field is to find other, more effective methods of volume reduction. All available methods have their own limitations, such as purity, scalability, and time/cost factor (Watson et al., 2018). Currently, tangential flow filtration (TFF) is increasingly discussed as an effective method for volume reduction (Nordin, Bostancioglu, Corso, & El Andaloussi, 2019). However, this requires specific equipment, and optimization for clinical grade production is still ongoing. Here, we present a scalable, easy-to-handle and cost-effective procedure for the preparation of EVs from larger MSC-CM volumes, which could potentially be used for the clinical-grade production of MSC-EVs (Figs. 1 and 2).

Facing the situation of a treatment-refractory graft-versus-host disease (GvHD) patient for whom no additional treatment options existed in 2011, we considered treating her with MSC-EVs and had to address the challenge of effectively extracting small EVs (<200 nm) from more than 4 L of MSC-CM. As EVs share many physical properties with viruses, especially lentiviruses (Nguyen, Booth, Gould, & Hildreth, 2003), which can effectively be prepared from larger supernatants by polyethylene glycol (PEG) precipitation, we established and optimized a PEG precipitation protocol for small EVs (Ludwig et al., 2018). Subsequently, the protocol was used to prepare MSC-EVs for the successful treatment of the GvHD patient (Kordelas et al., 2014). Notably, as demonstrated at the example of an ischemic stroke mouse model, PEG-prepared MSC-EVs showed comparable effects as their parental a MSCs (Doeppner et al., 2015).

PEG precipitation allows the scaled preparation of functional EVs

Briefly, after obtaining MSC-CM (Basic Protocol 1), the protocol (Basic Protocol 2) starts with a 2000 × *g* centrifugation step (Fig. 1). It is followed by a mid-speed centrifugation

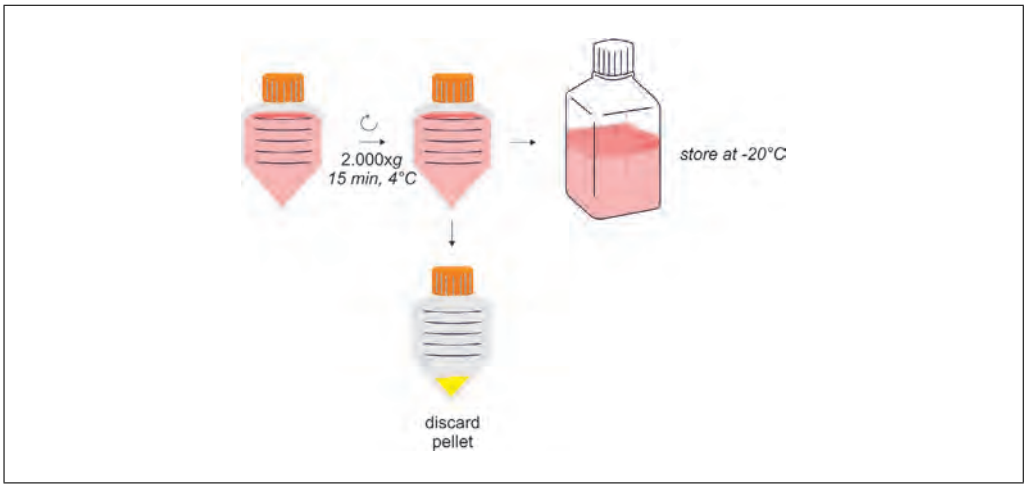

Figure 1 Harvested MSC-CM is collected and spun down for 15 min at 2000 × *g*, 4°C. Supernatant is transferred into storage containers and placed at −20°C until processing.

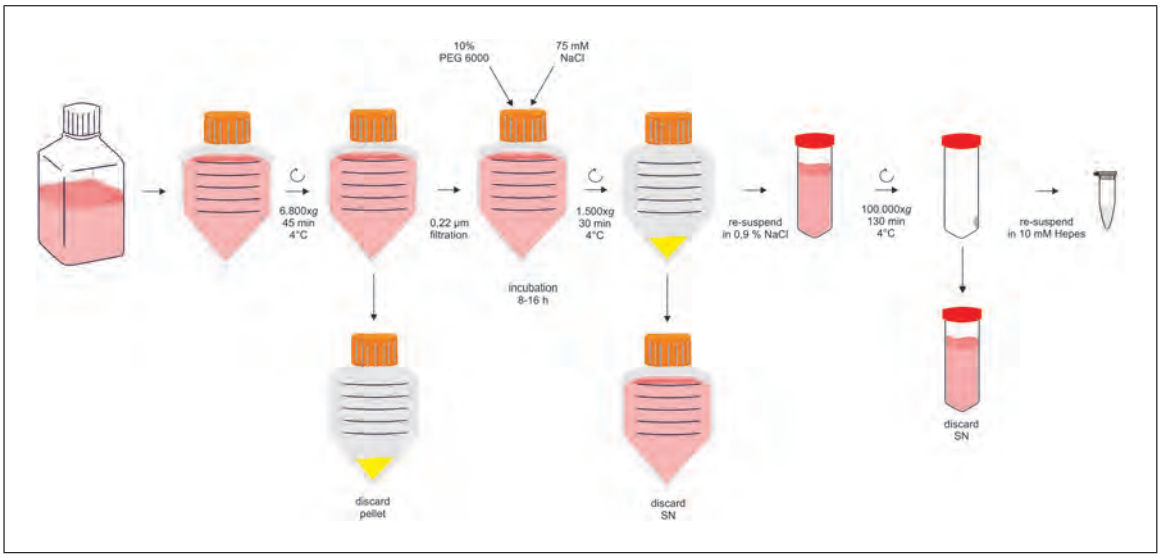

Figure 2 Harvested MSC-CM is pooled and spun down for 45 min at 6800 × *g*, 4°C. The supernatant (SN) is filtered through a 0.22-µm pore-size filter, adjusted to concentrations of 10% PEG 6000 and 75 mM NaCl, and incubated for 8-16 hr at 4°C. The suspension is centrifuged for 30 min at 1500 × *g*, 4°C. The pellet is resuspended and washed in NaCl. EVs are reprecipitated by ultracentrifugation for 130 min at 100,000 × *g*, 4°C. The obtained EV pellet is resuspended in buffer and stored at −80°C until use.

(depending on the maximum rotation speed of the rotor: 6800 to 10,000 × *g*) and a filtration step to successively remove contaminating cells, larger debris, and EVs that are larger than 200 nm (Fig. 2). Next, PEG precipitation occurs overnight, and the small EVs are pelleted at 1500 × *g*. To remove the PEG effectively, the precipitated EVs are washed with 0.9% NaCl and are pelleted again by ultracentrifugation. Thereafter, they are resuspended in the buffer of choice, e.g., HEPES or 0.9% NaCl, and stored −80°C until use (Fig. 2).

PREPARATION OF MSC-CONDITIONED MEDIUM FOR SCALED MSC-EV PRODUCTION

BASIC PROTOCOL 1

For scaled MSC-EV production, MSCs derived from bone marrow aspirates of healthy individuals are raised in 4-layered tissue culture stacks in human platelet lysate (hPL)−supplemented cultivation medium. During the expansion process, CM is harvested every 48 hr in a cumulative manner. Of note, to obtain optimal cell

expansion required for optimal MSC-EV yield, we do not remove the EVs from the hPL-supplemented cultivation medium.

NOTE: All steps should be performed under sterile conditions.

Materials

Mesenchymal stem/stromal cells (MSCs; see appropriate articles in *Current Protocols in Stem Cell Biology*)
Cultivation medium (see recipe)
1× trypsin/EDTA solution (see recipe) or other suitable enzymatic detachment reagent
Phosphate-buffered saline (PBS; Gibco, cat. no. 70013-016)
0.4% trypan blue (Sigma, cat. no. T8154)

4-layered tissue culture stack (Polystyrene Cell Factory System; ThermoFisher Scientific, cat. no. 140360)
Microscope
500-ml centrifugation tubes (Corning, cat. no. 431123)
Neubauer chamber (hemocytometer)
Medium-speed centrifuge (e.g., Avanti J26XP with rotor JS-5.3, Beckman Coulter)

Additional reagents and equipment for cell culture, including counting viable cells by trypan blue exclusion (see Current Protocols article: Phelan & May, 2015)

Cultivation of MSCs for scaled CM collection

1. Seed MSCs at a density of around 800 to 1500 MSCs/cm^2 in a 4-layered tissue culture stack containing 400 ml cultivation medium. Document the number of seeded cells.

   Proliferating MSCs, independent of their origin (including bone marrow, adipose tissue, perinatal tissue), should be raised in serum, or, if xeno-free settings are intended, in hPL-supplemented medium (10%). Because of their low protein content, which is not compatible with the PEG precipitation, chemically defined media are not applicable.

2. Examine the confluence of the cells with a microscope daily.

   Determine the approximate confluence of the cells; train participating co-workers to reach comparable outcomes.

3. Change medium when cells have reached ∼50% confluency.

   Discard the first harvest of CM. Because of low cell number, it contains only a low number of EVs.

4. Collect the CM every 48 hr from MSCs showing 50% to 90% confluency.

   MSCs normally need to be split once a week before reaching 100% confluency. Typically, collection of CM can be performed up to two times before each split.

5. For passaging, detach MSCs with suitable enzymatic detachment reagents and harvest the cells. For example, use 150 ml of 1× trypsin/EDTA for 5 min, at 37°C. Stop reaction by adding an equal volume of fresh cultivation medium; transfer to a 500-ml centrifugation tube, and spin down for 5 min at 900 × g.

   To save conditioned medium for use in Basic Protocol 2, do not stop the enzymatic reaction with CM. No enzymes should reside in the CM being used for the EV isolation, so it is critical to only use fresh cultivation medium prepared as in Reagents and Solutions for this step.

6. Resuspend pelleted cells in an appropriate volume of cultivation medium or PBS and determine the number of viable cells by trypan blue staining in a Neubauer chamber (hemocytometer; see Current Protocols article: Phelan & May, 2015).

7. Calculate the cell equivalents from which the CM was harvested:

$$n\,(t_z) = n_0 \cdot e^{k \cdot t_z}$$

$n(t_z)$: cell number during medium harvest at a time point t_z; n_0: number of originally seeded cells.

$$k = ln\left(\frac{n_1}{n_0}\right) \cdot \frac{1}{t_1}$$

n_1: cell number during passaging at time point t_1.

As an example, if 2×10^6 cells were seeded on day 0, and 4×10^7 cells are harvested on day 8 with the cells in exponential growth, the cell equivalent of day 6 CM is calculated as follows:

$$k = ln\left(\frac{4 \times 10^7}{2 \times 10^6}\right) \cdot \frac{1}{8}$$

$$k = 0.374$$

$$n\,(t_6) = 2 \times 10^6 e^{0.374 \cdot 6}$$

$$n\,(t_6) = 18.91 \times 10^6$$

Thus, at day 6, the CM can be considered to contain EVs from $\sim 1.89 \times 10^7$ cell equivalents.

Preparation of MSC-CM

8. Transfer the collected MSC-CM to a new centrifuge tube.

The size of the centrifuge tubes should be chosen based on the amount of cell culture. For small scale (up to 150 ml CM), 50-ml tubes are sufficient. For larger volumes (>150 ml) bigger tubes (e.g., 500-ml centrifugation tubes) are mandatory.

9. Centrifuge the CM 15 min at $2000 \times g$, 4°C.

10. Transfer supernatant to sterile storage containers.

The pellet contains detached cells and larger debris and should be discarded; only use the supernatant.

The supernatant can be stored either in centrifuge tubes or empty medium bottles.

11. Store CM at −20°C until further processing.

Supernatants of CM can be stored for up to several months at −20°C; however, freezing and thawing cycles should be avoided.

PEG PRECIPITATION OF MSC-EV FROM MSC-CONDITIONED MEDIUM

BASIC PROTOCOL 2

The protein content of the CM is critical; thus, only serum- or hPL-supplemented media should be used. CM is harvested in in a cumulative manner, and can be pooled after thawing.

Conditioned medium (CM; Basic Protocol 1)
3.75 M NaCl (see recipe)
50% (w/v) PEG 6000 (see recipe)
0.9% sodium chloride (B. Braun, cat. no. 151072)
Medium or buffer of choice: e.g.,10 mM HEPES buffer (see recipe)

Medium-speed centrifuge (e.g., Avanti J26XP with rotor JS-5.3, Beckman Coulter)
Rapid flow filter system (e.g., Nalgene, cat. no. 595-4520)
500-ml centrifuge tubes (Corning, cat. no. 431123)
Polycarbonate tubes for ultracentrifugation (Beckman Coulter cat. no. 355622)
Ultracentrifuge (e.g., L7-65 with rotor Ti45, Beckman Coulter)
Low-retention tubes (Kisker, cat. no. G017)

1. Thaw CM at 4°C or at room temperature.
2. Transfer CM to centrifuge tubes and—depending on the rotor—centrifuge 45 min at a minimum of 6800 × *g* and maximum of 10,000 × *g*, 4°C.

   The pellet contains larger debris and should be discarded; only use the supernatant.

   The maximum g-force that rotors can tolerate varies among available rotors. Rotors should be used that can be spun at least at 6800 × g, and if possible at 10,000 × g.
3. Perform a bottle-top filtration of the CM using 0.22-µm filters.

   Depending on the cultivation medium used, pores of the filters tend to clog.
4. Add PEG 6000 and NaCl to the filtered CM to a final concentration of 10% or 75 mM, respectively:

CM [ml]	50% PEG 6000 [ml]	3.75 M NaCl [ml]
10	2.56	0.26
40	10.26	1.03
100	25.64	2.56
400	102.56	10.26
1000	256.41	25.64
2000	512.82	51.28
4000	1025.64	102.56
10,000	2564.10	256.41

5. Incubate the suspension for 8-16 hr at 4°C (overnight).
6. Mix the suspension well before transferring to centrifuge tubes.

   Tubes from the preparation of the CM in Basic Protocol 1 can be re-used if they were kept sterile.

   Over time, the suspension will form layers. After mixing, the suspension should appear homogenous.
7. Centrifuge 30 min at 1500 × *g,* 4°C.
8. Remove and discard the supernatant carefully using a pipette; keep the white pellet.

   The supernatant should be removed from the pellet as completely as possible. With some exercise, residual supernatant also can be carefully rinsed off.
9. Resuspend the pellet in 10 ml 0.9% NaCl until the pellet is completely dispersed.

   The suspension should be clear and should show no PEG lumps.
10. Transfer the suspension to ultracentrifuge tubes.

    Ultracentrifuge tubes are available in different materials; we have experienced the best recoveries in polycarbonate tubes.
11. To transfer residual material, rinse the centrifuge tubes from the PEG precipitation with 25 ml of 0.9% NaCl.
12. Transfer each washing to the same ultracentrifuge tube as its original pellet.

13. Add 0.9% NaCl to the tubes to a final volume of 65 ml. Close the tubes with their lids.

14. Balance/tare tubes before loading tubes opposite to each other.

    For high-speed centrifugation, tubes need to be balanced according to the manufacturer's instructions. We tolerate a maximum discrepancy of 0.01 g. For odd numbers, load an empty tube with water to an equivalent weight and use it to balance the rotor.

    Mark the outer side of each tube on the lid to easily identify the pellets following centrifugation.

15. Ultracentrifuge 130 min at 100,000 × *g*, 4°C.

    Deceleration must be set without the brake. Total running time will thus increase to around 140 min.

16. Take the tubes out of the rotor and place on ice.

17. Carefully remove the supernatant with a pipette, and discard it.

    Avoid contact between the pipette and the pellet on the wall of the tube, which may be difficult to see. The mark on the lid allows localization of the pellet in case it is invisible. All of the supernatant should be removed from the pellet. The final residual amount can be carefully rinsed off; to avoid losing material, hold the pellet-containing side of the ultracentrifuge tube upwards.

18. Resuspend the pellet in an appropriate volume of the medium or buffer of choice.

    We resuspend the yield of the CM of 4 × 10^7 cells in 1 ml 10 mM HEPES buffer. For reproducibility, we recommend always resuspending the pellet in a defined volume per cell equivalent.

19. Add half of the calculated amount of buffer to the ultracentrifuge tubes, rinse the walls, and resuspend the pellet for a minimum of 4 min.

    Avoid air bubble formation during resuspension. It is best to resuspend by repetitive pipetting, keeping the tip always in the liquid.

20. Add buffer to the final volume.

21. Store small aliquots of the EV preparation in appropriate containers at −80°C.

    We have compared several commercially available tubes and currently store our samples in Kisker low-retention tubes. Other tubes can be used, but should be tested for their impact on particle recovery and EV characteristics after storage.

    Example results are shown in Table 1.

REAGENTS AND SOLUTIONS

Cultivation medium

DMEM, low-glucose (Pan Biotech, cat. no. P04-01500)
10% human platelet lysate (hPL; in house production; available also from Macopharma and PL Bioscience)
100 U/ml, penicillin-streptomycin-glutamine (Life Technologies, cat. no. 10378016)
5 IU/ml heparin (Ratiopharm, cat. no. N68743.06)
Store up to 4 weeks at 4°C

HEPES buffer, 10 mM

Add 1 ml of 1 M HEPES (Gibco, cat. no. 15630049) to 99 ml of 0.9% NaCl (B. Braun, cat. no. 151072). Sterilize by filtration using a 0.22-µm bottle-top filter. Store up to 6 months at 4°C.

Table 1 Example Results for Basic Protocol 2

Volume supernatant [ml]:	4300	Cell count:	6.45×10^8
Complete medium (after 6800-10,000 × *g* centrifugation)			
Protein concentration [ng/µl]: From BCA	5,224.69	Protein total [mg]: From BCA	22,466
Particle concentration [per ml]: From NTA	2.0×10^9	Particle total: From NTA	8.6×10^{13}
Particle size [nm]: From NTA_Average Size (×50 Value)	100.3		
EVs			
Resuspended in:	☒ 10 mM HEPES NaCl ☐ Other: _____	Volume [ml]:	16.1
Protein concentration [ng/µl]: From BCA	5518.45	Protein total [mg]: From BCA	88.85
Particle concentration [pro ml]: From NTA	2.5×10^{11}	Particle total: From NTA	4.03×10^{12}
Particle size [nm]: From NTA_Average Size (×50 Value)	116.3		
Recovery [%]: (Particle total EV/particle total CM)	4.69	Particle/mg protein:	4.5×10^{10}

NaCl, 3.75 M

Weigh 219 g sodium chloride (Sigma Aldrich, cat. no. 71376), transfer to a 1 L glass bottle, make up to 1000 ml with distillated water, and autoclave. Store up to 6 months at room temperature.

PEG 6000, 50% (w/v)

Weigh 250 g PEG 6000 (Sigma Aldrich, cat. no. 81260), transfer to a 1-L glass bottle, and make up to 500 ml with distilled water (50% w/v). Shake the bottle to mix the components, and use a magnetic stirrer until the PEG is completely dissolved, shaking from time to time. Autoclave, and store at room temperature for up to 6 months.

Trypsin/EDTA, 1×

50 ml 10× trypsin/EDTA (PAN Biotech, cat. no. P10-024100)
450 ml phosphate-buffered saline (PBS; Gibco, cat. no. 70013-016)
Store up to 4 weeks at 4°C

COMMENTARY

Background Information

The method given here is applicable for the large-scale isolation of EVs from CM of various cell types. EVs harvested with this method from MSC-conditioned media have been successfully applied to various preclinical models (Doeppner et al., 2015; Drommelschmidt et al., 2017; Gussenhoven et al., 2019; Ophelders et al., 2016; Wang et al., 2020). EVs share several features with viruses, such as their size and a comparable molecular assembly. Since viruses can be concentrated by PEG precipitation (Kanarek & Tribe, 1967; Kohno et al., 2002; Vajda, 1978), we adopted and optimized protocols originally developed for viruses to be used with EVs (Ludwig et al.,

Table 2 Troubleshooting Guide for Isolation of MSC-Derived EVs

Problem	Possible solution
Preparation of CM	
Filtration of MSC-CM causes filter clogging	Always centrifuge the CM first and filter it in a second step; alternatively, use different material for the filter membrane
PEG precipitation	
PEG incompletely dissolved	Increase the time and speed of magnetic stirring and check after autoclaving the solution for residual solids
Absence of white precipitate pellet after PEG precipitation and subsequent 1500 × *g* centrifugation step	Check for correct volumes of added reagents Check on cultivation medium used; was serum or hPL added? Incubation time of PEG precipitation should be between 8-16 hr; modified incubation times may affect the recovery
Pellet after 1500 × *g* cannot be resuspended	The precipitate from a maximum of 750 ml of CM should be applied to the 65 ml transferred to the ultracentrifuge tubes
Ultracentrifugation	
Precipitates become visible after filling ultracentrifuge tubes with samples of the resuspended PEG pellet	Check the volume equivalents of CM which was pelleted, resuspended, and transferred to the ultracentrifuge tube; do not load more than 750 ml original CM equivalents per ultracentrifuge tube Invest more effort to disperse the pellet correctly
Lack of visible EV pellet after 100,000 × *g* centrifugation	Check the seeded cell number; analyze obtained EVs with appropriate methods (like NTA or Western blot). If EVs are detectable, check the ultracentrifugation speed. It might be too low, or the run was interrupted.
High protein concentration in obtained EV preparations	Removal of residual supernatant after the 1500 × *g* step will decrease the protein concentration; EVs may not have been carefully resuspended following PEG precipitation (proteins stick to EVs)

2018). The principle of the PEG precipitation is based on replacement of water molecules that form a hydrate envelope around the EVs. Due to the hydrophobic effect, the EVs precipitate surrounded by PEG, leading to a massive volume reduction, mandatory for using subsequent ultracentrifugation-based methods. Our group established the method as a low-cost alternative to commercially available EV precipitation products.

Critical Parameters and Troubleshooting

Table 2 lists problems that may arise with the protocols in this article along with possible solutions.

For a scalable system with larger volumes (>10 L), the described method is limited. As an alternative method, TFF appears feasible. Depending on the system, TFF can be scaled to process hundreds of liters in relatively short time intervals. TFF devices from some companies are provided as automated, scalable systems, both as benchtop devices for research labs and as big machines for industry. First attempts to prepare EVs using TFF have already been published (Busatto et al., 2018; Heinemann et al., 2014). Notably, expensive hardware needs to be purchased. In contrast, for the PEG precipitation, only centrifuges are required, which should belong to each lab working with EVs.

Although the described method can be scaled for EV preparation, there are some bottlenecks to be discussed. The method needs to be considered an open system, including numerous handling steps that increase the risk of contamination. The method is only scalable to mid-range. The limiting factor is the centrifuge size. For example, only up to 5 L can be processed if only one ultracentrifuge run is going to be performed to remove residual PEG from the samples. In total, this is still 14-fold more than the amount that can be processed by conventional differential centrifugation protocols. For larger volumes, several runs need to be performed.

Time Consideration

The given method is applicable for large-scale isolation of CM from MSCs. With this approach, up to 10 L can be processed within

24 hr (including overnight incubation) on 2 subsequent days.

In detail, for the preparation of the medium in advance of the PEG precipitation, approximately 2 hr are needed, including the centrifugation and filtration of the CM. For the precipitation itself, we recommend overnight incubation. On the following day, another 5 hr need to be invested, including the 130 min for the ultracentrifugation run.

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Author Contributions

Verena Börger: Conceptualization; project administration; writing-original draft; writing-review & editing. **Simon Staubach:** Conceptualization; project administration; writing-review & editing. **Robin Dittrich:** Data curation; writing-review & editing. **Oumaima Stambouli:** Writing-review & editing. **Bernd Giebel:** Methodology; supervision; writing-review & editing.

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