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Comparison of the efficiency of ultrafiltration, precipitation, and ultracentrifugation methods for exosome isolation

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ABSTRACT

Extracellular vesicles (EVs) are enclosed by a lipid-bilayer membrane and secreted by all types of cells. They are classified into three groups: apoptotic bodies, microvesicles, and exosomes. Exosomes play a number of important roles in the intercellular communication and crosstalk between tissues in the body. In this study, we use three common methods based on different principles for exosome isolation, namely ultrafiltration, precipitation, and ultracentrifugation. We use field emission scanning electron microscopy (FESEM) and dynamic light scattering (DLS) analyses for characterization of exosomes. The functionality and effect of isolated exosomes on the viability of hypoxic cells was investigated by alamarBlue and Flow-cytometry. The results of the FESEM study show that the ultrafiltration method isolates vesicles with higher variability of shapes and sizes when compared to the precipitation and ultracentrifugation methods. DLS results show that mean size of exosomes isolated by ultrafiltration, precipitation, and ultracentrifugation methods are 122, 89, and 60 nm respectively. AlamarBlue analysis show that isolated exosomes increase the viability of damaged cells by 11%, 15%, and 22%, respectively. Flow-cytometry analysis of damaged cells also show that these vesicles increase the content of live cells by 9%, 15%, and 20%, respectively. This study shows that exosomes isolated by the ultracentrifugation method are characterized by smaller size and narrow size distribution. Furthermore, more homogenous particles isolated by this method show increased efficiency of the protection of hypoxic cells in comparison with the exosomes isolated by the two other methods.

1. Introduction

Extracellular vesicles (EVs) are lipid-bilayer membrane-enclosed vesicles which are grouped into two categories base on their size: small EVs (Exomeres, Exosomes, and Ectosomes) and large EVs (Migrasomes, Apoptotic bodies, and Oncosomes) [1]. Also, these EVs can be categorized into different groups, such as apoptotic bodies, microvesicles, and exosomes, based on their origin, distribution, and surface markers. Exosomes are secreted in the extracellular media by multivesicular bodies through exocytosis of live cells under all physiological conditions [2–4] and can be found in amniotic fluid, bile, blood, breast milk, bronchial fluid, cell culture media, cerebral spinal fluid (CSF),

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gastric acid, lymph, plasma, saliva, semen, serum, synovial fluid, tears, and urine [4–7]. Being a class of cell-derived EVs of endosomal origin, exosomes are released into the extracellular media upon the fusion of the multivesicular bodies to the cell membrane. The size of exosomes ranges from 30 to 150 nm, and theses vesicles contain DNA fragments, miRNAs, siRNA, mRNA, proteins, lipids, and other metabolites [8,9]. These compositions are affected by the origin and environment of the cells. Exosome secretion has been shown to increase in response to environmental insults, such as inflammation [10], hypoxia [11], and acidic conditions [12].

Exosomes play a number of important roles in the intercellular communication and are critical mediators of organ crosstalk. Exosomes can alter the behavior of target cells through autocrine and paracrine signaling, such as the miR-21's role in the hypertrophy [13] or miR-143's role in angiogenesis; exosomes derived from hypoxia cells were shown to induce proliferation, survival, and migration of fibroblast [14]. They show therapeutic potential for detection of pathological conditions and can be used for drug delivery. Targeted drug delivery using exosomes has been developed, especially in delivery of drug into tumor and heart tissues. Tumor cells can change their microenvironment by using exosome communication between cells and the immune system [15–18]. It was shown that exosomes isolated from biofluids can be efficiently used as liquid biopsy in clinical diagnosis in the fields of cardiovascular diseases, oncology, organ transplantation, and pregnancy disorders [19].

Exosomes have emerged as a promising source of biomarkers, which can be isolated from small volumes of samples and illustrate tissue-specific molecules representing disease-specific and disease-status of biomarkers for various chronic and acute diseases, such as CPNE3 in colorectal cancer EVs [20], miR-451a in non-small cell lung cancer EVs [21], and miR-451a in pancreatic ductal adenocarcinoma EVs [22].

With the increasing utilization of exosomes in clinical applications, selection of the best methods for maximizing the quality and yield of these EVs is needed. Several commercial exosome isolation methods, such as ultracentrifugation, precipitation, ultrafiltration, affinity purification based on magnetic beads, as well as microfluidics and size exclusion chromatography have been used to isolate exosomes [23,24]. The classical method for exosome isolation is ultracentrifugation, which uses centrifugal force to separate exosomes at a very high speed (100,000–110,000×*g*) to move them across the length of the tube in approximately 70–120 min. In this method, various sediment speed is used for separate different particles present in the culture medium; large debris and particles are removed from the culture medium by the use of lower centrifugal force, whereas exosomes are sediment and separated from supernatant at 100,000×*g* [25–27]. This technique requires specialized equipment and is also time consuming. For example, to increase the purity and reduce the amount of other extracellular vesicles, the pellet should be dissolved and centrifuged again for additional 70 min [28].

Several different precipitation approaches were developed for exosome isolation, including charge-based precipitation methods, as well as precipitation with organic solvents and neutral polymers, such as polyethylene glycol (PEG). Since exosomes are negatively charged, they can be precipitated by interaction with positively charged molecules, such as protamine [29]. In the PRotein Organic Solvent PRecipitation (PROSPR) approach, soluble proteins are removed from the plasma using precipitation in cold acetone, a process that leaves the lipid-encapsulated EVs [30]. In polymer-bases precipitation methods, PEG is primarily used to reduce the solubility of exosomes, then after overnight incubation, exosomes are subsequently isolated using low-speed centrifugation [31].

The ultrafiltration method uses porous membranes (or centrifugal filters) with different membrane types and pore sizes to trap particles of specific size. One or more filters, most often Amicon filters with a Molecular weight cut-off (MWCO) of 10 kDa or 100 kDa, were used to separate particles larger than 30 nm. In this method, exosomes are isolated based on their size differences. This method is time-consuming and requires Amicon or Vivaspin filters for isolation [32,33]. In a recent comprehensive study, the efficiency of five commonly used filters (Amicon Ultra-2 10 k (regenerated cellulose), Amicon Ultra-2 100 k (regenerated cellulose), Vivaspin 2 PES 10 k (polyethersulfone), Vivaspin 2 CTA 10 k (cellulose triacetate), and Vivaspin 2 HY 10 k (Hydrosart)) to recover EVs from plasma, urine, and EV-spiked PBS was compared [34]. This study revealed that the most efficient EV recovery can be achieved by using regenerated cellulose membranes with pore size of 10 kDa, Amicon Ultra-2 10 k [34]. Another method for exosomes isolation is affinity chromatography, which is based on utilizing the capability of monoclonal antibodies to bind specific markers of exosomes. In this method, antibodies against exosome-associated antigens, such as CD 81, CD 63, and CD 9 are used for separation of exosomes from media [28,35]. These antibodies can be immobilized on the magnetic beads or within the microfluidic devices [28].

One of the best method for exosome isolation in the view of their size homogeneity is size-exclusion chromatography (SEC), which isolates exosomes based on their hydrodynamic volume. This method provides highly purified and homogeneous exosomes, but regardless of high purity and homogeneity of the resulting samples, this method requires expensive equipment [36].

The goal of this study is to compare three common methods of exosome isolation, which are mainly used in research, such as ultrafiltration, precipitation, and ultracentrifugation. We hope that this study will serve as a guide for choosing the best exosome isolation method for downstream applications in medicine while considering different features, such as time, scalability, requirement of special equipment, and costs.

2. Materials and methods

2.1. Cell culture

H9c2 cells were purchased from the Institute Pasteur of Iran. H9c2 cells are myoblast, which serve as precursors of cardiomyocytes. This cell line is frequently used in various cardiovascular research [37–39]. Previous reports showed that these cells can be used to obtain exosomes for drug delivery into the heart [40]. In this study, cells were grown in 10^6 T 75 flask DMEM supplemented with 15% FBS (Biosera, Iran) and 1% penicillin–streptomycin (Biosera, Iran), and incubated at 37 °C with 5% CO_2 (Memmert, Germany) [40,41].

2.2. Ischemic preconditioning

To simulate ischemic conditions *in vitro* and investigate the effect of exosomes on cell viability, we used ischemic preconditioning. After 80% confluency, cells were washed three times with phosphate-buffered saline (PBS), and the medium was changed to media containing 15% free-exosome fetal bovine serum (FBS). To induce ischemic preconditioning, cells were subjected to repeated cycles of anoxia O_2 less than 0.1 mmHg in a hypoxic chamber for 30 min with further reoxygenation (10 min) for three cycles. After inducing ischemic preconditioning, cells were returned to the incubator for a further two days to produce exosomes [42].

2.3. Exosome isolation methods

After 80% confluency, the medium of cells was changed to free-exosome FBS media. Two days after incubation with free-exosome media, the culture supernatant was collected for exosome isolation.

2.3.1. Ultrafiltration

In this method, the supernatant of the media was collected in a 15 ml falcon tube, followed by centrifugation at 300×*g* for 10 min at 4 °C to remove dead cells. The supernatants were centrifuged at 3000×*g* for 30

min at 4 °C to eliminate cellular debris and large microvesicles, then the media was concentrated and buffer exchanged to PBS using a 100 kDa MWCO ultrafiltration column (Millipore, Germany) and stored at −80 °C till use [43].

2.3.2. Precipitation

To isolate exosomes from culture medium by the precipitation method, we used the Exocib kit (Cibzist, Tehran, Iran). Cell culture supernatants were processed according to the manufacturer's protocol. Briefly, the cell culture supernatants were centrifuged at 300×*g* for 10 min at 4 °C to remove dead cells. The supernatants were centrifuged at 3000×*g* for 30 min at 4 °C to eliminate cellular debris and large microvesicles, then transferred to a sterile vessel, and an appropriate volume of exosome precipitation solution was added, followed by refrigeration overnight (16 h). After refrigeration, the exosome precipitation/supernatant mixture was centrifuged at 3000×*g* for 30 min [44].

2.3.3. Ultracentrifugation

The exosomes were also isolated by ultracentrifugation. For ultracentrifugation, cell culture supernatants were collected after two days of culture, followed by centrifugation at 300×*g* for 10 min at 4 °C to remove dead cells. The supernatants were first centrifuged at 3000×*g* for 30 min at 4 °C to eliminate cellular debris and large macrovesicles and then additionally centrifuged at 100,000×*g* for 70 min at 4 °C (Beckman Coulter, USA). The sediment was resuspended in PBS, and the mixture was centrifuged at 100,000×*g* for 70 min to obtain relatively pure exosomes and stored at −80 °C till use [45].

2.4. Quantification of exosomes

Bradford assay was used for quantification of total protein content to evaluate the concentration of isolated vesicles. In order to reliably investigate the quality and effects of isolated vesicles on the damaged cells, we need to use identical conditions. To reach this goal, we used 10 μg/ml total protein content from each method in the experiment. The absorbance was read at 595 nm in UV–Vis spectrophotometer (Bio-Tek UV–Vis spectrophotometer, USA) [46].

2.5. Exosomes characterization

2.5.1. FESEM

To investigate the morphology and shape of isolated EVs, we used field emission scanning electron microscopy (FESEM). In these experiments, 100 μL of 10 μg/ml exosomes were fixed in a 2% paraformaldehyde aqueous solution. Then the sample was dehydrated in serial of ethanol from 100, 80, 70, 60, and 50%. After dehydration, to increase contrast and to make the surface conductive, the sample was coated with 2–5 nm of gold by sputtering in a SPI-Module Sputter Coater Unit (Structure Probe, Inc., West Chester, PA). SEM was conducted using low beam energies (10.0 Kv) on a Mira III TESCAN Field Emission Scanning Electron Microscope (Brno, Kohoutovice, Czech Republic) [47].

2.5.2. DLS

To investigate size distribution of isolated vesicles, we use dynamic light scattering (DLS). In these experiments, 10 μl of 10 μg/ml isolated exosomes for each method were diluted 100-fold with PBS and Brownian motion was analyzed with a 405 nm laser beam for 300 msec by single exposure, with a total exposure time of 92 s at room temperature. The size of each sample was measured using dynamic light scattering on a Scatteroscope particle size analyzer (K-One Nano, Seoul, South Korea) [48].

2.6. Functionality study of exosomes

2.6.1. Viability analysis (alamarBlue)

AlamarBlue analysis was performed as a cell viability indicator. The blue color of alamarBlue changes to red if the cells are viable. In this analysis, after inducing hypoxic conditions, we utilized exosomes purified by different methods to increase cell viability. 10% (v/v) alamarBlue was added to each well after treatment and the reaction mixture was incubated for 6 h in an incubator 37 °C. Then, we used 570–600 nm Elisa Absorbance Microplate Reader (BioTek ELx800, USA) for viability detection of exosomes [49].

2.6.2. Flow cytometry

To study the effect of exosomes on hypoxia cells, we used flow cytometry (annexin-PI) to show the effects of exosomes isolated by different methods on the percent of live cells % (live cell%). For flow-cytometry analysis, after culturing the cells and induction of the hypoxia, 10^5 cells were collected at 1000×*g* for 10 min and the supernatant was decanted. Then, the cells were resuspended in 1 ml PBS, and after additional centrifugation at 1000×*g*, the supernatant was decanted again. The cells were resuspended in 1 ml binding buffer at concentration of 10^6 cells/mL, then 100 μL of each sample was prepared and 10 μL of staining solution containing 5 μL Annexin V-FITC+ 5 μL PI were added. The resulting reaction mixture was incubated for 15 min at room temperature in the dark, then 400 μL binding buffer were added to each tube, and the final samples were analyzed with flow cytometry [50].

2.7. Statistical analysis

Data are represented as the mean SEM. The significance of differences between two groups was tested by Student's t-test and multiple groups by ANOVA. Differences were considered statistically significant at $p < 0.05$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. All experiments were repeated at least three times.

3. Results

3.1. Characterization of exosomes

The FESEM image results show that vesicles isolated using the ultrafiltration method have more variety in shape that ranges from sphere to ellipsoid, and also show large size variability. On the other hand, vesicles isolated by precipitation and ultracentrifugation methods have lower size variability, and mostly possess uniform spherical shapes (Fig. 1) (scale bare, 100 nm).

To analyze the size distribution of vesicles isolated by three different methods, DLS analysis was conducted. Results of this analysis showed that the isolation method have a noticeable impact on the size distribution of exosomes. Our results show that the size of vesicles isolated by the ultrafiltration method ranges from 40 to 420 nm, with a mean diameter being 122 nm. Results of the analysis of exosomes isolated by the precipitation method showed size distribution ranges from 30 to 220 nm with a mean diameter of 89.3 nm. The analysis of the results for exosomes isolated by the ultracentrifugation method showed that in this case, the exosome size ranged from 30 to 180 nm, with a mean diameter being 60.5 nm (Fig. 2).

3.2. Analysis of the functionality of exosomes isolated by different methods: effects on viability of damaged cells

To investigate the functionality of exosomes isolated by different method on damaged cells, we used viability with alamarBlue and live cell% with flow-cytometry analysis. The same concentrations (10 μg/ml) of isolated vesicles, as evaluated by the Bradford assay, were applied to investigate the efficiency of exosomes. We applied ischemic preconditioning to simulate ischemia injury in H9c2 cells. After inducing

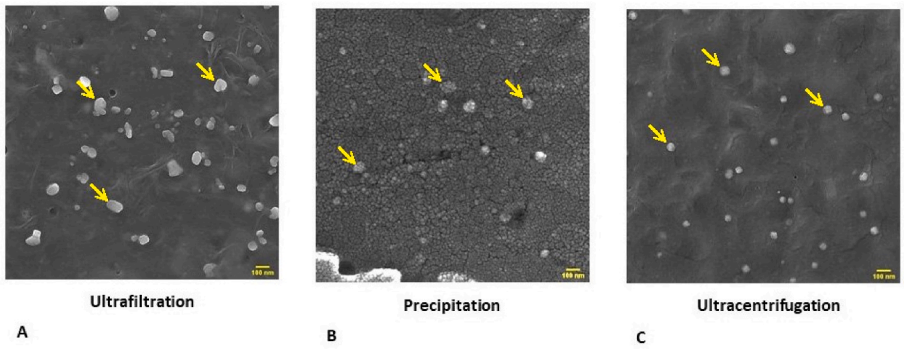

Fig. 1. Representative FESEM image of different exosome isolation methods. The left image shows ultrafiltration methods which show more variety in the shape and size of exosomes. The middle image shows the precipitation method. The right image shows ultracentrifugation methods which show less variety in the size and uniform exosomes with rounded shape. (Arrow heads show exosomes, scale bar = 100 nm).

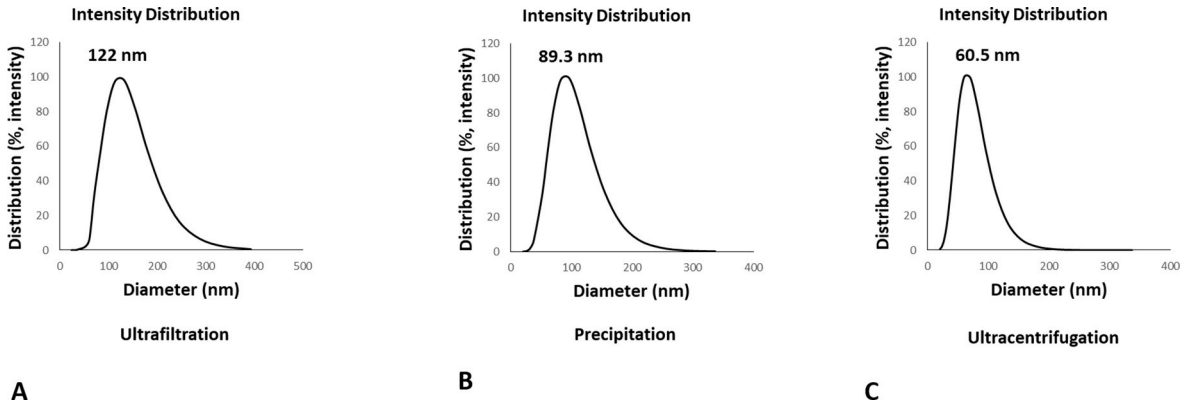

Fig. 2. Dynamic light scattering (DLS) analysis for identifying size distribution of isolated vesicles. The left plot shows ultrafiltration methods which show isolation vesicles from 40 to 420 nm with a mean diameter of 122 nm. The middle plot shows precipitation methods with polyethylene glycol, showing isolation vesicles from 30 to 220 nm with a mean diameter of 89.3 nm. The right plot shows ultracentrifugation methods which show isolation vesicles from 30 to 180 nm and a mean diameter of 60.5 nm.

hypoxia, the viability of damaged cell reached 34.0 ± 2.6%. Treatment of these cells by vesicles isolated from different methods show that viability significantly increases to 45.3 ± 3.1%, 49 ± 3%, and 56.3 ± 1.5% for ultrafiltration, precipitation, and ultracentrifugation methods, respectively (Fig. 3A). All three groups treated with isolated vesicles showed significantly increased viability when compared to that of the control group. Ultracentrifugation had the best effect on the viability of the hypoxic H9c2 cells by increasing viability by 22%. To confirm the viability assay, we used flow cytometry (annexin-PI) analysis to show the functionality of isolated vesicles by different isolation methods on the damaged cells. This analysis showed that after hypoxia live cells% reached 43 ± 2%, treatment of injured cells with exosomes significantly increased viability to 52.33 ± 2.08%, 55.66 ± 2.08%, and 63.33 ± 1.52% for ultrafiltration, precipitation, and ultracentrifugation methods, respectively (Fig. 3B). A representative plot of flow cytometry for evaluating the effect of exosomes from different methods is shown in (Fig. 3*C*–F).

3.3. Study of the industrialization potential of different exosomes isolation methods

In addition to the shape, size distribution, and functionality of exosomes isolated by different methods, their industrialization potential is an important issue. We will consider some parameters for potential industrialization of these approaches in the future. Those parameters include time, sample volume, scalability, cost, and requirement of special equipment. The ultrafiltration method is a simple and rapid approach (the total time requirement is 0.5–1 h). However, the scalability of this method is low, as the sample volume is restricted by 50, 25, and 2 ml tubes. This method also requires an Amicon filter, which makes it an expensive approach. The cost is further increased by the need to use several successive steps to isolate EVs of the precisely desired size [33]. Furthermore, the likelihood of filter plugging increases with the sample size, potentially resulting in lowering the yield [33,51].

Conducting the precipitation method requires longer time (12–16 h or overnight) for isolation. Despite this long time, sample volume and scalability are high. Furthermore, this method does not require special equipment, and its cost is very low.

Ultracentrifugation is the classical method for isolation of exosomes. The time required for this method is 3–4 h. Sample volume and scalability are more restricted than those of the precipitation method, but generally, ultracentrifugation is more scalable than the ultrafiltration method. Obviously, the use of this method requires an ultracentrifuge. A summary of industrialization potential of different exosome isolation methods is presented in Table 1.

4. Discussion

Exosomes are a subtype of EVs, which are membrane-embedded vesicles secreted by the cells into the medium. Exosomes play a number of important roles in the intercellular communication, being critical mediators of organ crosstalk. These vesicles alter the behavior of target cells through autocrine and paracrine signals. The method of exosome isolation might have a strong impact on the shape and size distribution of isolated vesicles. Our results showed that exosomes isolated by

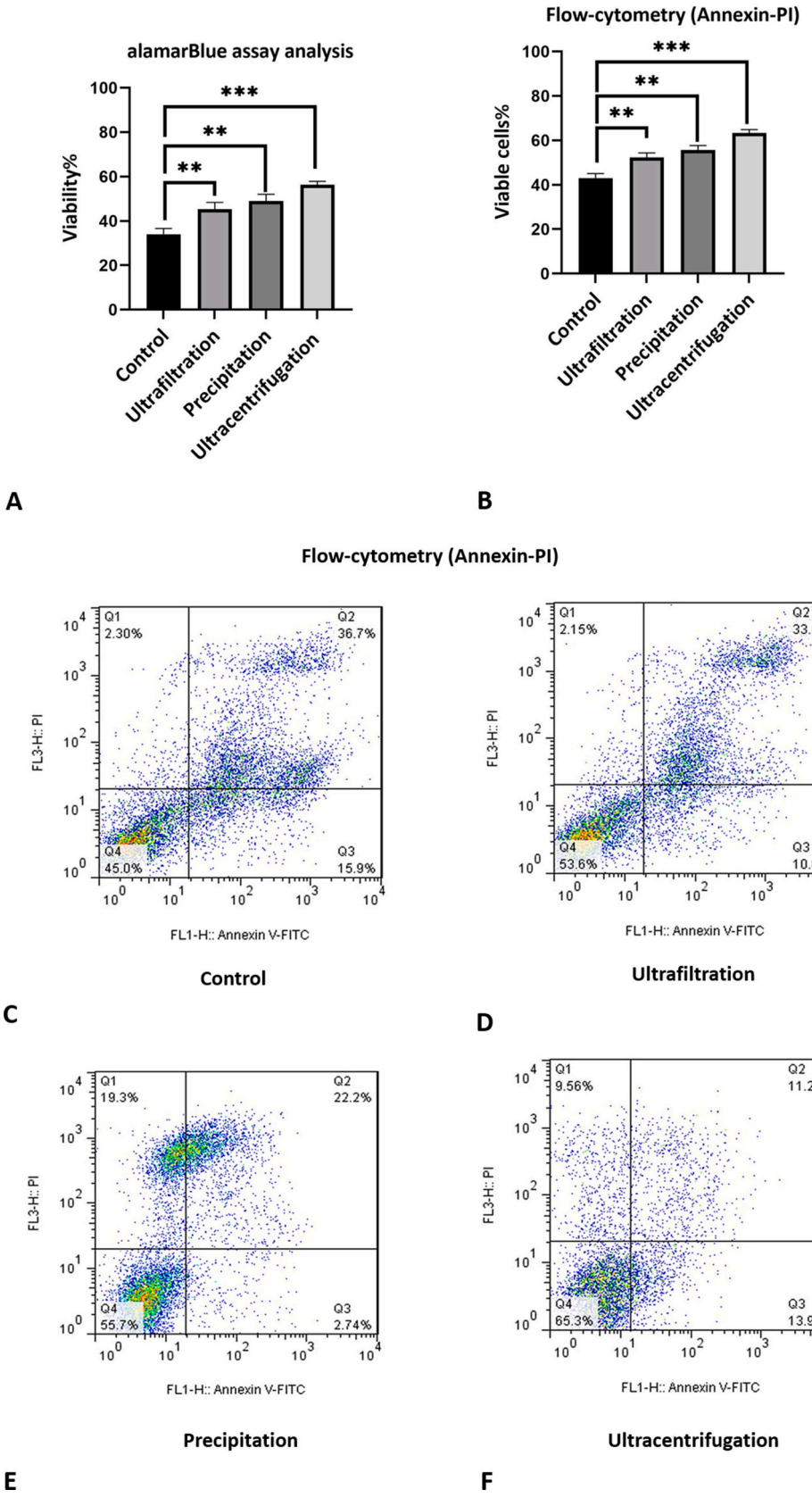

Fig. 3. Functionality assay of exosomes isolated by different methods on damaged cells. **A.** Quantitative alamarBlue results show viability of hypoxia cells is 34 ± 1.52% after treatment with exosomes viability significantly increase to 45 ± 2.64%, 49 ± 3.05%, and 56 ± 3% for ultrafiltration, precipitation, and ultracentrifugation methods, respectively. **B.** Quantitative flow cytometry (annexin-PI) results show live cell% of hypoxia cells is 43 ± 2% at first, and after treatment with exosomes the live cell% significantly increased to 52.33 ± 2.08%, 55.66 ± 2.08%, and 63.33 ± 1.52% for ultrafiltration, precipitation, and ultracentrifugation methods, respectively. ***C*–F.** Representative flow cytometry (annexin-PI) plots of the different treatment groups.

Table 1
Summary table of the industrialization potential of different exosome isolation methods.

	Principle	Time Required	Sample volume	Scalability	Cost	Specialized equipment	Potential for use in industry (Grade)
Ultrafiltration	Based on filtration and size under centrifugal force	0.5–1 h	Low	No	Medium	Yes (Amicon filter)	Low
Precipitation	Differential solubility based precipitation	12–16 h	High	Yes	Low	No	High
Ultracentrifuge	Based on density and size under centrifugal force	3–4 h	Medium	No	High	Yes (Ultracentrifuge)	Medium

ultrafiltration vary noticeably in their size and shape that range from sphere to ellipsoid. On the other hand, exosomes isolated by the precipitation and ultracentrifugation methods show less variety in their size and mostly have spherical shape. This is in line with previous reports, which revealed that the shape of exosomes ranges from sphere to ellipsoid [52–54].

One of the reasons for the higher shape variability of vesicles isolated by ultrafiltration method can be the presence of some other vesicles (and not only exosomes) in the isolated samples. In this method, all the particles larger than 30 nm including protein aggregates and other EVs can be co-separated with exosomes, which possibly reduces the quality and purity of the corresponding exosome samples. Lobb et al. used transition electron microscopy analysis to show that the exosomes isolated by the ultrafiltration method had more size and shape variability than the exosomes isolated by the ultracentrifugation method [55]. Extracellular vesicles are characterized by the high size heterogeneity and include exomers (less than 50 nm), exosomes (30–150 nm), ectosomes (100–1000 nm), migrasomes (500–3000 nm), and apoptotic bodies (1000–5000 nm) [56]. This highly heterogeneous nature of the EV population emphasizes the important impact of the isolation method on the quality of exosomes. Therefore, the use of techniques allowing isolation of vesicles in a narrow size distribution window is highly desirable.

Our results show that ultracentrifugation and precipitation methods isolated vesicles in the range of exosomes 30–180 nm and 30–220 nm respectively, but the ultrafiltration method isolated a wide range of vesicles (40–420 nm). In line with our observations, previous reports showed that ultrafiltration method recovers 1.5 times more particles and produces samples with a wider range of size distribution in comparison with the ultracentrifugation method [55]. Our result are also in line with the data reported by Patel et al. who showed narrower size distribution of exosomes isolated by the ultracentrifugation method than by the precipitation method [57]. In the precipitation method, proteins and particles with the solubility similar to exosomes are isolated from the media [57].

As mentioned above, exosomes contain many metabolites, proteins, and miRNA, which can alter function of target cells. A pervious study showed that exosomes were able to increase the viability of cells when using increasing H_2O_2 content as stress conditions [58]. Such increased viability of cells is due to the autocrine and paracrine factors that exist in the exosomes, such as short fragments of DNA, microRNA, siRNA, and mRNA [59]. Induction of hypoxia in H9c2 cells caused changes in the growth and morphology of the cells, increased cell apoptosis, and reduced cell viability. Zhang et al. [40] showed that 92 miRNA in the exosomes are involved in the HIF-1 signaling pathway or are related to the cell apoptosis pathways, such as MAPK, TNF, and mTOR. Furthermore, this study showed that three miRNA, such as miR-21–5p, miRNA152–3p, and miRNA 378–3p have anti-apoptotic and pro-viability effects in H9c2 cells under hypoxia stress [40]. The results of this study revealed that the H9c2 derived exosomes increased the viability of hypoxia cells by more than 20% [40]. Another study showed that transfer of exosomal miR-21 to the H9c2 cells decreased the level of the oxidative stress-induced apoptosis *in vitro* [13]. Reports also showed that the exosomes derived from the hypoxic cells and enriched in the miR-143 induced migration, proliferation, and survival of cells under stress [14,40].

Analysis of the functionality of exosomes in term of their effect on the cell viability showed that the exosomes extracted by the ultracentrifugation method increased the viability of the hypoxia damaged cells by more than 22%, while exosomes isolated by the precipitation and ultrafiltration methods increased viability by 15% and 11%, respectively. Flow-cytometry results also showed that vesicles isolated by the ultracentrifugation method increased viable cells% of damaged hypoxia cells by 20%, while in the presence of the exosomes isolated by the precipitation and ultrafiltration methods, viable cells% increased by 15% and 9%, respectively. These results show that although exosomes isolated by all three methods significantly increased the viability of the hypoxia damaged cells and their live cell%, in comparison with other methods, the ultracentrifugation allowed extraction of exosomes with higher functionality.

Patel et al. reported that the total protein obtained by the ultracentrifugation method corresponds to half of the total protein isolated by the precipitation method under the same conditions [57]. However, the concentration of miRNA and CD marker was higher in the samples obtained by the ultracentrifugation method. These observations supported high functionality and purity of samples obtained by the ultracentrifugation method in comparison with the precipitation method. These authors also reported that the vesicles isolated by the ultracentrifugation method were characterized by a smaller size than the vesicles isolated by the precipitation method [57]. Furthermore, analysis of the zeta potential as a stability factor of the isolated particles also showed that the ultracentrifugation method generated more stable samples than the precipitation method [57].

In another report, Coumans et al. showed that the precipitation method has a high potential of the exosome contamination [60]. Also, Carnino et al. reported high protein contamination potential of the ultrafiltration method [51]. All these results showed that when compared to precipitation and ultrafiltration, the isolation of vesicles by the ultracentrifugation method generates samples with smaller size, more homogenous shape and size distribution, and higher functionality as tested in terms of the effects on the viability of damaged cells.

In the view of industrialization potential, each methods has its own advantages and disadvantages. Ultrafiltration separates vesicles based on their size and molecular weight. The advantages of this method include low time consumption and easiness of use. The disadvantage of this method is the requirement of the special filter membranes, which are expensive and restrict the scalability of this method [61]. Therefore, the overall industrialization potential of this method is low.

The precipitation method is based on differential solubility of separated vesicles. The advantages of this method are its operational easiness, suitability for small and large sample volumes, overall low cost, and high yield. Some reports showed that the scalability of this method is high and can be used in high throughput sample preparation. The disadvantages of this method include the long time required for isolation (12–16 h) and precipitation of EV and non-EV materials that causes reduction of the purity and quality of the isolated exosomes [60,62]. Since this method is inexpensive and does not require special equipment, it has a high potential for use in industry, especially if the potential impurity issue will be resolved in the future.

The ultracentrifugation method uses centrifugal force to isolate

exosomes. The advantages of this method are high purity, homogeneity, and functionality of the isolated exosomes. The disadvantages are the inability to handle samples with small volumes and the utilization of ultracentrifuges, which are expensive and highly specialized instruments [27,63].

5. Conclusions

Since the use of exosomes in diagnostics and drug delivery is rapidly developing, the elaboration of specialized isolation methods represents a very important task. There are several different approaches that can be used for the exosome isolation, each with its own advantages and disadvantages. In this study, three common exosomes isolation methods, namely ultrafiltration, precipitation, and ultracentrifugation, were compared in the context of shape, size distribution, and functionality of the exosomes they purify.

The ultrafiltration method isolated vesicles with a wide range of shape and size distribution and lower homogeneity than the samples isolated by the precipitation and ultracentrifugation methods. Furthermore, samples isolated by this method had lower functionality than the exosomes purified by other methods. In the view of potential industrialization, this method shows low potential, because its low scalability and requirement of the use of expensive Amicon filters. In the precipitation method, isolated vesicles are smaller in size and are characterized by higher homogeneity than the vesicles isolated by the ultrafiltration method. In comparison with ultrafiltration, this method also generated vesicles with higher functionality as evidences by the higher viability and live cells% in the samples of damaged cells treated with the corresponding exosomes. In the view of industrialization, precipitation method shows high potential, because it is cheap, scalable, and does not require special equipment. Finally, the ultracentrifugation method allowed for isolation of highly homogeneous exosomes with narrow size distribution. Results of the viability and live cells% assays showed that particles isolated by the ultracentrifugation method are more functional than vesicles isolated by the two other methods. In the view of industrialization, this method showed medium potential, because it requires an ultracentrifuge as special equipment, and the scalability of this method is low.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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