Cost-effective Strategies for Depletion of Endogenous Extracellular Vesicles from Fetal Bovine Serum

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Abstract

Despite the prominent therapeutic potentials of stem cells, their use in cell therapy has been challenged with some unreproducible and inconsistent outcomes in addition to the risk of rejection and tumorigenesis. Gaining novel insights to the importance of the conditioned medium, secretory factors and extracellular vesicles as the functional components of the cultured stem cells, suggested the idea of substituting the cells with their cell-free counterparts. Biological properties of these products are influenced by the cues received from their microenvironment. Hence, providing optimal and fully defined culture conditions is essential for their preparation. Fetal bovine serum (FBS), one of the most routine supplements of cell culture, is enriched by endogenous extracellular vesicles (EVs). These EVs will affect the yield, purity and functional features of the cell-free products. Here, we endeavored to examine and compare three different methods including ultrasonication, ultrafiltration and polymer-based precipitation, to deplete EVs from FBS. We chose easy to perform and fast methods with the capacity for high-throughput applications. Based on our observations, although all examined methods were able to deplete EVs from FBS to some extent, polymer-based precipitation could be considered as the method of choice with minimal consequences on the biological requirements of FBS to support cell growth and characteristics. Due to similarities between FBS and some other biological solutions, this strategy would be suitable for EV-depletion from other liquids with high concentrations of proteins and nutrients. Moreover, it could be applied for preparation of optimal culture conditions for nanoparticle applications.

Introduction

Although, in recent decades stem cell therapy as an advanced scientific research topic has been declared to hold a great promise for untreatable diseases, the fluctuations in the outcome of cell therapy procedures, put some struggle in their application (Choi et al., 2019; Lukomska et al., 2019; Zakrzewski et al., 2019). In recent years, there has been a trend to take advantage of stem cells' benefits by substituting them with their secretome or active components of their conditioned media such as extracellular vesicles (EVs) (Gurunathan et al., 2019; Weiss and Dahlke, 2019; Zhang et al., 2019). These vesicles transfer a wide range of bioactive components such as RNAs, DNAs and proteins while protecting them from environmental insults

due to presence of a bilayer membrane (Colombo et al., 2014).

There are a wide range of techniques for isolation of extracellular vesicles from various bioliquids or conditioned media (*see* Gurunathan et al., 2019; Li et al., 2019). The method of choice affects the purity of the final preparations which should be carefully examined according to the guidelines of the International Society of Extracellular Vesicles (ISEV) (Théry et al., 2018).

It is known that application of FBS, as an essential supplement in cell culture, bears a risk of transferring bovine EVs to the culture (Czapla et al., 2019; Eitan et al., 2015; Mannerström et al., 2019; Shelke et al., 2014). A brief literature review indicates the significance of this problem as most studies investigating the functional roles of EVs

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have been performed under serum-free circumstances, which are not optimal (Lehrich et al., 2019; Théry et al., 2006). In some other studies, exosome-depleted serum prepared by more than 18 h ultracentrifugation at 100000 to 120000 *g* has been applied. Despite the large number of experiments performed based on this method, particle removal was reported to be inefficient (Lehrich et al., 2018). Moreover, long-term ultracentrifugation, by itself, will also remove most of the active components of the serum. It is labor intensive, time consuming and needs expensive infrastructures. Commercial exosome depleted serum batches are also available. However, their application is extremely limited due to the high cost.

Hence, in this study, we investigated the possibility of removing the nano-sized extracellular vesicles from FBS based on the application of cost-effective methods including ultrasonication, ultrafiltration and polymer precipitation to decrease the expenditures of research in the field of EVs, while maintaining its quality. Unlike ultracentrifugation, these methods have the proper capacity for large-scale applications.

Materials and Methods

EV depletion strategies

Probe Sonication (Ultrasonication)

Commercial fetal bovine serum samples (Gibco, Germany), thawed to room temperature, were probe sonicated on ice for 120 sec (10 sec on, 10 sec off) with 20 kHz and 50 W (Ultrasonic probe, Topsonics; Iran). Samples were not heat-inactivated or diluted before sonication. Serum samples were then centrifuged at 600 *g* for 4 min at 4°C, to remove components created during sonication.

Ultrafiltration procedure

Briefly, FBS samples without any modifications or dilutions were centrifuged (400 *g*, 6 min) to remove large suspended particles. Then, they were concentrated from 35 ml to 5 ml using pressure-driven concentrating protocols via Stirred Cell Model 8050 with the application of 100 kDa disposable membranes (PLHK04310, Millipore, USA) using nitrogen gas (<10 psi) at room temperature. The concentrates were applied for downstream applications. EV-depleted serum samples were syringe-filtrated and collected in clean vessels as the final products.

Polymer-based precipitation

In order to deplete EVs from FBS samples, based on the polymer precipitation method, (polyethylene glycol) PEG 4000 (Merck, Germany) sterile solutions were applied. 16% PEG solutions were prepared in sterile deionized water and homogenized via ultrasonic-bath (35 Hz, 4 min, RT; BANDELIN SONOREX, Germany). Fetal bovine sera were thawed and homogenized by mechanical mixing, before the addition of NaCl (0.9%) at a ratio of 1:30 V/V. Then, PEG solutions were added to fetal bovine samples (final ratio of 1 to 4 V/V) and shaken to obtain homogenous solutions. Following 90 min incubation at 4°C, samples were cold-centrifuged (5000 *g*) for 50 min (Sigma, 3-16pk, Germany). The supernatants were collected in new tubes under sterile conditions as the exosome-depleted serum, while white pellets were discarded.

Nanoparticle characterization strategies

Dynamic Light Scattering

EV-depleted serum samples, prepared using different methods, were loaded on Particle Size Analyzer (0.5 nm-10 μm; Vasco3, Cordouan, France) to investigate the distribution pattern of the remaining particles (0-1000 nm) in comparison to the control samples. Measurements were performed based on Cumulants and Pade Laplace modes (wavelength 657 nm) and 3 to 5 repeats were considered for each sample. Measurements were carried out at time intervals of 10.000 μs and number of channels of 400, as device settings. Refractive index of 1.33 and viscosity of 0.891 were considered as dispersant properties during all studies. All particle size analytical experiments were performed at 25°C and data were analyzed using NanoQ Report software.

Zeta potential analysis

Zeta potential was measured to investigate the physical properties of different FBS samples following EV depletion procedures (Zeta Compact, CAD, France). At least three independent measurements were carried out for each sample and reported as mean of zeta potential+/-SEM in each case.

Atomic Force Microscopy

Atomic force microscopy (AFM) was performed to demonstrate the presence of EVs in EV-enriched fraction following the application of polymer-based precipitation procedure. Briefly, diluted EV-

containing solutions in each case were loaded on freshly cleaved mica and air-dried. They were investigated in non-contact mode at different scan size ranges including 3, 5 and 10 μm based on the protocols provided previously (Parisse et al., 2017; Ridolfi et al., 2019; Skliar and Chernyshev, 2019) via Ara Research atomic force microscope (model: Full).

Bicinchoninic Acid Assay

Bicinchoninic Acid (BCA) assay was performed using the BCA protein quantification kit (DNAbioTech life Sciences) to investigate the total protein content of different samples according to manufacturer protocol. Optical densities were recorded at 545 nm by an ELISA reader (Awareness, USA) following 1 h incubation at 60°C. Same procedures were performed for unknown samples in addition to the serial dilutions of albumin as the standard protein.

FBS biosufficiency evaluation Strategies

Mesenchymal stem cells derivation and characterization

Human adipose tissue aspirates, provided by surgeons from healthy volunteers with informed consents, were transferred to the lab and applied for derivation of primary cultures of human MSCs, based on our previously described protocol (Ahmadian Kia et al., 2011). Briefly, after removing the extra blood, adipose tissues were gently washed with phosphate-buffered saline (PBS 1X) and digested with collagenase type I in the absence of bovine serum albumin (BSA) and $CaCl_2$ for 45 min at 37°C. Digestion was stopped by the addition of heat-inactivated-serum-containing medium, and mononuclear cells were separated after centrifugation and washing steps. Cell suspensions were cultured for 48 h at 37°C in low glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS in the presence of penicillin/streptomycin. Upon reaching 90 to 100% confluency, cells were sub-cultured. MSCs were applied for downstream applications from passage numbers 3 to 5.

Primary adipose tissue derived mesenchymal stem cells (Ad-MSCs) were also applied for immunophenotyping experiments following 5 days of culture in the presence of medium supplemented with 10% of PEG 4000-mediated EV-depleted FBS. Briefly, for flow cytometric analysis, Ad-MSCs were trypsinized following two rounds of washing

with PBS 1X, and after centrifugation steps they were applied for staining procedures with single color antibodies (Supplementary *Table 1*).

MTT assay

Human mesenchymal stem cells were seeded in 96-well plates (8000 cells per well) and 24 h later, upon reaching proper confluency, their media were exchanged with the complete media prepared by application of ultrafiltration- or PEG 4000-mediated EV-depleted serum. In control wells the media were exchanged with the normal FBS-containing media. 24, 48 and 72 h following the treatments, 25 μl of the MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Germany), was added per well (final concentration of 5 mg/ml). After 3.5 h incubation, the media were discarded from each well and formazan crystals were solved in 200 μl dimethyl sulfoxide (DMSO). Cell viabilities were calculated by dividing the mean of optical densities in the treated-cell groups to the mean of ODs for the cells cultured in the presence of 10% unmodified FBS and reported as the percentage of cell viabilities.

Examination of sample contamination

PEG 4000-mediated exosome-depleted serum was applied to investigate the presence of any contamination using the BHI (Brain Heart Infusion) broth medium (HiMedia, Germany). Serum samples were inoculated in BHI media and incubated for 48 h at 35°C incubator. This medium with high concentrations of nutrients supports the proliferation of a variety of pathogens including aerobic bacteria, anaerobic bacteria, yeast and molds (Atlas, 1993; MacFaddin, 1985; Roseno, 1919; Salfinger and Tortorello, 2015).

Methods presented in this article for removal of EVs from fetal bovine serum samples were registered in the Intellectual Property Center of Iran under Declaration No. 13985014000300973 on 13 December 2019.

Results

Removal of EVs from FBS by probe sonication

Particle size analysis was performed for FBS samples, following the probe sonication method. The efficient removal of endogenous particles in the range of 50-1000 nm was confirmed and compared with commercial FBS (Figure *1A,B*). It was inferred from results that sonication decreases the amount of

poly dispersity index as an indicator of homogeneity from 0.4+/-0.04 to 0.2+/-0.01. Furthermore, a considerable decrease in the count of particles (kcps) was observed following the sonication experiments, based on particle size analysis (laser power 50%; Ctrl: 4735.708+/-962; Sonicates: 1354.931+/-93;

70% depletion; data reported as mean of three independent experiments+/-SEM). Zeta potential means, measured at 23-24°C were equal to -19.83+/-0.596 and -23.27+/-0.384 for control and sonicated serum samples, respectively. This is an indication of homogenously dispersed solutions (Table 1, data are
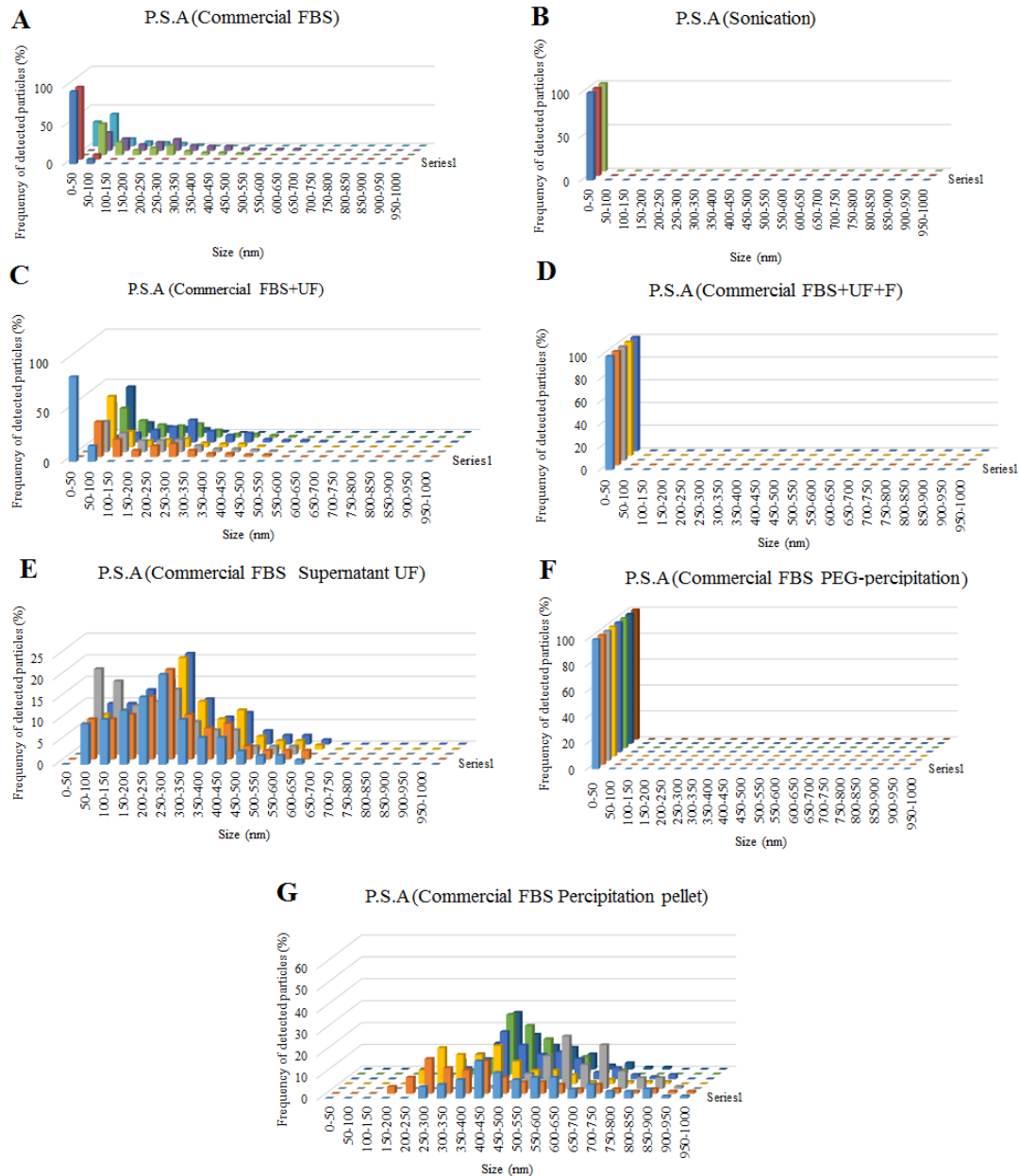

Figure 1. Particle size analysis experiments indicated the removal of desired particles from fetal bovine samples (A) and following the probe sonication (B). Furthermore, consequences of ultrafiltration on the distribution of particles from different size range (0 to 1000 nm) were demonstrated by particle size analysis via dynamic light scattering device: (C) commercial FBS ultrafiltrates, (D) commercial FBS ultrafiltrates followed by one round of syringe filtration (0.22 μm), and (E) supernatant of ultrafiltration. EV depletion was also evident in FBS samples following one round of polymer-based precipitation: (F) commercial FBS following EV depletion steps via PEG 4000 solution. Enrichment of larger particles including extracellular vesicles can be observed in the pellet of the polymer-precipitation procedure (G). As demonstrated 3 to 5 replicates were considered for each experiment.

reported as mean of three independent experiments+/-SEM). No changes were observed in the pH which remained 7.6 for both samples.

Table 1. Comparative biophysical analysis for FBS samples following EV depletion via ultrasonication in comparison to commercial control FBS.

Mean of +/-SEM	FBS Ctrl	EV-depleted FBS
Temperature (°C)	24.42+/-0.02	23.54+/-0.00
Electric Field (V/cm)	8.49+/-0.003	8.57+/-0.003
Conductivity (mS/cm)	0.764+/-0.001	0.335+/-0.00
Dielectric Constant	78.62+/-0.00	78.94+/-0.00
Viscosity (mPas)	0.90+/-0.00	0.92+/-0.00
pH	7.6	7.6
Mobility (µm/s/V/cm)	-1.53+/-0.04	-1.77+/-0.02
Zeta Mean (mV)	-19.83+/-0.59	-23.27+/-0.34
Coefficient	12.96+/-0.00	13.17+/-0.00

Table 2. Comparative biophysical analysis for FBS samples following EV depletion via ultrafiltration in comparison to commercial control FBS.

	FBS Ctrl	EV-depleted FBS
Temperature (°C)	21.96+/-0.031	22.48+/-0.059
Electric Field (V/cm)	8.54+/-0.013	8.57+/-3.834
Conductivity (mS/cm)	0.255+/-0.002	0.268+/-0.000
Dielectric Constant	79.51+/-0.01	79.32+/-0.02
Viscosity (mPas)	0.95+/-0.00	0.94+/-0.00
pH	7.35	7.40
Mobility (µm/s/V/cm)	-1.42+/-0.35	-1.05+/-0.28
Zeta Mean (mV)	-19.29+/-0.49	-14.22+/-3.89
No. of Tracking	62+/-14.53	12+/-2.21
Coefficient	13.57+/- 0.00	13.44+/-0.01

Depletion of EVs from FBS by ultrafiltration

Based on particle size analysis, pressure-mediated ultrafiltration accompanied by a round of syringe filtration resulted in efficient depletion of nano-sized particles (50-1000 nm size range; Figure *1C-E*). Ultrafiltrated serum samples indicated significantly lower kcps (17.77+/-0.40) in comparison to control samples (1541.011+/-21.574), which indicates more than 85% particle depletion following ultrafiltration. This method led to some changes in the physical appearance of the samples, i.e. increased light transparency and decreased density of the serum. Furthermore, polydispersity index was increased from 0.28+/-0.016 to 0.63+/-0.037 following the procedure. Mean of zeta potential, measured at 21-22°C, was equal to -19.29+/-0.497 and -14.22+/-3.894 for FBS samples before and after the ultrafiltration, respectively (Table 2). During the Zeta potential analysis, similar noise level of 1-1.05 was considered for all samples. In accordance with probe-sonication, adjacent pH values were measured for ultrafiltrates (7.40) in comparison to the intact FBS samples (7.35). Remarkable depletion of nano-sized particles was observable in the videos recorded by zeta size analyzer (Supplementary Videos, *V1*).

PEG 4000 solution as the method of choice for EV-depletion

Comparative particle size analysis performed based on the dynamic light scattering detection of particles demonstrated the efficient and reproducible depletion of EVs from FBS samples by PEG 4000 (Figure *1F-G*). Based on the DLS results, more than 91% depletion was observed in kcps for serum samples following the PEG-precipitation (380.958+/-60.456) in comparison to naïve FBS samples (4563.949+/-1187.615). As demonstrated in Figure *1F*, FBS samples were completely cleared from extracellular vesicles following a short-time (90 min) incubation with PEG 4000 solution and low-speed centrifugation (50 min, 5000 *g*). The reproducible depletion of EVs was accompanied by no obvious modification in the physical appearance of FBS samples. In addition, as confirmed by zeta potential analysis, the stability of the samples and their physical properties were not decreased or deteriorated, however, as we expected, number of tracking was decreased in EV-depleted serum samples due to removal of particles from 50 to 1000 nm size range (Table 3). Mean of polydispersity index (PDI) which was variable between 0.234 and 0.920 for unmodified serum samples, was equal to 0.253+/-0.038 for PEG 4000-mediated EV-depleted FBS. This is an indication of a proper level of stability. pH value, recorded at 23°C for control FBS, PEG 4000-mediated EV-depleted FBS and

collected EVs, were equal to 7.75, 7.61 and 6.70, respectively. 10-second videos recorded for EV-depleted FBS samples indicated EV removal as compared to the samples prepared from the pellets (Supplementary Videos, *V2*). Extracellular vesicles enriched from fetal bovine samples were detected via atomic force microscopy as demonstrated in Figure 2.

BCA assay, to investigate the total protein content of FBS samples following the probe sonication, ultrafiltration or PEG-precipitation, demonstrated that while the protein content of FBS remained constant in comparison to control FBS (5163.294+/-0.011) following ultrasonication (5142.223+/-0.004), it was considerably decreased following one round of ultrafiltration accompanied by syringe filtration (1459.223+/-0.018).

EV-depleted FBS samples prepared by polymer precipitating method were negative for bacterial, fungal or mold contaminations at the end of the process, as confirmed by inoculation of the samples in BHI broth medium. Moreover, culturing the cells in the presence of the EV-depleted FBS for 5 days had no considerable effect on cell viability and immune phenotype as demonstrated by morphological observations (Figure *3A*), MTT assay (Figure *3B*) and flow cytometric analysis of the cells.

Table 3. Comparative biophysical analysis for FBS samples following EV depletion via PEG 4000 in comparison to commercial control FBS.

Mean of +/-SEM	FBS Ctrl	EV-depleted FBS
Temperature (°C)	24.45+/-0.023	23.54+/-0.00
Electric Field (V/cm)	8.48+/-0.003	8.57+/-0.003
Conductivity (mS/cm)	0.763+/-0.001	0.335+/-0.000
Dielectric Constant	78.60+/-0.00	78.94+/-0.00
Viscosity (mPas)	0.90+/-0.00	0.92+/-0.00
pH	7.75	7.61
Mobility (µm/s/V/cm)	-1.52+/-0.04	-1.71+/-0.02
Zeta potential (mV)	-19.74+/-0.59	-22.60+/-0.34
No. of Tracking	105+/-7.05	30+/-4.09
Coefficient	12.95+/-0.00	13.17+/-0.00

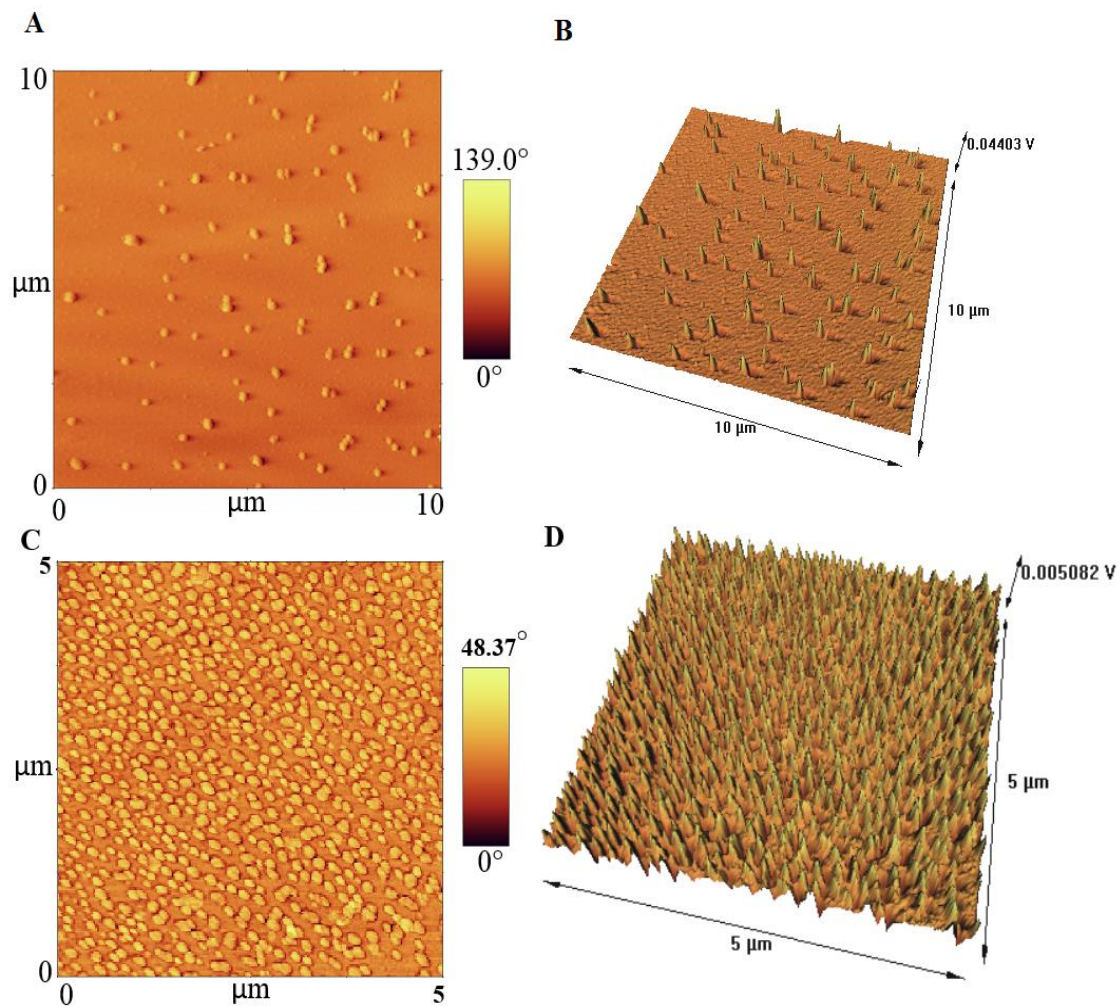Cell viabilities of mesenchymal stem cells, treated with EV-depleted serum prepared by PEG method or ultrafiltration were assessed in comparison to reference controls. 91 percent of the primary cells remained viable after 24 h of both treatments. Cell viabilities were equal to 80.85+/-0.60 and 86.72+/-2.16 for PEG group and 86.95+/-3.74 and 93.17+/-0.98 for ultrafiltration method, after 48 and 72 h, respectively. Furthermore, Ad-MSCs showed the common spindle-like morphology of mesenchymal stem cells, when they were exposed to the PEG 4000-mediated EV-depleted FBS. During our experiments cells exposed to these media were frequently sub-cultured and propagated without any technical problem, similar to cells grown in control medium.

Figure 2. Atomic force microscopic detection of extracellular vesicles enriched from FBS samples. Samples were diluted at least 50 folds before loading on freshly cleaved mica. (A, C) 2D phase and (B, D) 3D amplitude pictures of EV-containing fractions following the application of polymer (PEG 4000) precipitation (A, B) and ultrafiltration (C, D) methods for removal of EVs from FBS.

As demonstrated in Figure 4, primary adipose tissue derived cells indicated high expression levels for CD44 (99.7%), CD105 (99.8%) and CD29 (97.2%), while they were negative for the expression of CD45 (0.93%) and CD11b (0.60%), as markers for hematopoietic and endothelial cells. They were also positive for expression of CD34 (Colter et al., 2000; Harvanová et al., 2011; Pittenger et al., 1999; Riekstina et al., 2008; Tran et al., 2012; Vaculik et al., 2012).

Discussion

Although first cell-based products have been approved by the European Medicines Agency (EMA) in 2018 and despite emerging very recent approvals for therapeutic applications of MSCs, still there are major concerns regarding their short- and long-term consequences. Among which risk of pro-tumorigenic events, stimulation of immune response and reduced differentiation capacity have been highlighted (Chu et al., 2019; Hoogduijn and Lombardo, 2019). Such unique and undeniable superiority of cell-based therapies in comparison to classic therapeutics, led the scientists to find a way for bringing the benefits of the cells to off-the-shelf and cell-free products, by using extracellular vesicles. As this field is yet in its infancy, providing the basic requirements, in an affordable manner, is not easy for many researchers throughout the world.

The presence of endogenous EVs in fetal bovine serum, as well as other biological fluids, is important from different aspects. As previously reported, they

have significant influence on different features of the cells in culture (Beninson and Fleshner, 2015) and encounter with their normal functional properties (Angelini et al., 2016). In this study, we aimed to deplete endogenous EVs present in the FBS by application of widely accessible methods to be applied for large-scale depletion of EVs in an affordable manner. Ultrasonication, ultrafiltration and polymer-precipitation methods were investigated comparatively to achieve this goal.

Ultrasonication was used as a proper method to destruct the lipid bilayer membrane of the extracellular vesicles and was followed by one round of centrifugation to remove the artifacts. Ultrafiltration is considered as a method of choice for isolation of extracellular vesicles from conditioned media or EVs from liquid biopsies including urine, saliva or plasma samples (He et al., 2019; Lobb et al., 2015; Yu et al., 2018). In a previous study, depletion of EVs from FBS was shown, based on ultrafiltration method via the application of ultra-15 centrifugal filters for 55 min at 3000 *g* (Kornilov et al., 2018). Although this method is economically preferred over the commercially available EV-depleted FBS, it does

not yet meet the requirements for large-scale EV preparation. Ultrafiltration chambers, working under high pressure conditions, are proper substitutes to deplete the EVs under sterile conditions. In addition, this method is faster than common ultracentrifugation method, which takes more than 18 h. However, due to agglomeration of the vesicles following ultrafiltration of different liquids under pressure, it becomes necessary to add another filtration step to remove larger aggregates. Ideal ultrafiltration membranes are the ones with strong mechanical properties, hydrophobicity, durability, chemical stability and low polymer cost (Dobosz et al., 2017).

The third method investigated here is polymer based precipitation of the EVs by PEG 4000. PEG as a water-excluding polymer, has the capacity for conducting less soluble extracellular vesicles out of the media via binding to water molecules (Li et al., 2017; Patel et al., 2019; Ramasubramanian et al., 2019; Willis et al., 2017; Witwer et al., 2013). Normally PEG 6000 is the chemical of choice for isolation of extracellular vesicles from different biological liquids and conditioned media with an acceptable efficiency (Ludwig et al., 2018). To avoid
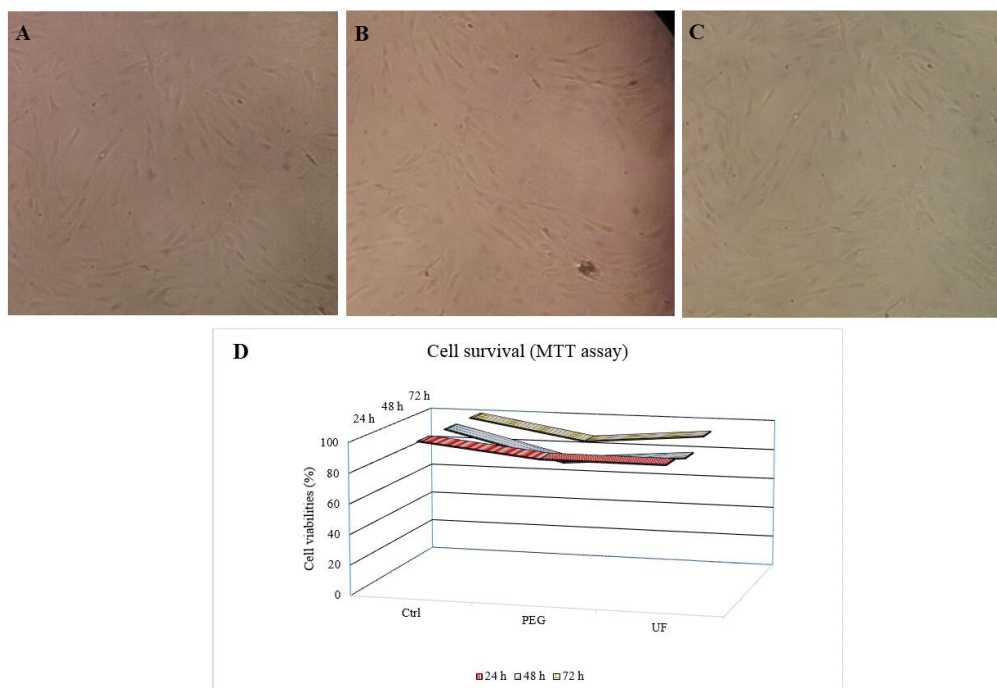

Figure 3. Spindle-like morphology of mesenchymal stem cells propagated in (A) normal medium in comparison to the cells which were conditioned with EV-depleted supplemented media prepared by (B) polymer precipitation or (C) ultrafiltration strategies, as evidenced by light microscopy. Panel (D) demonstrates cell viabilities of Ad-MSCs grown in different media (exosome-depleted FBS prepared based on the PEG precipitation or ultrafiltration methods) after 24, 48 and 72 h, as obtained by MTT assay.

strong protein precipitation in FBS samples PEG 6000 was substituted by PEG 4000 and the incubation time was decreased to 90 min. The

observations, FBS samples depleted from EVs by PEG 4000 were closely similar to normal FBS regarding their stability in comparison to the ones
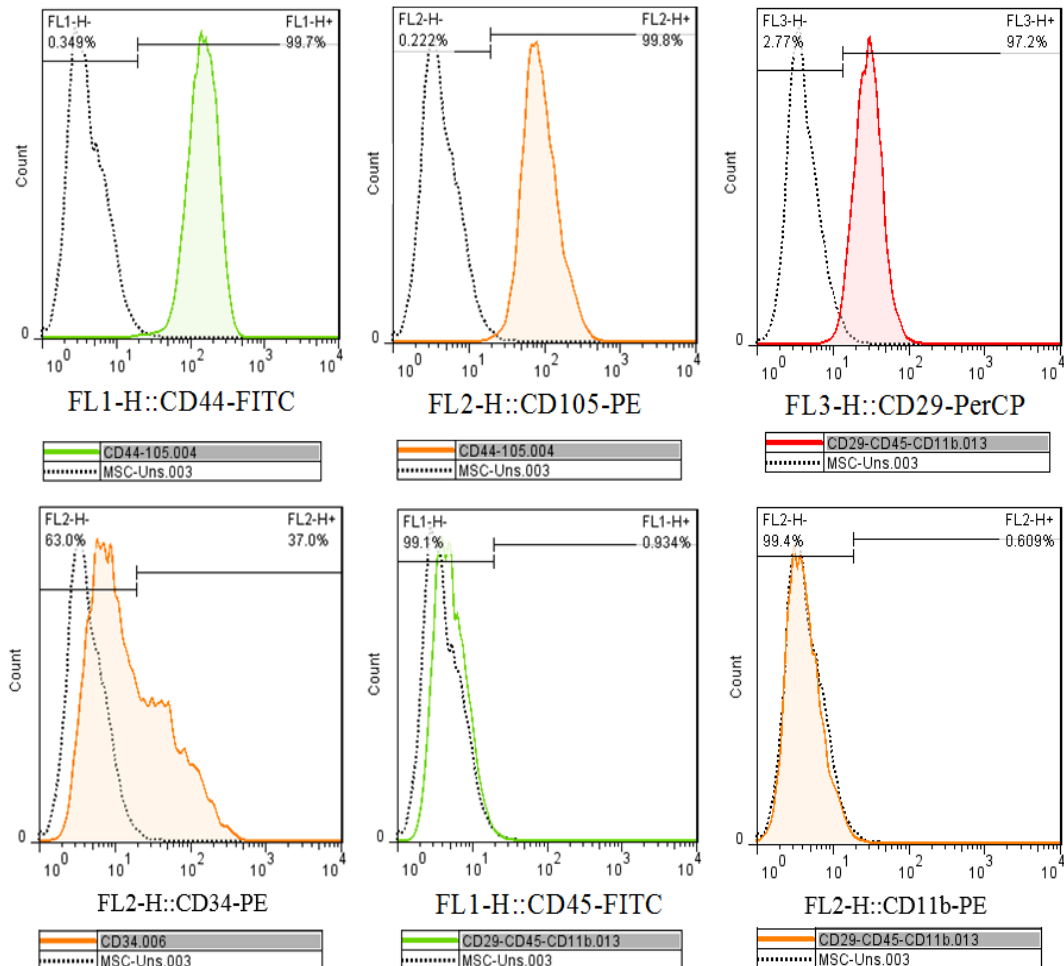

Figure 4. Flow cytometric analysis of primary adipose tissue derived mesenchymal stem cells from passage 3 following 5 days of culture in the presence of the medium supplemented by 10% PEG 4000-mediated EV-depleted serum. As evidenced by these experiments mesenchymal stem cells retained high expression levels for their typical markers including CD44, CD105 and CD29, while, they were negative for CD45 and CD11b as non-mesenchymal cell origin CD markers. 37% of the cells express CD34 (BD FACSCalibur, BD biosciences, USA). Data analysis was performed by FlowJo (version 7.6.1).

efficacy of EV removal was explored based on particle size analysis. Zeta potential experiment was performed to monitor the physico-chemical properties and stability of the FBS samples. Results indicated no considerable change in the primary features of the samples. Solutions with higher magnitude of zeta potential (mV) are less competent for sedimentation or agglomeration during the time (Helwa et al., 2017; Wang et al., 2015). Based on our

exposed to rounds of ultrafiltration/filtration.

Our morphological analysis in addition to MTT assay and immunophenotyping of the Ad-MSCs following exposure to the media supplemented with EV-depleted FBS, prepared based on the polymer-precipitation method, demonstrated normal growth and phenotype of the cells. Furthermore, the presence of EVs in the precipitants was approved by atomic force microscopy.

In conclusion, we propose both polymer-precipitation and ultrafiltration can be applied as proper and efficient methods to deplete EVs from FBS samples. These methods are faster and less labor-intensive in comparison to the ultracentrifugation method. Furthermore, unlike more complicated methods such as microfluidics or tangential flow filtration, there is no need for high-tech or advanced instruments and expertise. Based on our experiments application of PEG 4000 for depletion of EVs would simplify the procedure and decrease the expenditures, while, would provide higher efficiency of EV depletion without any side effects on the quality of the FBS. This method would be of special interest for removing the EVs as it supports large-scale requirements and is not dependent on frequent rounds of filtration, centrifugation, preparation of serial dilutions, and application of disposable materials. Due to less overall sensitivity, this method is also proper for culturing a wide range of cancerous cell lines under exosome-depleted condition and its application could be attributed to other fields of nano-therapeutics and targeted drug delivery procedures.

Acknowledgements

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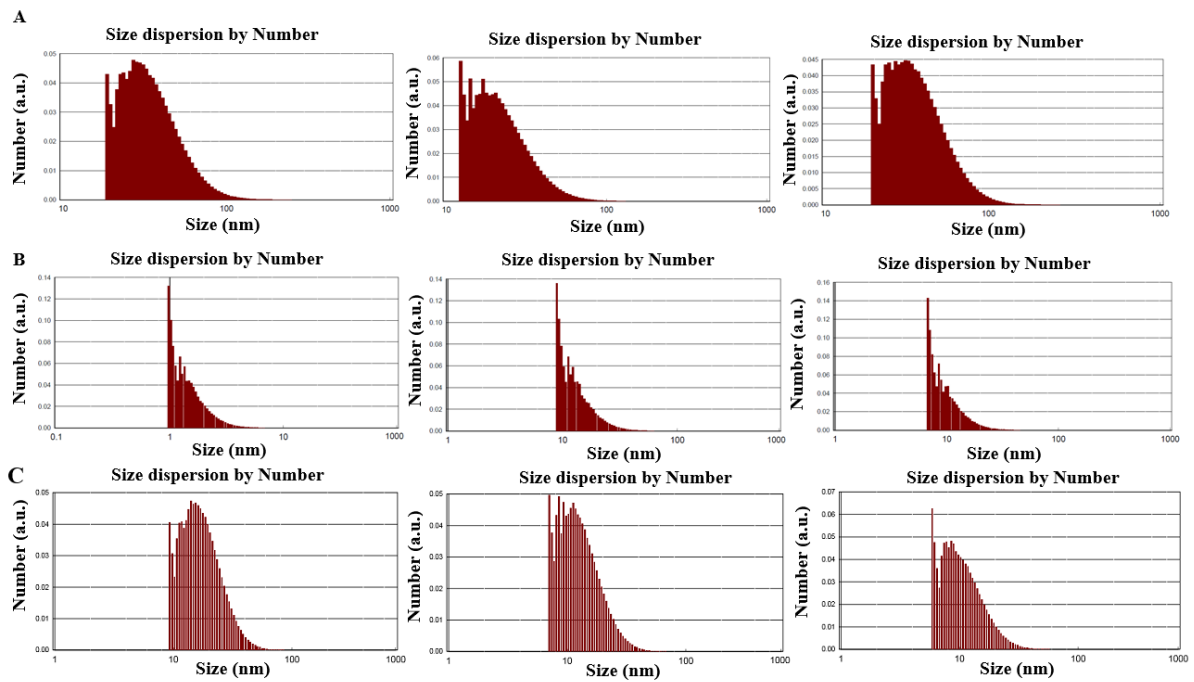
Supplementary Materials:

Figure S1. Size distribution of detected particles based on their frequencies (number) during particle size analysis, as demonstrated by DLS device. As seen a wide range of particles detected inside unmodified serum samples in the first row (A), were removed in the second and third rows, which represent ultrafiltration-mediated exosome-depletion (B) and PEG-mediated-exosome-depletion (C), following three rounds of analysis.

Table S1. Antibodies applied during flow cytometric analysis.

No.	Antibody	Company	Cat No.	Isotype	Clone	Reactivity
1	CD44-FITC	Immunostep	114659	IgG2a	HI44a	Human
2	CD105-PE	Exbio	1P-298-T025	IgG2a	MEM-226	Human
3	CD29-PerCP	Immunostep	29PB-100T	IgG2b	VJ1/14	Human
4	CD34-PE	Immunostep	34PE-100T	IgG1	581	Human
5	CD45-FITC	BD Bioscience	560976	IgG1, κ	-	Human
6	CD11b-PE	Biolegend	101207	IgG2b, κ	-	Mouse, Human

Supplementary Videos S1. Depletion of EVs from FBS samples (Ctrl) following the ultrafiltration procedure (UF) was shown for three different samples, as evidenced by zeta potential analysis. (Videos: Ctrl 1, Ctrl 2, Ctrl 3, UF1, UF2, UF3).

Supplementary Videos S2. Endogenous EVs isolated from FBS samples (EVs collected by PEG precipitation) in addition to EV-depleted serum samples (PEG 4000-mediated EV-depleted FBS) were shown for three different samples. Videos were recorded by Zeta Analyzer device. (Videos: endogenous FBS EVs-1, endogenous FBS EVs-2, endogenous FBS EVs-3, EV-Depleted serum-1, EV-Depleted serum-2, EV-Depleted serum-3).