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Original Article

A simple, economical, and high-yield method for polyethylene glycol-based extraction of follicular and serum-derived extracellular vesicles

Aye Aye Khine[a], Pao-Chu Chen[b], Ying-Hsi Chen[b], Sung-Chao Chu[c,d], Yu-Shuan Chen[e,f,g], Hsuan-Shun Huang[a], Tang-Yuan Chu[a,b,d,h]**

[a]Center for Prevention and Therapy of Gynecological Cancers, Department of Research, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan, [b]Department of Obstetrics and Gynecology, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan, [c]Department of Hematology and Oncology, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan, [d]School of Medicine, College of Medicine, Tzu Chi University, Hualien, Taiwan, [e]Department of Medical Research, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan, [f]Bioinnovation Center, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan, [g]Center for General Education, Tzu Chi University, Hualien, Taiwan, [h]Institute of Medical Sciences, Tzu Chi University, Hualien, Taiwan

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Abstract

Objectives: The optimization of polyethylene glycol (PEG)-based extracellular vesicles (EVs) extraction from human follicular fluid (FF) and serum was investigated, and their functional analysis was confirmed. The PEG-based EV results were compared to the ExoQuick (ExoQ)-based EV. **Materials and Methods:** FF-EVs and serum-EVs were extracted by using different concentrations of PEG (8000). Nanoparticle tracking analysis was used to count the particles, and electron microscopy of EVs was performed for visualization. Exosomes were confirmed by the western blot analysis with exosome-specific markers. RNA and microRNA were extracted from exosomes and quantitative polymerase chain reaction analysis was performed. Fallopian tube epithelial (FTE) cells were used for the EV uptake experiment and an anchorage-independent growth test to confirm that extracted EVs harbor transformation activity. **Results:** The PEG 8% enriched method produced the highest yield and the lowest carry-over protein. Salt containing PEG 8% produced a higher yield than nonsalted PEG 8%. Overnight enrichment increased four times and 18 times for PEG 8% and ExoQ-based EV extraction from FF. For serum EV, the same overnight enrichment moderately increased yield for both PEG 8% and ExoQ methods. Less carry-over protein resulted in more EV-promoted transformation activity. **Conclusion:** This study overcomes the time-consuming, expensive, laborious, and complicated machine-dependent EV extraction methods. The study highlights that longer incubation time is needed for EV extraction from FF. PEG 8000-based EV extraction provided a higher yield and less carry-over protein than ExoQ-based EV extraction.

Keywords: *Extracellular vesicles, Follicular fluid, Polyethylene glycol*

Introduction

Extracellular vesicles (EVs) are diverse, nano-sized, lipid bilayer-enclosed structures released from cells and recycled in body fluids. Unlike the cells, EVs are not able to self-replicate, thus their purpose is cell-to-cell communication [1-6]. The size of EVs is wide-ranged, from 30 nm to 2000 nm in diameter depending on their origin. EVs are classified according to their size, produced from cellular origin, and their biological function. Three main classes of EVs are exosomes, microvesicles, and apoptotic bodies. Exosomes are 30–150 nm in diameter, produced from intraluminal budding of multivesicular bodies and fusion of multivesicular bodies with cell membrane. Exosomes transport mRNA, microRNA (miRNA), and other noncoding RNAs, cytoplasmic and membrane proteins including receptors and major histocompatibility complexes. Microvesicles are 50–1000 nm in diameter and produced by cell membrane outward budding.

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**Address for correspondence:* Dr. Hsuan-Shun Huang, Center for Prevention and Therapy of Gynecological Cancers, Department of Research, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, 707, Section 3, Chung-Yang Road, Hualien, Taiwan.
E-mail: huang.sansam@gmail.com
Dr. Tang-Yuan Chu, Department of Obstetrics and Gynecology, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, 707, Section 3, Chung-Yang Road, Hualien, Taiwan.
E-mail: hidrchu@gmail.com

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They carry similar molecules such as exosomes, such as mRNA, miRNA, noncoding RNAs, cytoplasmic proteins, and membrane proteins including receptors. The large-sized apoptotic bodies are 500–2000 nm in diameter, produced from outward blebbing of the apoptotic cell membrane and contain nuclear fractions and cell organelles [7].

Exosomes are extensively investigated in different medical fields for their biological and pathological functions and applications as disease biomarkers, therapeutic carriers, and therapeutic targets [8]. The isolation and separation of exosomes are particularly important for research and clinical application. Various methods for exosome isolation are now available, including ultracentrifugation, ultrafiltration, size-exclusion chromatography, precipitation, immunomagnetic bead capture, aptamer-based isolation, and microfluidic technology-based isolation. However, many of these methods have limitations, such as being time-consuming, costly, labor-intensive, low-yield, and requiring complex machinery [9]. Therefore, the quick and cost-effective polyethylene glycol (PEG)-based precipitation method becomes considerable for exosome separation from biological fluid and cell culture medium [10].

Follicular fluid (FF) is an important tissue fluid that incubates the development of oocyte within the ovarian follicle. More importantly, we have proved that FF played an essential role in the transformation of fallopian tube epithelial (FTE) cells into high-grade serous carcinoma of the ovary [11-14]. We have also discovered that FF-EVs carried the majority of the transforming activity and the transforming factors in FF (manuscript under review).

In this study, using FF and serum as the starting sample, we tested the different conditions for EV purification by PEG partition and compared it with the commercially available ExoQuick (ExoQ) reagent.

Materials and methods

Cell lines and drugs

PEG 8000 (40 g; Sigma-Aldrich, St. Louis, MO, USA) was used for EV extraction. To detect exosome makers, cluster of differentiation (CD) 9 (A1703), CD81 (GTX637265), Hsc70 (A2487), beta-actin (#4970), and albumin (GTX102419) were purchased. For the EV uptake and anchoring-independent growth (AIG) test, fallopian tube epithelial cell lines, FE25, in which disrupted p53 and Rb pathways by HPV16 E6/E7 were used [15]. The cells were cultured in MCDB105 and M199 media (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin.

Follicular fluids

The recruitment of FF samples was done from an *in vitro* fertilization program during the oocyte retrieval process from women. A transvaginal needle was introduced to aspirate one follicle after another under the guidance of sonography after HCG injection. FF specimens were checked through spectrophotometry at OD418 nm of hemoglobin absorption to avoid blood contamination [16]. FF from five subjects were pooled, samples ID 230064-14, 230066-36, 230068-12, 230071-12, and 230122-21, centrifuged at 1500 g for 15 min to remove the cells. Then pooled FF was filtered by 0.22 μm size and stored at −80°C. The serums were obtained from the subjects who underwent an *in vitro* fertilization program. This study was conducted in accordance with the Declaration of Helsinki. The collection and use of the specimens were approved by the Institutional Review Board of Tzu Chi Medical Center, Taiwan (Approval IRB-106-07-A, and IRB108-12-A). Informed consents were obtained from all participants.

Polyethylene glycol preparation, extracellular vesicle extraction, and nanoparticle tracking analysis

Polyethylene glycol preparation and extracellular vesicle extraction

PEG 8000 (Sigma-Aldrich, St. Louis, MO, USA) was first prepared in 40% concentration by dissolving 4 g of PEG in 10 mL of PBS, with or without 0.5 M NaCl. Air bubbles formed after mixing of PEG and PBS, which were resolved after incubation at 4°C overnight. A clear 40% PEG solution was then added to 100 μL of FF to a final concentration of 4%, 5%, 6% 7%, 8%, 9%, or 10% PEG for the EV extraction. The commercialized ExoQ precipitation reagent (# EXOQ5A-1, SBI, USA) was used to extract EVs as the control method. ExoQ solution was added into FF in a 1:4 ratio. The mixture was incubated at 4°C overnight or 1 h. EVs were pelleted down by centrifugation at 2200 g for 15 min and dissolved in 100 μL of exosome-depleted FBS for further use.

Nanoparticle tracking analysis

EVs were subjected to nanoparticle tracking analysis (NTA) to determine EV size and concentration. A NanoSight NS300 instrument (Marvell Panalytical, Malvern) with a 488 nm laser and sCMOS camera module (Malvern Panalytical) was used according to the manufacturer's instructions. Three videos of 60 s were recorded for each sample at a camera level of 14–15. Data analysis was done by NTA 3.2 analytical software (Malvern) with a detection threshold of 13.

Western blot analysis

PEG-based and ExoQ-based EVs in PBS were measured by total protein concentration by Bradford assay dye (BIO-RAD, 500-006). About 10 μg of total protein of EVs in PBS were subjected to the SDS-PAGE for protein separation. An equal volume of ×2 Laemmli sample buffer was added, heated at 95°C for 5 min, and cooled on ice for 15 min. After running SDS-PAGE to separate the proteins, the bands were transferred onto a polyvinylidene fluoride membrane. The membrane was blocked for 1 h with 5% skim milk in Tris-Buffered Saline with 0.05% Tween (TBST) to prevent nonspecific binding, then incubated overnight at 4°C with primary antibodies targeting CD9 (A19655, Abclonal), CD81 (GTX637265, Gene Tex), Hsc70 (A2487, Abclonal), beta-actin (#4970, Cell Signaling), and albumin (GTX102419). After incubation, the membrane was washed with TBST, followed by exposure to appropriate horseradish peroxidase-conjugated secondary antibodies and staining with the ECL Western blot detection reagent (GE Healthcare, RPN2209).

RNA extraction and quantification

RNA extraction and purification kit (DPT-BD30) was used to extract RNA from EV samples. The pelleted EVs were dissolved with 1 mL of RBZ buffer and incubated at the room temperature for 5 min. Then, 200 μL of chloroform was added and incubated at the room temperature for 10 min, followed by centrifugation at 13,000 g for 15 min at 4°C. The clear upper layer was carefully transferred to a new Eppendorf and an equal 0.5 volume of ice-cold 96%–100% ethanol was added. After mixing, the sample was transferred into a spin column, and centrifuged at 13,000 g for 10 min at 4°C. The column was washed with 500 μL of RBD buffer and the same amount with RBW buffer and centrifuged as mentioned above. To remove ethanol, an empty spin column was centrifuged at 13,000 g for 2 min. Thirty microliters of RNAase-free ddH_2O were added and incubated at room temperature for 2 min. RNA was eluted by centrifugation as mentioned above. The RNA samples were converted to cDNA, analyzed for quantity and purity using a Thermo Nanodrop, and then stored at −80°C.

MicroRNA extraction and quantification

Exosomal miRNA was extracted using the SeraMir Exosomal RNA amplification kit (#RA800A-1). The commonly referenced miRNAs, RNU6B and miRNA-16, were converted to cDNA by reverse transcription-polymerase chain reaction (RT-PCR) using the TOOLS miRNA RT kit (TTH-mi250) with specific stem-loop primers. The RT-PCR was conducted under a thermocycler at 42°C for 15 min and 95°C for 3 min. The quantity and purity of the miRNA and cDNA were analyzed using a Thermo Nanodrop. Real-time PCR was then performed using specific forward and reverse primers along with TOOLS Easy SYBR qPCR Mix (#FPT-BB01-4) on an ABI QuantStudio 5 system (A28138) with an initial 95°C for 15 min, followed by 40 cycles of 95°C for 10 s and 60°C for 20 s. The primer sequences are shown in Table 1.

Extracellular vesicle uptake assay

The experiment began by culturing 1 × 10^5 FE25 cells in a 6-well plate. PEG-EVs and ExoQ-EVs isolated from 500 μL of FF were stained with 1 μM calcein AM (Cat# BMD00064) in 500 μL PBS at room temperature for 1 h, following a modified method from the literature [17]. After staining, the EVs were washed and resuspended in 500 μL PBS. The stained EVs were then added to the FE25 cells in each well, using a volume equal to 10% of the well's total volume. The cells were incubated at 37°C for 1 h, followed by washing with PBS to remove any EVs remaining in the supernatant. Finally, fresh medium was added to the cells, and EV uptake was observed using an inverted fluorescence microscope with differential interference contrast and enhanced green fluorescent protein (EGFP) channels.

Table 1: The sequence of microRNA primers

miRNA type	Primers	Sequence
miR-16	Forward	TAGCAGCACGTAAATATTGGCG
miR-16	Reverse	ATCCAGTGCAGGGTCCGAGG
miR-16	Stem loop primer	GTCGTATCCAGTGCAGGGTCCGA GGTATTCGCACTGGATACGACCGCCAA
RNU6B	Forward	GCAAATTCGTGAAGCGTTCCA
RNU6B	Reverse	ATCCAGTGCAGGGTCCGAGG
RNU6B	Stem loop primer	GTCGTATCCAGTGCAGGGTCCGA GGTATTCGCACTGGATACGACAAAAAT

miRNA: MicroRNA

Anchorage-independent cell colony formation assay

The principle of this experiment was to test the single-cell ability to transform into three-dimensional colonies under attachment-free conditions. In a 96-well plate, two layers of agarose gel were prepared: a lower layer with 0.8% gel and an upper layer with 0.4% gel. 1000 FE25 cells in 0.4% agarose gel were seeded above 0.8% gel. Both layers were prepared in a serum-free medium. PEG-based and ExoQ-based extracted exosome pallet was dissolved by exosome-free FBS (Gibco™ A25904DG) into their original volume of FF. Ten microliters of exosome was added and serum-free medium was replenished every 2 days to maintain gel moisture. The spheroid colonies >50 μm in the well were counted under the microscope after 10 days.

Statistical analyses

All the experiments were performed in duplicate or triplicate and repeated in three independent replicates. Data presented as mean ± standard deviation represent three or more independent experiments from technical replicates. Statistical analyses were performed using GraphPad Prism (ver. 5.0c; GraphPad Software, Boston, MA), Excel (Microsoft, Redmond, WA), or SPSS 19.0 (IBM SPSS Statistics, Armonk, NY). Differences between groups were examined using unpaired Student's *t*-tests. Statistical significance was set at $P < 0.05$.

Results

Optimization of the polyethylene glycol-based method of extracellular vesicle extraction from follicular fluid

FF-EV was extracted by different concentrations of PEG and the commercial kit ExoQ. In the manner of the PEG, we referred to the literature, adding 0.5 M NaCl to increase the affinity of PEG with EV [18,19]. Figure 1 shows the PEG-partitioned EV pellet enriched by different preparations. Other than the 4% PEG, the other preparations of 5%–10% all showed a visible white pellet comparable to the ExoQ. Total proteins in EV-rich pellet (EV-R) and EV-poor supernatant (EV-P) from the FF specimen and serum specimen were resolved by SDS-PAGE. The albumin protein was abundantly present in the EV-P but was scarce in EV-R in both samples, especially in the more efficient preparations with PEG 7%, 8%, 9%, 10%, and ExoQ [Figure 2a-c]. Western blot analysis confirmed the presence of exosome-specific markers, Hsc70, CD9, and CD81 in EV-R with less abundance of albumin as compared to the original FF [Figure 2d]. The opposite distribution of these bands in the original FF further indicated the efficacy of the purification. Interestingly, two major bands around 50 kD and 54 kD were specifically present in EV-R and absent in EV-P. The nature of them remains to be clarified. In addition, the faint 25 kD band coincided with the sizes of CD9 (24 kD) and CD81 (26 kD) [Figure 2a, Arrow]. The total RNA yield was higher in the PEG 6% (183 ng/μL) and

PEG 8% (172 ng/μL) extractions compared to the ExoQ extraction (137 ng/μL) [Figure 2e]. We also compared the amount of miRNA, which was 16.5 ng/μL for PEG6%-EV, 15.6 ng/μL for PEG8%-EV, and 16.1 ng/μL for ExoQ-EV. In addition, miRNA was reverse transcripted into cDNA by using Stem loop primers of commonly referenced miRNA-16 and RNU6B, and the values were found to be 285, 274, and 260 ng/μL, for miRNA-16, and the values of RNU6B were 268, 274, and 266 ng/μL across the EVs extracted under the three conditions. The data suggested a significant amount of miRNA in EVs, with PEG and ExoQ purification methods yielding similar quantities [Figure 2e]. After operating real-time PCR, we ran the 4% agarose gel electrophoresis of the qPCR product to confirm the presence of the specific bands. For RNA, the band of beta-actin was seen in all enrichment methods. For miRNA, including the stem-loop primer length, a clear band is observed around the 50–75 bp position for all three EV purification methods [Figure 2f]. Figure 2g provided electron microscopic pictures of PEG8%-EV and ExoQ-EV with the evident particle size close to the 100 nm diameter.

Eight percent polyethylene glycol yielded the highest concentration of exosome-like extracellular vesicle with the least carry-over proteins

By NTA, the concentration and size of EV particles in different preparations were measured. As shown in Table 2 and Figure 3, ExoQ and PEG 8% preparations yielded the highest EV particle number ($2.7 \times 10^{11} \pm 4.13 \times 10^{10}$, $2.2 \times 10^{11} \pm 2.17 \times 10^{10}$ particles/mL, respectively) [Figure 3a], and lower carry-over protein (30.97 ± 5.7 and 24.05 ± 1.2 pg/particle, respectively) [Figure 3b]. The range particle size coincided with that of the exosome, as revealed by NTA [Figure 3c]. The mean particle size was slightly larger in PEG 8% (127.3 ± 3.9 nm) than in ExoQ (117 ± 3.5 nm) [Table 2]. In contrast, preparations with PEG 9% and PEG 10% yielded significantly lower EV particle numbers ($8.73 \times 10^{10} \pm 4.23 \times 10^{9}$, $1.34 \times 10^{11} \pm 1.52 \times 10^{10}$ particles/mL, respectively) [Figure 3a] and higher carry-over protein amount (75.22 ± 6.6 and 63.39 ± 5.8 pg/particle, respectively) [Figure 3b].

Extracellular vesicle extraction with polyethylene glycol can be enhanced by adding salt

It has been reported that salt could attract water molecules and enhance the partition of the EV from its water environment [18,19]. We also compared the optimization of adding salt to the PEG purification of FF-EV. Figure 4a, b showed an increased yield of EV and a decrease in carry-over protein in FF-EV extracted by both PEG 6% and PEG 8% under 0.5 M NaCl. Particularly, adding salt in PEG 8% increased the yield by 1.3 times than no salt ($2.2 \times 10^{11} \pm 2.2 \times 10^{10}$ vs. $1.75 \times 10^{11} \pm 1.0 \times 10^{10}$ particles/mL), it decreased carry-over protein by 0.7 times than no salt (24.05 ± 1.25 vs. 31.51 ± 3.26 pg/particle). The mean particle size was indifferent in PEG 8% with

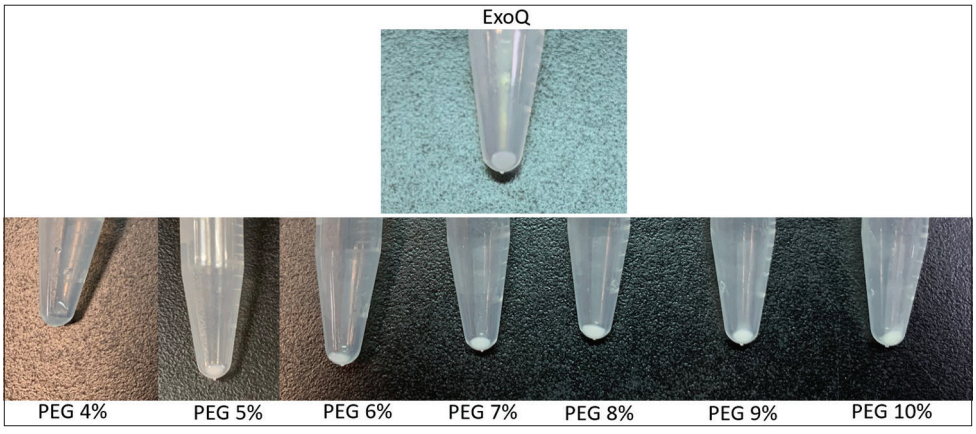
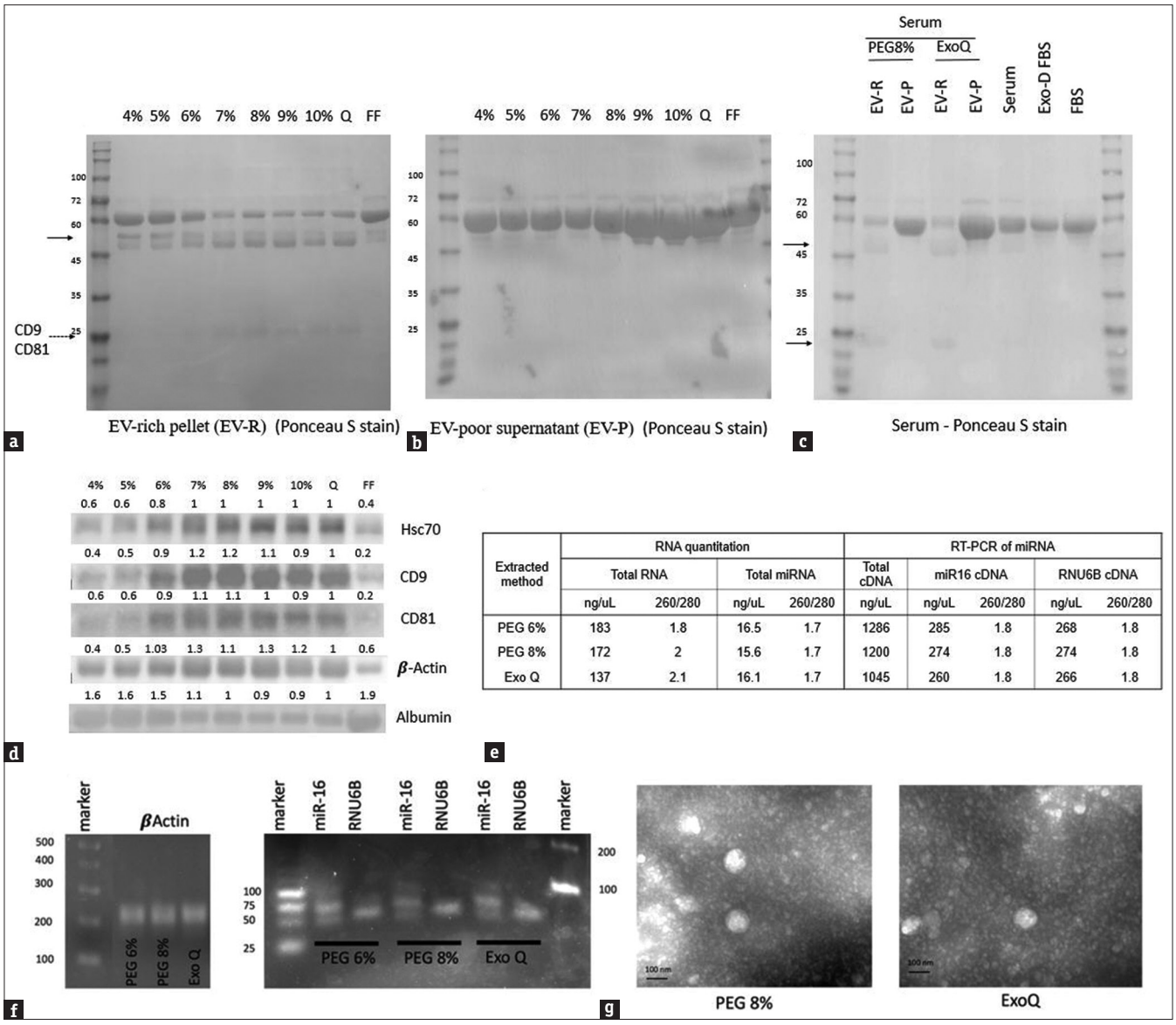


Figure 1: Partitioning of follicular fluid-extracellular vesicles after overnight incubation at 4°C in different concentrations of polyethylene glycol and ExoQuick. Enriched EVs were pelleted down by centrifugation at 2200 g for 15 min. PEG: Polyethylene glycol, ExoQ: ExoQuick

Table 2: Concentration and size of extracellular vesicle particles in different preparations, as analyzed by nanoparticle tracking analysis

Sample	Methods	0.5 M NaCl	PEG (%)	Particles/mL, mean±SD	pg/particle, mean±SD	Size (nm)	
						Mean±SD	Mode±SD
FF	PEG	Yes	6	1.85E+11±1.23E+10	15.49±1.4	137.7±6.9	146.9±8.6
			7	1.46E+11±1.70E+10	32.46±4.2	122.9±1.8	117.0±7.2
			8	2.20E+11±2.17E+10	24.05±1.2	127.3±3.9	96.9±27.6
			9	8.73E+10±4.23E+09	75.22±6.6	114.1±3.6	130.2±3.1
			10	1.34E+11±1.52E+10	63.39±5.8	105.0±1.1	76.7±30.7
	PEG	No	6	1.45E+11±3.40E+09	19.29±0.5	114.1±3.6	117.6±39.6
			8	1.75E+11±1.06E+10	31.51±3.2	124.2±8.9	99.8±19.3
	ExoQ		Q	2.70E+11±4.13E+10	30.97±5.7	117.1±3.5	84.4±29.3

FF: Follicular fluid, PEG: Polyethylene glycol, SD: Standard deviation, ExoQ: ExoQuick



Extracted method	RNA quantitation				RT-PCR of miRNA				
	Total RNA		Total miRNA		Total cDNA	miR16 cDNA		RNU6B cDNA	
	ng/uL	260/280	ng/uL	260/280	ng/uL	ng/uL	260/280	ng/uL	260/280
PEG 6%	183	1.8	16.5	1.7	1286	285	1.8	268	1.8
PEG 8%	172	2	15.6	1.7	1200	274	1.8	274	1.8
Exo Q	137	2.1	16.1	1.7	1045	260	1.8	266	1.8

Figure 2: Protein, RNA, microRNA contents, and morphology of follicular fluid-extracellular vesicles (FF-EVs) extracted by different polyethylene glycol (PEG) preparations and ExoQuick (ExoQ). (a and b) Total proteins in EV-rich pellet (EV-R, a) and EV-poor supernatant (EV-P, b) from FF were resolved by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Ponceau S. (c) Total proteins in EV-R and EV-P from serum, in exosome depleted fetal bovine serum (Exo-D FBS) and FBS. (d) Western blot analysis of exosome-specific markers, heat-shock cognate protein 70, cluster of differentiation (CD) 9 and 81 CD9, CD81, as well as the protein controls, β-actin, and albumin. (e) Quantity and quality of total RNA and miRNA, and the reverse transcription-polymerase chain reaction (PCR) products of two marker miRNAs, miR-16 and RNU6B. (f) 4% agarose gel electrophoresis of real-time-PCR products of RNA (left) and microRNA (right,) from EV-R by different enrichment methods. (g) Electron microscopy view of FF-EVs purified by PEG 8% and ExoQuick. Scale bar: 100 nm. EV-R: EV-rich, EV-P: EV-poor, PEG: Polyethylene glycol, Exo-D: Exosome depleted, RT-PCR: Reverse transcription polymerase chain reaction, CD: Cluster of differentiation

or without salt (127.3 ± 3.9 nm vs. 124.2 ± 8.9 nm) [Table 2 and Figure 4c].

Overnight incubation highly increased extracellular vesicle yield from follicular fluid while decreasing the carry-over protein

We wondered whether an increase in the partition time would increase the yield of FF-EV. As shown in Figure 5a, compared to 1-h incubation, overnight incubation with PEG 8% or ExoQ increased the EV yield by four times and 18 times, respectively. In terms of protein carry-over, overnight incubation significantly reduced protein levels compared to 1-h incubation in both PEG and ExoQ manner. For PEG 8%, carry-over protein decreased from 124.61 ± 10.3 pg/particle to 24.05 ± 1.25 pg/particle, and for ExoQ, it decreased from 467.65 ± 23.38 pg/particle to 30.97 ± 5.7 pg/particle [Table 3 and Figure 5b]. In addition, overnight incubation compared to the 1 h also decreased the average size of FF-EV with PEG 8% or ExoQ extraction [Table 3 and Figure 5c].

Overnight incubation only moderately increased the yield of extracellular vesicles extracted from serum with ExoQuick and did not increase the yield with polyethylene glycol 8%

In contrast to FF-EV extraction where overnight incubation markedly increased the yield and decreased the carry-over

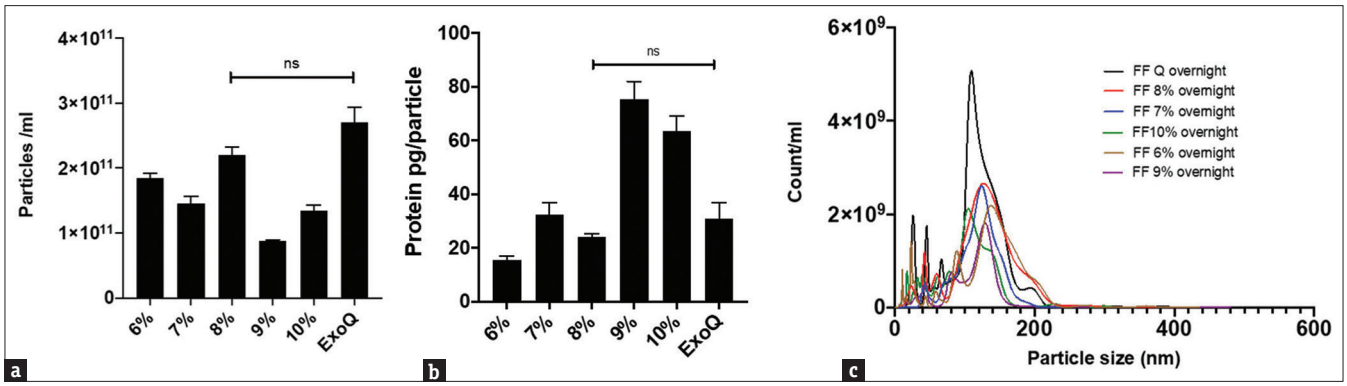


Figure 3: Eight percent polyethylene glycol (PEG) yielded the highest concentration of extracellular vesicle (EV) with less protein carry-over. (a) Nanoparticle tracking analysis (NTA) analysis of follicular fluid-extracellular vesicle (FF-EV) yields extracted by overnight incubation with PEG of different concentrations (with 0.5M NaCl) and ExoQuick. (b) Protein amount of FF-EV particle, analyzed by NTA. (c) Particle size distribution of FF-EV, analyzed by NTA. Data was shown in mean ± standard deviation. ExoQ: ExoQuick

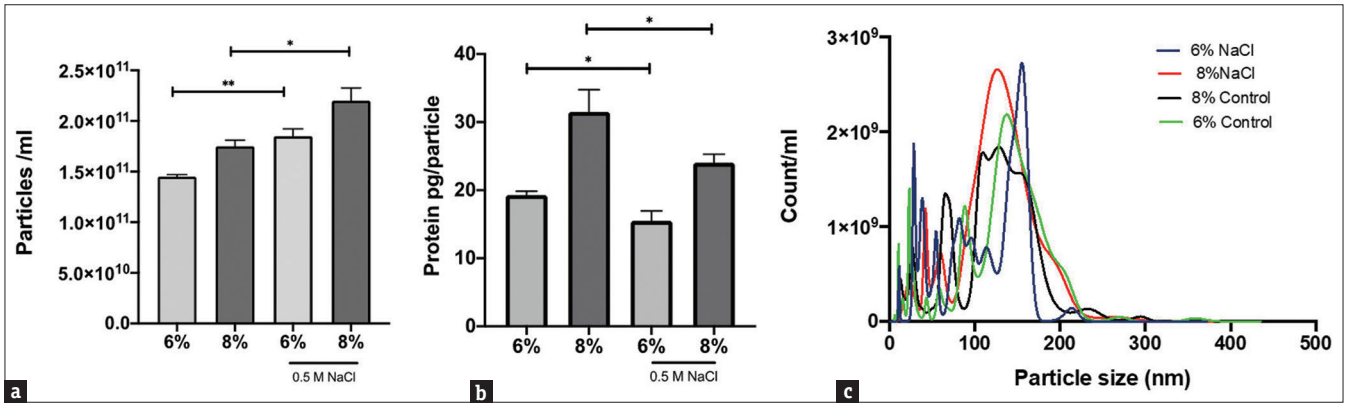


Figure 4: Adding salt to polyethylene glycol (PEG) increased extracellular vesicle (EV) yield while decreasing the carry-over protein. (a) Nanoparticle tracking analysis (NTA) analysis of follicular fluid-EVs (FF-EVs) amount extracted by overnight incubation by using 6% or 8% PEG and with or without 0.5M NaCl added. (b) Protein amount of FF-EV particle, analyzed by NTA. (c) Particle size distribution of FF-EV, analyzed by NTA. Data was shown in mean ± standard deviation. *$P < 0.01$, **$P < 0.001$

protein, the same overnight extension of incubation only moderately increased the yield by ExoQ partition and did not increase in the PEG 8% partition of the serum specimen [Figure 5d]. Again, the increase in yield was associated with a less carry-over protein in the overnight incubation with ExoQ extraction [Figure 5e]. Meanwhile, the average size of serum-EV was decreased in overnight incubation compared to the 1 h with PEG 8% or ExoQ extraction [Table 4 and Figure 5f].

Follicular fluid-extracellular vesicle are readily up taken by fallopian tube epithelial cells and promote anchorage-independent growth

Previously, we had characterized the transforming activities of FF [12,13]. The promotion of AIG of the fallopian tube epithelial cells (FE25) was the most characteristic transforming activity [11,13], and the AIG-promoting activity mostly resided in FF-EV (manuscript under revision). As a final functional test, we tested the uptake of FF-EV by FE25 cells and the AIG promotion effect. As shown in Figure 6a, after 1 h of incubation, FF-EVs extracted by 6% PEG, 8% PEG and ExoQ were all readily up taken by FE25 cells. After overnight incubation, the PEG 8%-extracted EVs induced the highest number (107.5 ± 3.5) of AIG colony, more than that by ExoQ (91 ± 1.4) and PEG 6% (59 ± 1.4) in the treatments of 100 μg amount. The activities positively correlated with the amount of EV added. Moreover, under the same 100 μg amount, EVs extractions by overnight incubation promoted more AIG colonies than those by 1-h incubation (107.5 ± 3.5 vs. 67.5 ± 0.71) with 8% PEG extraction, and with ExoQ extraction (91 ± 1.4 vs. 54 ± 1.4) [Figure 6b and c]. Since EVs extracted by overnight partition had less carry-over protein, more EV particles per 100 μg weight was expected. Therefore, more AIG colonies were resulted.

Discussion

The optimized polyethylene glycol concentration for extracellular vesicle extraction is 8%

A long chain highly hydrophilic polymer, PEG, has been used to precipitate viral particles, nucleic acids, and other biomolecules for a long time [20]. The presence of PEG polymers results in a decrease in hydration and a change in osmotic pressure, both helping to partition the bioparticles [21,22]. This study optimized the conditions including PEG concentration, salt in solution, and incubation time of the PEG partition to extract EVs from FF and serum. The purity check showed that PEG 8% works equally well with ExoQ to carry minimal carry-over proteins including the 60 kD

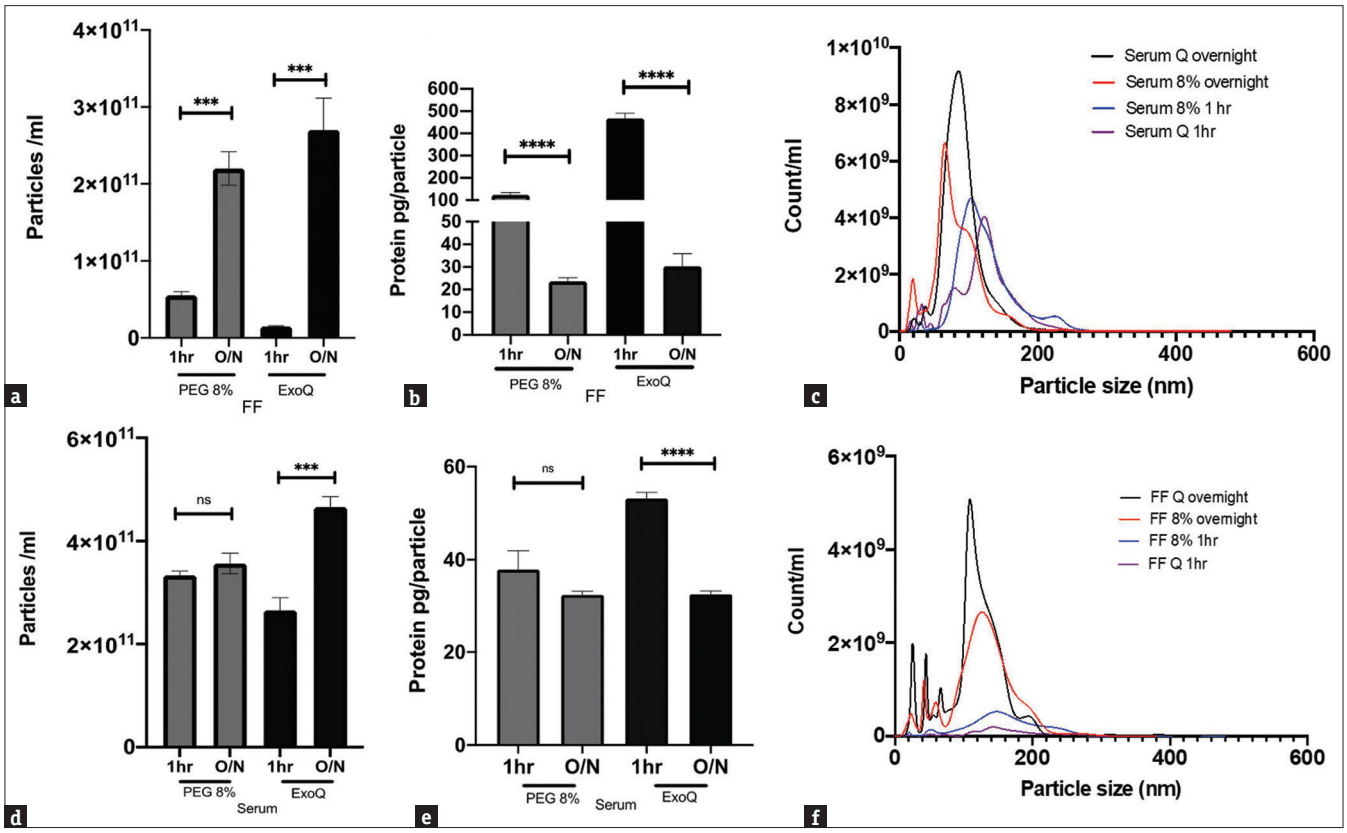


Figure 5: Different increases in extracellular vesicle (EV) yield by the extension of incubation time. (a and d) Yields of follicular fluid (FF) and serum EVs following 1-h or overnight incubation with 8% polyethylene glycol (containing 0.5M NaCl) or ExoQuick extraction, analyzed by nanoparticle tracking analysis (NTA). (b and e) Protein content of FF and serum EV particles, analyzed by NTA. (c and f) Particle size distribution of FF and serum EVs, analyzed by NTA. Data was shown in mean ± standard deviation. ***$P < 0.0005$, and ****$P < 0.0001$. PEG: Polyethylene glycol, ExoQ: ExoQuick, FF: Follicular fluid

Table 3: Yield and size of extracellular vesicle and protein carry-over in follicular fluid with polyethylene glycol or ExoQuick extraction in two partition times

Sample	Time	Particles/mL, mean±SD	pg/particle, mean±SD	Size (nm)	
				Mean±SD	Mode±SD
FF	Overnight				
	PEG 8%	2.20E+11±2.17E+10	24.05±1.2	114.1±3.6	117.6±39.6
	ExoQ	2.70E+11±4.13E+10	30.97±5.7	124.2±8.9	99.8±19.3
	1 h				
	PEG 8%	5.53E+10±4.47E+09	124.61±10.3	159.3±3.0	160.2±11.4
	ExoQ	1.49E+10±6.99E+08	467.65±23.38	159.2±6.2	133.7±1.9

FF: Follicular fluid, PEG: Polyethylene glycol, SD: Standard deviation, ExoQ: ExoQuick

Table 4: Yield and size of extracellular vesicle and protein carry-over in serum with polyethylene glycol or ExoQuick extraction in two partition times

Sample	Time	Methods	Particles/mL, mean±SD	pg/particle, mean±SD	Size (nm)	
					Mean±SD	Mode±SD
Serum	Overnight	PEG 8%	3.57E+11±2.00E+10	32.31±0.4	84.8±1.7	66.5±0.8
		ExoQ	4.66E+11±2.10E+10	32.55±0.3	91.1±1.3	85.0±2.0
	1 h	PEG 8%	3.34E+11±8.00E+09	37.88±2.4	126.0±0.7	98.6±5.4
		ExoQ	2.66E+10±2.84E+09	53.16±0.9	122.2±4.2	123.1±3.3

PEG: Polyethylene glycol, SD: Standard deviation, ExoQ: ExoQuick

albumin. Contrarily, exosome-related proteins, including Hsc70, CD9, and CD81, were enriched in the EV extracted by PEG 6% to 10%. As revealed by NTA, PEG 8% particularly yielded the highest concentration of EV with the least carry-over proteins.

Salt enhanced the extracellular vesicle partitioning

NaCl is a highly polar molecule and strongly adsorbs water molecules. In previous studies, different molarity of NaCl was used to enrich PEG-based exosome extraction [18,19]. Our

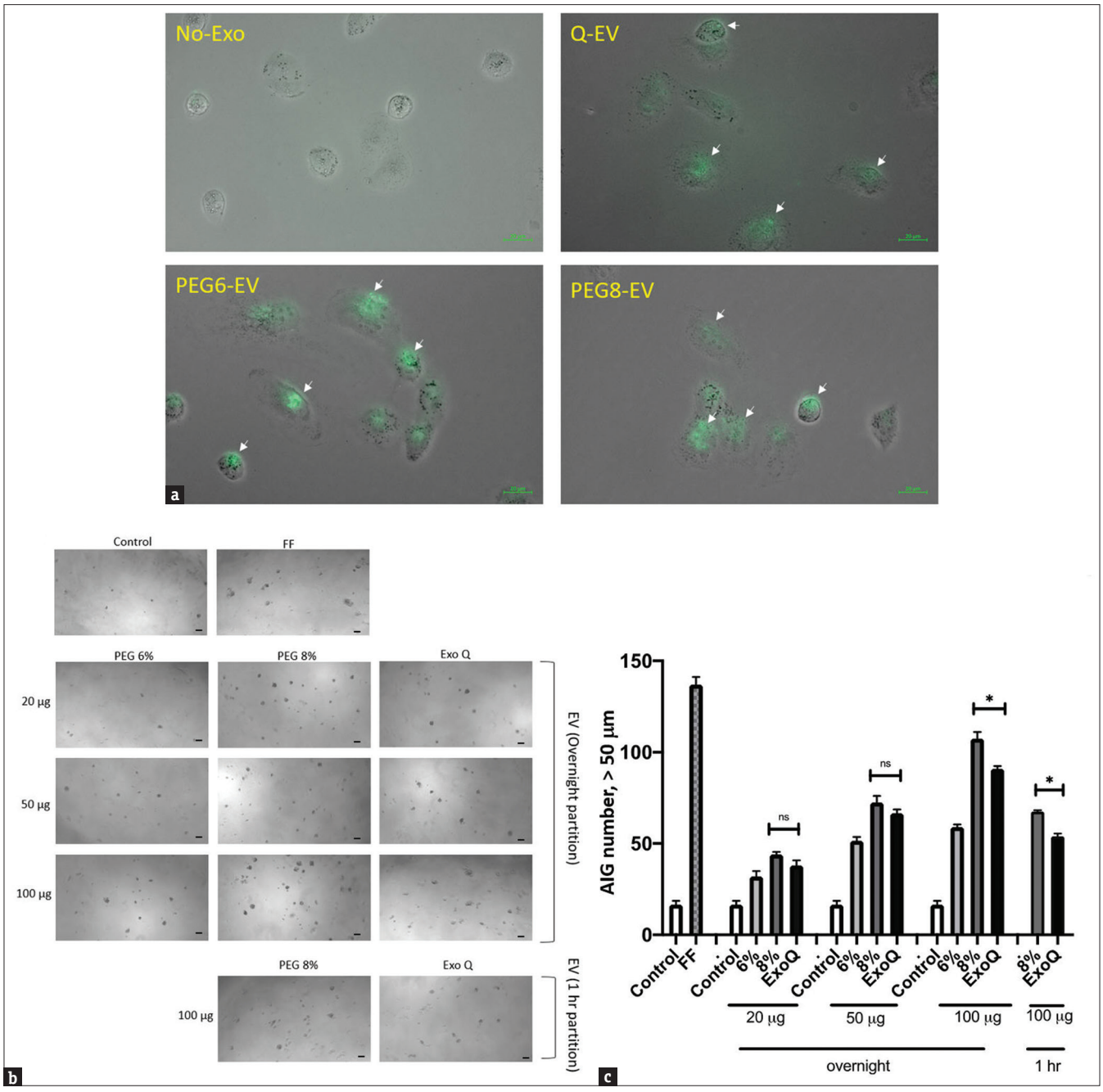


Figure 6: Functional assays of follicular fluid-extracellular vesicles (FF-EVs) extracted showed that 8% polyethylene glycol (PEG) in salt outperformed ExoQuick (ExoQ). (a) Fluorescence microscope image of FE25 cells 1 h after treatment with 6% and 8% PEG (containing 0.5M NaCl) and ExoQ-purified EVs stained with 1 µM calcein AM (green). Scale bar: 20 µm. (b) Images showing the colonies from the anchorage-independent growth assay of FE25 cells treated with varying amounts of FF-EVs extracted using either PEG (containing 0.5M NaCl) or ExoQ. Scale bar: 100 µm. (c) Quantification data of cell colonies. Data is shown in mean ± standard deviation. *$P < 0.01$

test results showed that EVs and water partitioning worked better under 0.5 M NaCl with a 26% increase in EV yield and 24% decrease in protein carry-over, and a negligible influence on the particle size distribution in PEG8% extraction.

Extension of time has different effects on serum and follicular fluid exosome

This study highlights the importance of the incubation time for EV yield in PEG-based extraction, especially for the FF sample. The yield increased by 18 and 4 times when incubation was prolonged from 1 h to overnight in ExoQ and PDG 8% based extraction, respectively. Importantly, the suggested incubation time in the ExoQ manual was 1 h. We assume this short incubation time was based on purification from cell culture-conditioned media or other tissue fluid such as serum. Indeed, we found no improvement of EV yield for the serum sample by overnight culture under the PEG 8% and only a moderate increase of yield under ExoQ. FF differs from serum by containing a large amount of osmotic molecules such as heparan sulfate proteoglycan [23]. Therefore, it may take a longer time to partition exosomes from surrounding water molecules. Meanwhile, we also found the increase in

EV yield was associated with a decrease in carry-over proteins when comparing 1-h to overnight extractions.

Polyethylene glycol 8% is superior to ExoQuick

The EVs carry the cargo for RNAs, miRNAs, and proteins. Total RNA extracted from FF-EVs enriched by PEG 8% was higher than that by ExoQ. We initially mentioned that the FF-EVs extracted by the ExoQ method had transformation activity of FTE cells (manuscript under review). Here, we proved that under the same 1-h incubation, FF-EVs extracted by PEG 8% under 0.5 M NaCl had a higher yield and less carry-over protein [Table 3] as well as a higher AIG-promoting activity than ExoQ [Figure 6].

Conclusions

In summary, using PEG 8000, we optimized a protocol of EV extraction with a higher yield, less protein carry-over, and higher activity from FF and serum samples. This simple and efficient approach would save time and cost in EV and exosome extraction from the biological fluid.

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Data availability statement

The dataset is not available for public access but is available from the corresponding author upon reasonable request.

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Conflicts of interest

There are no conflicts of interest.

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