RESEARCH PAPER

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Isolation and characterization of human amniotic fluid and SH-SY5Y/ BE(2)-M17 cell derived exosomes

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The application of stem cells as a therapy for degenerative disease holds great promise. Substantial evidence suggests that stem cell derived exosomes are a novel cell-free therapy for the corresponding cells. Exosomes are less complex as compared to their parental cells, due to the fewer number of membrane proteins. In addition, the smaller size and lower risk of immunogenicity makes exosomes potentially safe therapeutic nano-carriers. A large number of ongoing research studies are focused on characterizing exosomes that were derived from different sources, for their potential use in various therapeutic applications. In the present study, we focused on characterizing human amniotic fluid stem cell derived exosomes for future therapeutic applications, such as paracrine therapy/nano carrier. In addition, we characterized exosomes derived from SH-SY5Y and BE(2)-M17 cells, which are a known neuronal model, for further characteristic analyses of neuronal differentiation and neurobiology. Finally, we compared various exosome isolation techniques and procedures and evaluated exosome yield.

Key words: exosome, characterization, human amniotic fluid stem cell, SH-SY5Y, BE(2)-M17

INTRODUCTION

Regenerative medicine has burgeoned, largely due to recent advances in the field of stem cell transplantation. In particular, advances in stem cell transplantation allow for the replacement of damaged tissues with autologous or allogeneic stem cells derived from adipose, blood, bone, and brain tissue, to facilitate the regeneration of damaged or lost tissues. This is in contrast to the only option 20 years ago, which involved grafting the whole tissue, such as bone marrow (Vishnubhatla et al., 2014). Despite the remarkable results reported so far from the early phase trials of stem cell therapy (Matsa et al., 2014), it should be noted that the therapeutic benefits cannot be necessarily attributed to stem cells (Hicks et al., 2013). Indeed, recent studies show that the efficiency of cell implants cannot be achieved without long-term cell survival and cell-conditioned culture medium, suggesting an essential role of secreted paracrine factors (Bi et al., 2007). The paracrine hypothesis not only includes secreted soluble factors, but also extracellular vesicles such as exosomes, which contain similar content and elicit similar biological activity to the stem cells (Camussi et al., 2013).

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Exosomes are nanometer-sized vesicles with a unique morphology, bilayer membrane protein, and lipid composition, and are actively secreted into the extracellular space under normal and pathological conditions (Mincheva-Nilsson et al., 2016). Recent data suggest that exosomes link stem cells to the neighboring diseased or damaged cells and shuttle protein, bio-active lipid, and nucleic acid cargos into the recipient cells (Ratajczak et al., 2006). This process subsequently induces functional and phenotypic changes in the recipient cells.

The advantages of exosomes over cell-therapy, and their similar function to cells (e.g., stem cells), pave the way for the clinical application of stem cell secreted exosomes. Stem cell derived exosomes have been isolated and characterized from cell culture supernatants of several stem cell types, namely human neuronal stem cells (Drago et al., 2013). Despite the fact that secreted exosomes from a variety of cell types exhibit some shared contents, they also express distinctive molecules that identify their producer cells (Taylor et al., 2011). Therefore, characterizing stem cell derived exosomes with different sources will broaden the knowledge of their application in paracrine therapy, and will offer the opportunity to find promising therapeutic approaches. In this study, we aimed to characterize exosomes derived from human amniotic fluid (hAF) stem cells. Based on several previous studies demonstrating that stem cells constitute the majority of human amniotic fluid, we speculated that exosomes isolated from hAF contain a high percentage of stem cell derived exosomes (De Coppi et al., 2007; Dev et al., 2012; Yang et al., 2013; Dziadosz et al., 2016). We also used SH-SY5Y(Walton et al., 2004; Ferlemann et al., 2017) and BE(2)-M17 (Andres et al., 2013; Ross et al., 2015), which are two well-known neuronal models, to explore characteristics of their exosomes and to further understand and explore neurodifferentiation and neurobiology. In addition, we used different sources to compare yield and purity of various exosome isolation methods.

METHODS

Cell culture and sample collection

SH-SY5Y and BE(2)-M17 are human neuroblastoma cell lines that are commonly used for modelling neurodegenerative diseases, and for studying basic mechanisms in neuroscience. SH-SY5Y and BE(2)-M17 cell lines were obtained from Ege University (Department of Medical Biology, İzmir, Turkey) and Pasteur Institute of Iran, respectively. The cells were maintained in a 1:1 mixture of Eagle's minimum essential medium (EMEM) (ATCC, Cat. No. 30-2003) and F12 medium (Merck, Cat. No. M4655) supplemented with 10% fetal bovine serum (FBS) (Gibco, Cat. No. 11573397), 100 U/mL penicillin, and 100 mg/mL streptomycin (Merck, Cat. No. P4333) and incubated at 37°C in 5% CO2. hAF samples, containing stem cells and stem cell derived exosomes, were collected from patients recruited for genetic tests at the Avicenna Clinic (Tabriz, Iran) during the fifteen and sixteen weeks of pregnancy. Samples were collected following written informed consent, and approval by the Tabriz University of Medical Sciences Ethics Committee (no. 5/D/72903).

Exosome harvest

For all experiments, cells were cultured in 1:1 mixture of EMEM and F12 medium 10% FBS with defined cell density (4×10E5 cell/ml) to yield a confluent monolayer of each cell line. Next, the supernatant was replaced with EMEM supplemented with serum, which was free of exosomes (i.e., exosome depleted serum) (Gibco Cat. No. A2720801) for 48 h. After 48 h, the conditioned medium was collected and subjected to Exosome isolation methods.

Exosome isolation

Ultracentrifugation

A two-step centrifugation was carried out to obtain exosomes from the collected conditioned media (from ≈ 16×10E6 cell, 20 ml)/hAF (18 ml). First, the media was centrifugation for 30 min at 48298 × g to dispose of debris and cell residues. Next, the supernatant was filtered through a 0.22 μm filter (Millipore Sigma). Then, ultracentrifugation (60 min at 584401 × g) was used on the filtered supernatant to pellet the exosomes. The pelleted exosome was subsequently re-suspended in 1 ml phosphate buffered saline (PBS). All ultracentrifugation steps were performed using the Beckman Optima TLX Ultracentrifuge (CA, USA) at 4°C.

Exosome isolation kit

Exosomes were isolated from the collected conditioned media (from ≈ 16×10E6 cell, 20 ml)/hAF (18 ml) using the ExoQuick exosome precipitation kit (System Biosciences, Cat. No EXOQ20A-1), following the manufacturer's instructions. The ExoQuick kit contains a nondenaturing water-soluble polymer which, similar to a high salt concentration, can precipitate exosomes. Briefly, cultured conditioned media or collected amni-

otic fluid were mixed with ExoQuick reagent and then incubated for at least 1 h at 4°C. Then, exosomes were pelleted using low speed centrifugation (1500 × g) for 10 min at 4°C. The exosome pellet was resuspended in 1 ml PBS.

Characterization of exosomes

Micro BCA protein assay

The concentration of exosomal proteins was quantified using the Micro BCA protein assay (SMART micro BCA protein assay kit, Cat. No. 23235), according to the manufacturer's instruction. In brief, 200 µl of isolated exosomes were incubated overnight at room temperature with 200 µl lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 1% NP40 and 1 mM EDTA, one protease inhibitor tablet; Merck, Cat. No. 11697498001; PH=7.4). Following the incubation, exosomes were centrifuged for 5 min at 25155 × g and then the supernatant was extracted for the assay. 100 µl of Micro BCA protein assay working solution was added to 100 µl exosome samples (unknown concentration) or standards (known concentration), and then mixed thoroughly. Prepared samples were incubated at 37°C for 2 h, and absorbance was subsequently measured at 562 nm with a spectrometer. Next, we calculated a calibration curve by plotting the absorbance at 562 nm against the concentration of prepared standards. The unknown concentrations of the released exosomes were then determined from the calibration curve.

Flow cytometry

Exosomal surface markers were analyzed using flow cytometry. First, exosomes were isolated as described above and then subjected to staining with antibodies that are specific to exosomal cluster of differentiation (CD) markers. Isolated exosomes were incubated for 1 h at 4°C with the following two antibodies: 4 µg of PE conjugated anti CD63 monoclonal antibody (H5C6, Mouse / IgG1, kappa) (eBioscience, Cat. No. 12-0639-42) and 4 µg of FITC conjugated anti CD9 monoclonal antibody (SN4 C3-3A2, Mouse/IgG1, kappa) (eBioscience, Cat. No. 11-0098-42). Next, the samples were fixed with 5% paraformaldehyde, and the stained and fixed samples were subsequently diluted in 500 µl of filtered PBS and FACS buffer was added. To reduce the impact of nonspecific binding to exosomes, we also had staining with an isotype control targeting irrelevant antigen. Flow cytometry data were acquired using a BD FACSCalibur (BD Biosciences) and analyzed using the FlowJo Software.

SDS PAGE and Western blot

Isolated exosomes (derived from the aforementioned ultracentrifugation protocol) were submitted to a western blotting analysis to examine exosomal surface markers. Exosome samples (derived from cell/hAF) were lysed in 500 µl of RIPA extraction buffer (Thermo Scientific, Cat. No. 89900) and one protease inhibitor tablet (Merck, Cat. No. 11697498001). Protein concentration was quantified by the SMART Micro BCA protein assay kit. Then, approximately 60 µg of total protein per sample was subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at a constant 60 V at room temperature for 2 h. Next, the separated proteins were blotted onto polyvinyl-difluoride (PVDF) membrane (Merck, Cat. No. 3010040001) at a constant 150 mA for 1 h. Membranes were then blocked with 10 ml of 5% non-fat dry milk (Merck, Cat. No. W236101) for 1 h at room temperature, and then washed and incubated with 5 µg of the corresponding primary antibodies (i.e., mouse monoclonal IgG1 targeting CD9 [Ts9], CD63 [Ts63] and D81 [M38], for Cat. No. 10626D, 10628D, 10630D respectively; eBioscience) overnight at 4°C. Lastly, the membrane was probed with 1 µg of horseradish peroxidase-conjugated secondary antibody, and Goat anti-Mouse IgG Fc Secondary Antibody (eBioscience, Cat. No. A16084). Protein detection was carried out by exposing the membrane to an enhanced chemiluminescence (ECL) western blotting detection system (INTAS Advanced Fluorescence Imager, INTAS Science Imaging).

Dynamic light scattering

Exosomes were isolated from SH-SY5Y and BE(2)-M17 conditioned medium and hAF via ultracentrifugation. Next, to estimate the exosome's diameter distribution, we performed a dynamic light scattering (DLS) analysis on the isolated exosomes. The mean hydrodynamic diameter of exosomes was calculated by fitting a Gaussian function to the measured size distribution. Prior to the measurements, exosome samples were shaken at 4°C for 10 min to avoid possible inter-particle interaction and aggregation. About 500 µl of each sample was added to a cuvette and DLS measurements were conducted at 25°C using a Zetasizer Nano ZS90 (Malvern Instruments, UK). The sample temperature was equilibrated with room temperature for 10 min before each measurement. Light scattering was recorded for 10 s.

Statistical analysis

Differences between groups were analyzed by means of a Student's *t*-test or one- way ANOVA using Graph

Pad Prism 6.01. A *p* value of 0.05 (one-tailed) or less was considered to be statistically significant. All values are presented as mean ± SD (three independent repeat).

RESULTS

Exosomal yield varies with isolation method but not with the cell passage's number

Exosomal yield using different isolation methods was defined as the concentration of exosomal proteins. There was a significant difference between the two isolation methods in terms of exosomal yield, such that higher yield was found for the ultracentrifugation isolation method as compared to commercial reagents. The protein concentration of isolated exosomes via ultracentrifugation/reagent method for SH-SY5Y, BE(2)-M17 and hAF is as follow: 818.5± 75.9/139.6±29.4, 223.7±47/106.6±10.3, and 558.9±53.8/352.7±32.8 (see Fig. 1). Given these results, we chose ultracentrifugation as an isolation method for the rest of the experiments.

Exosomes were isolated from the conditioned medium of SH-SY5Y and BE(2)-M17 cell cultures at various passage numbers (i.e., P2, P3, P4, P5 and P6) using ultracentrifugation. Of note, the cell passage for cell lines were designated as P1 upon arrival from the corresponding cell bank. Then, the concentration of produced exosomes was quantified via Micro BCA protein assay. We found that exosomal protein concentration, which reflects exosome production rate, did not significantly differ between the cell passage numbers (p>0.05, see Fig. 2). This experiment was performed in triplicate.

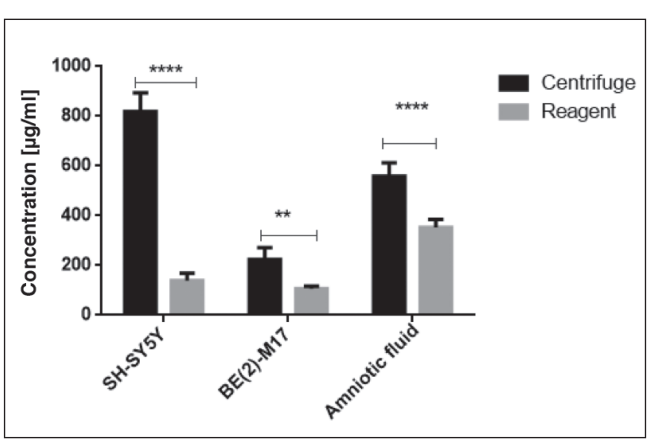

Fig. 1. Quantitative comparison between different exosome isolation methods. Note that ultracentrifugation was associated with higher exosomal yield as compared to polymer based precipitation. The y axis shows total protein concentration, as measured by a Micro BCA protein assay. Statistical analyses were performed using a one-way ANOVA, (n=5). ****p<0.0001, **p=0.0016.

hAF /SH-SY5Y, BE(2)-M17 cells exosomal surface markers: CD9, CD63

The presence of CD63 and CD9 on the surface of exosomes derived from SH-SY5Y and BE(2)-M17 cells and hAF was confirmed by flow cytometry (Fig. 3A). To better characterize the exosomes derived from different sources (i.e., SH-SY5Y and BE(2)-M17 cells and hAF), we performed a flow cytometry-based evaluation of CD9 and CD63 markers. Flow cytometry results showed that exosomes derived from all three tested sources (i.e., SH-SY5Y and BE(2)-M17 cells and hAF) were positive for all investigated markers. Further, we found less CD63 and CD9 in SH-SY5Y and BE(2)-M17 cells (p<0.0001) and CD9 (p<0.0001) as compared to in hAF derived exosomes (Fig. 3B).

Immunoblotting confirms flow cytometry results

To confirm the flow cytometry results, we probed for CD63 and CD9 markers using western blot analysis. Protein lysates were prepared from the isolated exosomes (i.e., SH-SY5Y and BE(2)-M17 cells and hAFs sample). In accordance with the results obtained by flow cytometry (Fig. 3), we detected expression of both CD63 and CD9 in 60 µg of protein lysates. Of note, exosomes were isolated via the ultracentrifugation method and lysed with RIPA buffer. In addition to these markers, we tested protein lysates of exosome samples for the expression of another well-known exosome marker, CD81. Results showed the expression of this protein marker in all samples (i.e., SH-SY5Y, BE(2)-M17, and hAF; see Fig. 4). Anti-CD63 staining showed stronger expression of CD63 in hAF samples as compared to the other

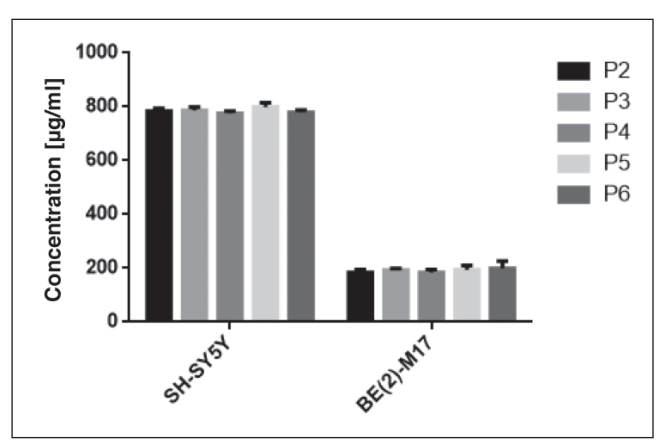

Fig. 2. Cell culture passage number was not associated with differences in exosomal production rate. Note that no difference in exosomal production rate between passage numbers P2, P3, P4, P5, P6. ****p<0.0001, **p=0.0016.

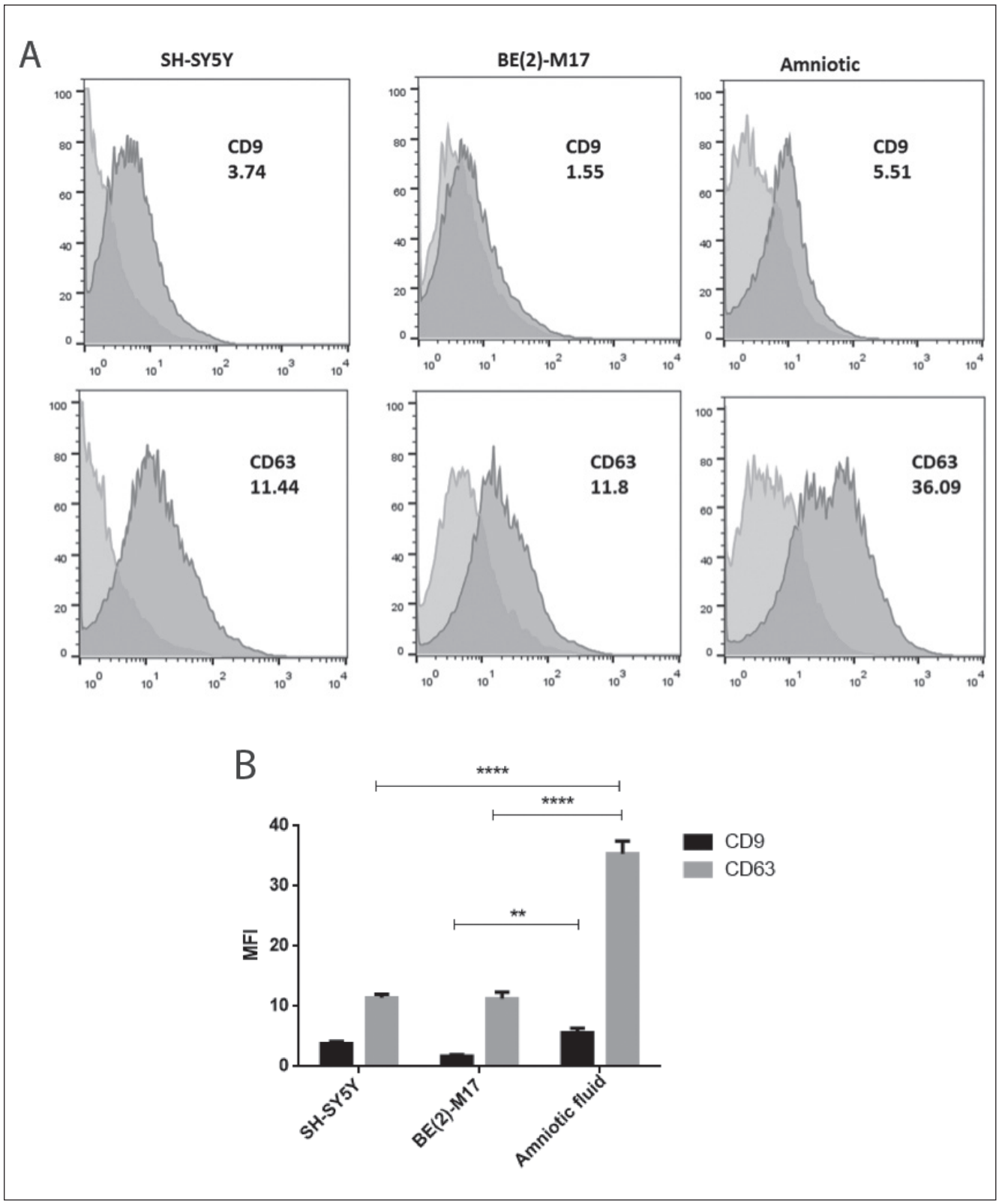

Fig. 3. Flow cytometry detection of exosomal surface markers. (A) Flow cytometry analysis showed the expression of both exosomal markers (i.e., CD9 and CD63) on the surface of exosomes derived from all three samples (i.e., SH-SY5Y, BE(2)-M17, and hAFs). (B) Comparison of mean fluorescent intensity (MFI) for all three samples. Results show higher expression of CD63 expression on the surface of exosomes derived from hAF than those derived from both SH-SY5Y and BE(2)-M17 cells. ** p=0.002, **** p<0.0001, n=3.

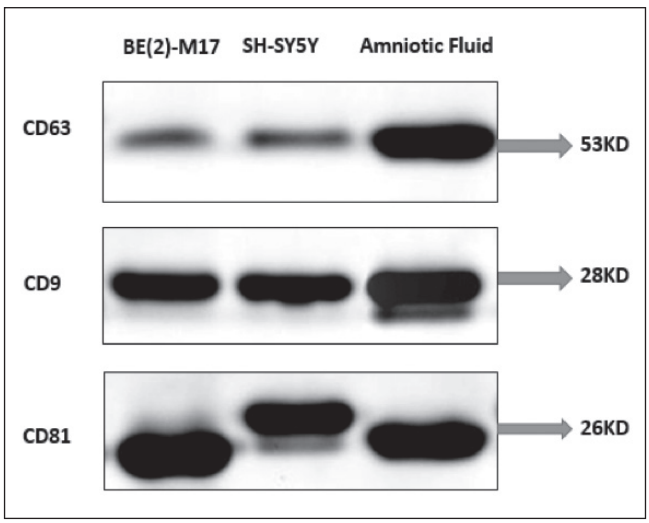

Fig. 4. Western blot analysis of CD63, CD9, and CD81 expression on the surface of exosomes. Data showed higher expression of CD63 and CD9 in hAF samples as compared to exosomes derived from SH-SY5Y and BE(2)-M17 lines. Expression levels for CD81, in contrast, did not significantly differ between samples, using an equal amount of protein lysates from samples.

two cell lines (i.e., SH-SY5Y and BE(2)-M17), which was consistent with the fluorescent intensity analysis via flow cytometry. Similarly, we found slightly higher (not significantly different) CD9 expression in hAF derived exosomes compared to SH-SY5Y and BE(2)-M17 cells. However, CD81 was detected equally across all three samples (i.e., SH-SY5Y, BE(2)-M17, hAF).

Physical characterization of exosomes by DLS

Sample size distribution of hAF-cell derived exosomes was measured using DLS. We found that average exosome diameter was 54.33±20.35, 49.78± 3.43, 21.44±6.11, respectively, for isolated exosomes from hAF, SH-SY5Y, and BE(2)-M17 (Table I). As shown in Fig. 5, hAF-cell derived exosomes formed a bell-shaped size distribution profile. The size distribution profiles were similar for exosomes from the aforementioned sources, with a polydispersity index ranging from 0.433 to 0.483, which is indicative of homogeneous exosome preparations (Table I).

Table I. Physical characterization of exosomes derived from human neuroblast cell lines and hAF.

Exosome source	Size (nm)	Polydispersity Index (PdI)	*Z*-potential (mV)
hAF	54.33 ± 20.35	0.471	-23.6 ± 6.42
SH-SY5Y	49.78 ± 23.43	0.483	-23.6 ± 6.42
BE(2)-M17	21.44 ± 6.11	0.433	-23.6 ± 6.42

DISCUSSION

The last decade has witnessed a growing number of research studies on exosomes. Exosomes are considered to be important intercellular communicators that are capable of transporting and transferring all types of biomolecules. This capacity makes exosomes valuable tools for diagnostic purposes, or as potential nanocarriers (Soria et al., 2017). A large number of studies have been conducted on exosomes, and these studies have answered many questions regarding the biogenesis, characteristics, functions, and physiological role of exosomes. However, their role and importance in degenerative diseases – and in particular, neurodegenerative disease – is yet to be completely understood (Quek et al., 2017). Given that exosomes protect their cargoes from degradation, screening of exosomes, particularly in cerebrospinal fluid (CSF), may offer a diagnostic tool and may provide insights into pathogenic processes in the central nervous system (CNS), which is typically difficult to access. On the other hand, the ability of exosomes to cross the blood-brain barrier and to pass into CSF highlights their potential application as drug delivery vehicles targeting the CNS (Jan et al., 2017). Recently, it has been shown that exosomes secreted from neuronal stem cells play a critical role in the CNS niche (Luarte et al., 2016). These results highlight the merit of neuronal stem cell derived exosomes as diagnostic tools and therapeutic delivery vehicles, and demand more research in this field. In this study, we focused on two well-known neuronal models (i.e., SH-SY5Y and BE(2)-M17 (Alberio et al., 2012)), as well as, human amniotic fluid stem cell, and characterized exosomes derived from each source. A large number of studies have reported that the majority of cells in human amniotic fluid are stem cells, and based on these results, we speculated that the majority of exosomes are from stem cells (Dev et al., 2012). In addition, the present study highlighted some technical points regarding isolation method using three different sources.

Our data showed that exosome yield varies with isolation method, but not with cell passage. Using two different isolation methods (i.e., ultracentrifugation and commercial reagent) on three different exosomal sources (including two neuronal cell models and hAF), we showed that exosomal yield is higher following ultracentrifugation as compared to commercial reagent. These results are in agreement with Gheinani et al. (2018), who used urinary samples to demonstrate that the ultracentrifugation method produced the highest exosomal yield. However, the authors also noted high variability in this method. Nonetheless, it is noteworthy to mention that the ultracentrifugation method

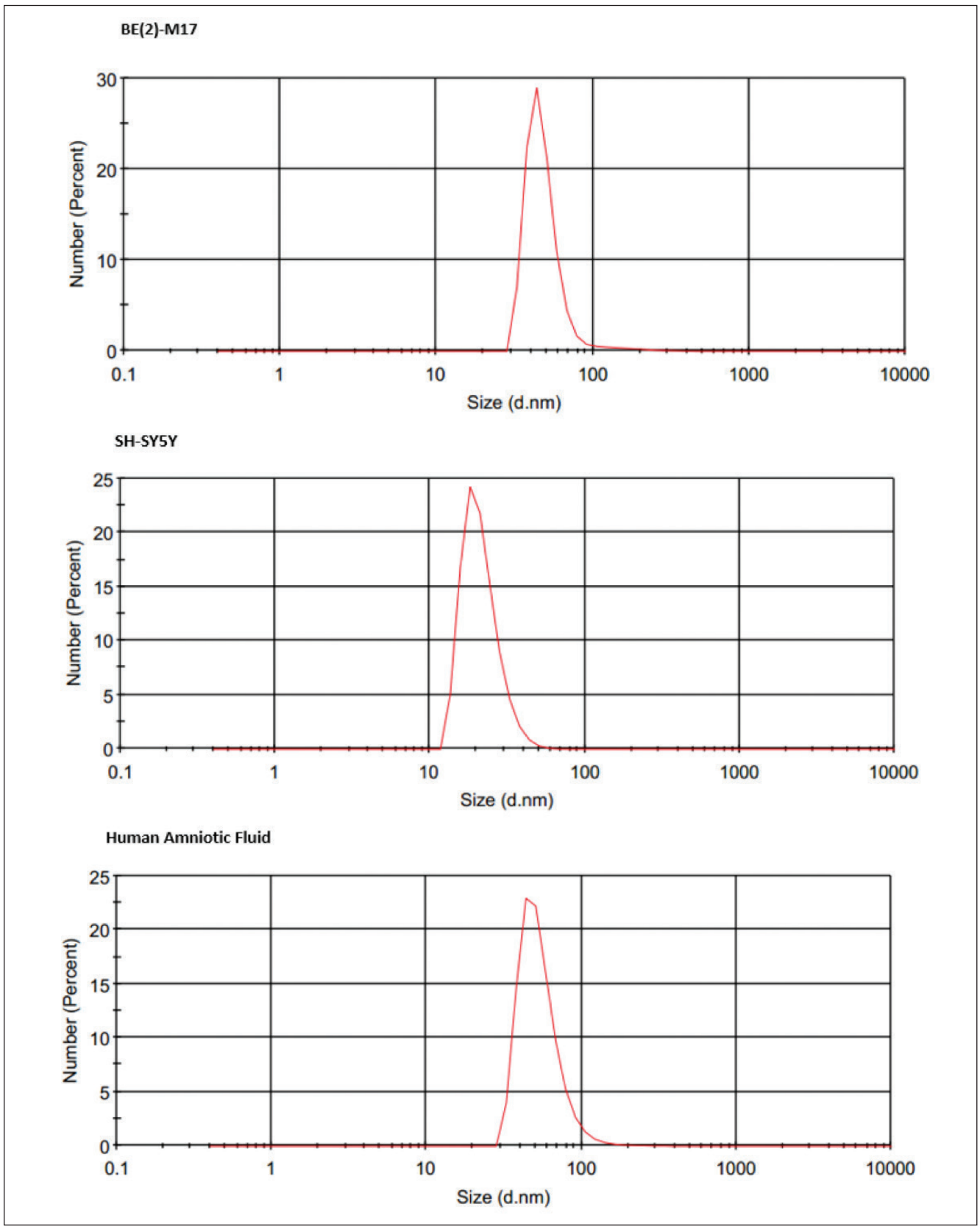

Fig. 5. Size distribution of exosomes derived from different sources. Of note, exosomes were isolated using the ultracentrifugation method. Each curve shows means ± SD from three replicates.

has not been proven to be an ideal method to isolate exosomes from serum. For example, albumin contamination was shown to be higher with ultracentrifugation compared to commercial reagents and size exclusion chromatography (Baranyai et al., 2015; Helwa et al., 2017). Our results also demonstrate that the effect of cell passage number can be ignored, as we observed no significant differences between exosomal production rate for different passage numbers. These null results are in agreement with another study that assessed the potential impact of cell passage number on mesenchymal stem cell exosome production. In that study, nanoparticle tracking analysis was used, and no difference was observed between different passage numbers (Patel et al., 2017).

Further, immunoblot and Flow cytometry analysis revealed that all samples (i.e., SH-SY5Y, BE(2)-M17, and hAF) were enriched with CD63 (Logozzi et al., 2009), CD9 (Yoshioka et al., 2013) and CD81 (Welker et al., 2012), which are protein markers associated with exosomes,. Our data also show uniform expression of CD81 in exosomes derived from all sources investigated in this study. However, there was higher expression of CD9 and CD63 in exosomes derived from hAF as compared to those derived from SH-SY5Y and BE(2)-M17.

The expression of conventional exosome CD markers (CD63, CD81, CD9) has shown for the first time by Balbi et al. (2017) who performed fluorescence-activated cell sorting (FACS) analysis on conditioned medium from hAFs. In their study, they kept hAFs in culture to enrich the medium and isolate the extracellular vesicles. In the present study, in contrast, we isolated exosomes directly from hAF without manipulating conditions to obtain *in vivo* stem cell secreted exosomes and characterize them by determining protein markers. This approach insight to naturally occurring exosomes in human fluids. Nonetheless, results of the present study together with Balbi et al. provide *in vivo* and *in vitro* evidence that secreted exosomes from hAFs express conventional exosome markers, including CD63, CD9, and CD81. In addition, a comparison of hAF derived exosomes with two established human neuronal cell models showed higher expression of these markers in hAF.

Physical characterization via DLS showed homogenous size distribution for exosomes isolated using ultracentrifugation for all three samples. The observed exosomal diameter ranged on average between 20–55 nm, which is in agreement with prior research on exosome diameter range. In another study done by Park et al. (2015) SH-SY5Y derived exosomal diameter was measured using dynamic light-scattering and showed a diameter range of 50–170 nm.

CONCLUSION

Although a large number of research studies have focused on functional analysis and characterization of exosomes secreted by different stem cells, few studies have reported on hAF derived exosomes. Our results may offer meaningful initial insights on the characterization of hAF derived exosomes for future therapeutic applications. Indeed, hAF derived exosomes can be easily isolated from left over samples of amniotic fluid from amniocentesis, or during cesarean delivery without ethical concern. On the other hand, characterizing SH-SY5Y and BE(2)-M17 derived exosomes as well-known neuronal models will help to advance our understanding of neurobiology and neuronal differentiation, and develop new therapeutic approaches for neurodegenerative disease. In addition, major challenges need to be addressed to bring exosome technology into clinic, such as developing efficient standardized isolation and purification methods.

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