3 Biotech (2018) 8:139
https://doi.org/10.1007/s13205-018-1157-7

SHORT REPORTS

Simple isolation and characterization of seminal plasma extracellular vesicle and its total RNA in an academic lab

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Received: 26 April 2017 / Accepted: 5 February 2018

Abstract

Extracellular vesicles (EVs) are small membrane-bound sacs, identified in many body fluids of humans. Standard extracellular vesicle separation methods such as differential and ultracentrifugation are very expensive, not affordable in academic labs. So, the current research tried to isolate seminal plasma EVs using polyethylene glycol (PEG) precipitation process. Normospermia semen from "Milann - The Fertility Center" processed to isolate EVs by PEG method. Nanodrop spectrophotometer showed presence of EVs by indirectly measuring protein content of precipitated EVs. EVs isolated by PEG precipitation showed a wide size range from 30 to 1000 nm with *Z* average of 75.4 nm and a PI of 0.464, whereas ultracentrifuge sample showed size range of 60–1000 nm with *Z* average of 501.3 nm with a PI of 0.692. Edax analysis also showed good elemental pattern. Total RNA extraction from PEG EVs analysed with nanodrop spectrophotometer, showed presence of RNA content in varying concentrations obtained from different ratios in nanograms. Thus, the current study concludes that seminal plasma EVs isolated by PEG precipitation is simple, reproducible and non-sensitive to carry out at academic labs.

Keywords Normospermia · Polyethylene glycol method · Infertility · Motility of sperm · Scanning electron microscope

Introduction

Extracellular vesicles (EVs) are small membrane-bound sacs that are secreted by almost all cell types from prokaryotes to eukaryotes. EVs were identified in many body fluids (blood, urine, saliva, breast milk, follicular fluid and semen) of humans. Extracellular vesicles are of three different types such as microvesicles or ectosomes formed by outward budding by plasma membrane fission, exosomes that origin from multivesicular bodies through endosome network finally fuse with plasma membrane, is released out of the cell and apoptotic bodies, in the form of blebs are shed out from cells that entered apoptosis (Yanez-Mo et al. 2015).

Human seminal plasma is composed with mixtures of extracellular vesicles which originate from different male reproductive accessory organs such as prostate, epididymis and seminal vesicles. Extracellular vesicles not only immunomodulate female genital tract (Tarazona et al. 2011) but also helps sperm in enhancing its motility (Park et al. 2011), semen liquefaction, protects from infection, and aids blood coagulation (Andersson et al. 2002; Fernández et al. 1997). Extracellular vesicles as such or its cargos are widely exploited as a noninvasive biomarker in identification and detection of prostate cancer (Tavoosidana et al. 2011).

Vojtech et al. (2014) found that the seminal plasma exosomes contain 20–100 nucleotides long RNAs. This mixture is a combination of microRNAs (21.7%), YRNAs, tRNAs and protein-coding mRNAs that are involved in immunosuppression activities in the female reproductive organ, aiding inhibition of antisperm antibody production and other immune reactions that could render sperm its efficiency to fertilise egg. Hoog and Lötvall (2015) found different varieties of extracellular vesicles with five major categories in the seminal plasma of humans using cryo-electron microscopy.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13205-018-1157-7) contains supplementary material, which is available to authorized users.

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Published online: 16 February 2018

Standard extracellular vesicle separation using old standard techniques such as differential centrifugation and ultracentrifugation is very expensive and is not affordable under a normal academic lab conditions. So, in this research we tried to isolate seminal plasma extracellular vesicle using polyethylene glycol (PEG) precipitation process and try to show that the isolated vesicles are in its native shape with its surface molecules along with intraluminal RNA molecules.

Methodology

Semen collection

Semen samples were collected from "Milann - The Fertility Center" Bangalore, Karnataka, India, from patients who were attending andrology lab for semen analysis. Samples were differentiated into different categories as normospermia or infertile according to WHO standard protocols (Cooper et al. 2009). Samples were then transported using cryovials with liquid nitrogen to VIT University, Vellore, Tamil Nadu, India. In the laboratory, normospermia semen samples were diluted with equal volume of sterile phosphate buffered saline (PBS, pH 7.4). Diluted plasma samples were initially subjected to centrifugation for 3000 rpm for 20 min to separate sperm and the plasma. Seminal plasma supernatant was transferred into a new Eppendorf tube and centrifuged at 15,000 rpm for 30 min to remove debris. Supernatant was filtered through 0.45-µm syringe filtre and dispensed into aliquots, stored at − 80 °C for further processing.

Isolation of seminal plasma extracellular vesicles with polyethylene glycol (PEG) and analysis of extracellular vesicles

Seminal plasma from normospermia males were pooled and used for further analysis. Polyethylene glycol (PEG) (6000 kDa) (Andreu et al. 2016) (Hi-media) of 500 mg/ml concentration was prepared with sterile PBS (pH 7.4). Exosome isolation mixture was prepared by adding different volumes of seminal plasma (SP) to a stable volume of PEG (PEG:SP) of 1:1, 1:2, 1:3, 1:4 and 1:5 (50 µl:50 µl, 50 µl:100 µl, 50 µl:150 µl, 50 µl:200 µl, 50 µl:250 µl). Final volume of all reaction mixture was made up to 300 µl with PBS. Control tubes were prepared with varying PBS instead of seminal plasma (Madison et al. 2014). Duplicates were prepared for all the ratios and control. Reaction mixture was mixed gently and incubated at 4 °C for 12 h. After incubation, mixtures were centrifuged to pellet EVs at 3000 rpm for 30 min at 4 °C. Supernatant discarded and the pellets suspended in PBS, aliquoted and stored at − 80 °C. For comparison, seminal plasma EVs were isolated by ultracentrifugation (UC) method with Optima L-100 K (Beckman Coulter) ultracentrifuge by centrifugation at 10,000×*g* (35,000 rpm) for 2 h at Christian Medical College Hospital, Vellore, Tamil Nadu, India, as per Thery et al. (2006) with minor modifications.

Quantification of isolated extracellular vesicles

Quantification of isolated extracellular vesicles was done with nanodrop spectrophotometer (Thermo Fisher). About 1 µl of the control, from the respective reaction mixture was read as blank followed with sample at 280 nm for protein quantification and the results are presented with a graph.

Dynamic light scattering (DLS) and zeta analysis

Extracellular vesicles were analysed for its average size by means of HORIBA SZ-100 nanoparticle analyser at VIT University, Vellore, Tamil Nadu, India, with temperature set at 25 °C, with a scattering angle of 90°. Zeta analysis was performed to analyse the integrity and stability of the isolated extracellular vesicles. All the experiments were performed with duplicates (Rider et al. 2016).

Scanning electron microscopy (SEM)

Extracellular vesicle samples obtained from both processes of PEG precipitation and ultracentrifugation were placed on a clean glass coverslip and allowed to dry for about 18 h in 37 °C incubator. Dried samples were further processed with gold coating, viewed with EVO scanning electron microscope of *Carl Zeiss microscopy Ltd* (UK) at School of Bio Sciences and Technology, VIT University, Vellore.

EDX analysis

Edx values of the observed vesicles were also analysed with *OXFORD* instruments for energy dispersive X-ray analysis to study elemental pattern of the vesicles at the same Scanning Electron Microscopy facility at the School of Bio Sciences and Technology, VIT University, Vellore.

Total RNA extraction

Total RNA extraction was done in RNAase-free labwares which were dipped in DEPC water, overnight and autoclaved before use. Total RNA from isolated extracellular vesicles extracted using *HiPurA*™ total RNA Miniprep purification kit (HiMedia). Extracellular vesicles were lysed with the lysis buffer which denatured and disrupted the vesicle lipid bilayer. Lysed vesicles were made to run through silica column, addition of ethanol to the lysate aids in the selective RNA binding to silica membrane. Contaminating polysaccharides, proteins, many other small molecular substances

including salts were removed by washing. Finally, RNA was eluted and quantified with nanodrop spectrophotometer. All the solutions in the kit contained DNAase, so the resulted RNA is free of DNA molecules.

Data analysis

Data were analysed with Graphpad Prism version 5.0 and graphs were constructed with the same.

Result and discussions

Semen parameters of normospermia

Semen analysis was done for all the samples and only normospermia semen samples were taken for this research ($N = 25$). The parameters such as sperm count, motility, morphology was analysed and found to be normal according to WHO standards.

Isolation of seminal plasma extracellular vesicles with PEG and analysis of extracellular vesicles

PEG precipitated and Ultracentrifugation EVs were aliquoted, stored at – 80 °C and analysed.

Quantification of isolated extracellular vesicles

Quantification of isolated extracellular vesicles was done by measuring total protein content by nanodrop spectrophotometer. Nanodrop observations showed the presence of extracellular vesicles by indirectly measuring the protein content of the sample as PEG precipitation would isolate extracellular vesicles. Vlassov et al. 2016 has showed that PEG6000 is good in isolation of exosomes without proteinase K treatment, with less albumin. PEG precipitation of seminal plasma extracellular vesicles showed good yield of extracellular vesicles in the 1:5 ratio (4.583 × 2 (dilution) = 9.166 mg/ml of seminal plasma), whereas all other ratio mixtures have also isolated EVs but with reduced quantity. Addition of PBS in the phase-separation process apparently enhances the separation of EVs which possesses a charged membrane. By Flory–Huggins mean-field theory for polymer solutions, this PEG in PBS renders a simple technique for separation and concentration process (Johansson et al. 1998) The main criteria to isolate extracellular vesicles lies in the gentle sample processing which could finally result in the isolation of these delicate structures. Nanodrop spectrophotometer protein quantification (Fig. 1) indirectly revealed the isolation of EVs by above-mentioned PEG precipitation method. Further analysis of the isolated EVs by different techniques confirmed extracellular vesicle isolation

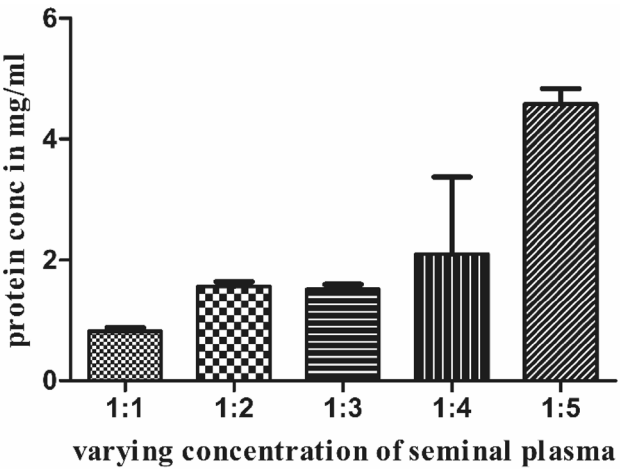

Fig. 1 Protein concentration for isolated extracellular vesicles from seminal plasma of normospermia semen samples quantified by nanodrop spectrophotometer. Protein concentration in mg/ml increase with increasing concentration of seminal plasma like 1:1, 1:2, 1:3, 1:4, 1:5 and maximum concentration was found at 1:5

from the seminal plasma by PEG precipitation protocol. EVs isolated with UC were also quantified using Nanodrop spectrophotometer which showed an average yield of 7.564 mg/ml of seminal plasma. Yield of EVs with PEG and UC is calculated by the average protein concentration measured with Nanodrop spectrophotometer. PEG protocol yielded 9.166 mg of EVs/ml of seminal plasma ± 0.51, whereas UC yielded 7.564 mg/ml ± 1.439.

Dynamic light scattering (DLS) and zeta analysis

DLS measure is a cumulant analysis which gives information on mean particle size or *Z* average and width of the distribution which is expressed as a polydispersity index (PI). So, in the present study, size distribution range of extracellular vesicles is 0.464, which falls in the best operation range of DLS. Extracellular vesicles isolated from seminal plasma of normospermia samples by PEG precipitation showed a wide size range from 30 to 1000 nm with mean size or *Z* average of 75.4 nm. It also showed a PI value of 0.464, whereas DLS of ultracentrifugation sample showed size range of 60–1000 nm with the *Z* average of 501.3 nm with a PI of 0.692. Both of the methods resulted in EVs with expected size range. Obtained DLS values were compared with SEM for size and structure similarities as a verification to validate protocol for extracellular vesicle isolation (Fig. 2). Zeta potential directly depends on electrophoretic mobility of the particles. It is measured with laser Doppler electrophoresis methodology where a laser beam which pass through a sample under electrophoresis is shifted to a different frequency, detected as the zeta potential of the sample under study. Zeta potential analysis of the PEG-isolated EVs

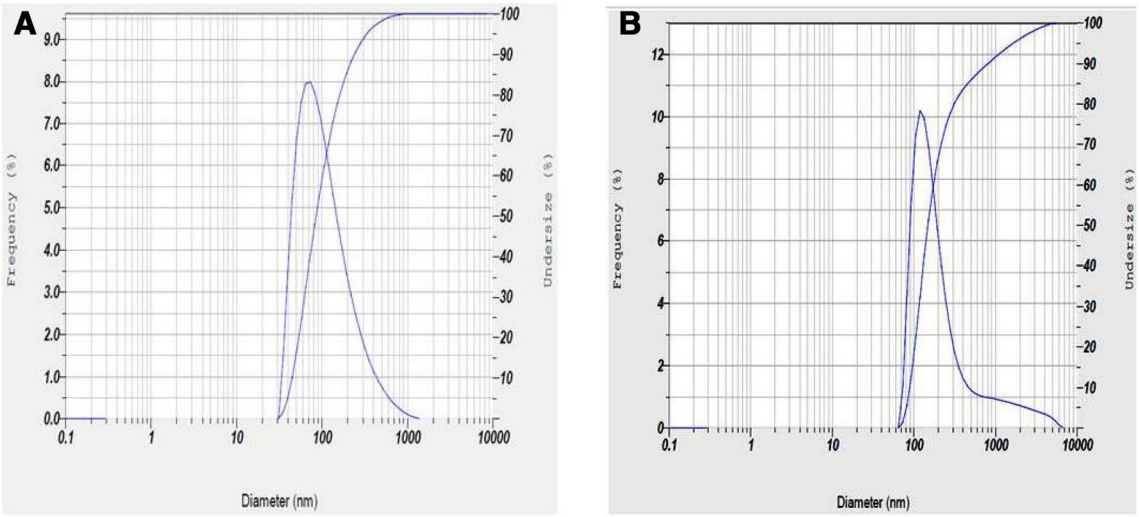

Fig. 2 **a** DLS of PEG-isolated EVs, **b** DLS of UC-isolated EVs. Extracellular vesicles isolated from seminal plasma of normospermia samples by PEG precipitation showed a wide size range from 30 to 1000 nm with mean size or *Z* average of 75.4 nm, whereas DLS of ultracentrifugation sample showed size range of 60–1000 nm with the *Z* average of 501.3 nm. Both of the results are highly related and comparable

revealed a mean zeta potential of − 64.1 Mv, confirmed that extracellular vesicles are good in integrity and stability (Supplementary Fig. 1).

Scanning electron microscopy (SEM)

Current study has identified mixtures of extracellular vesicles with SEM field showing different shell-shaped vesicles both from PEG-isolated EVs and UC-isolated EVs. As Sharma et al. (2011) and Chernyshev et al. (2015) showed that the electron dense staining followed with vacuum-mediated dehydration for electron microscopy could potentially result in artefacts in the form of cup structures resembling exosomes, we have followed a simple drying process at 37 °C for 18 h, followed with gold coating and it has resulted in typical bulged round vesicles. Size and shape of EVs isolated from normospermia samples by PEG methods and UC were found to be similar with previous studies related to seminal plasma exosomes and prostasomes with size range from 100 to 1000 nm. So, these results confirmed DLS analysis and Zeta potential measurements and finally propounded that PEG isolation of EVs are stable in their shape and integrity (Fig. 3a, b).

EDX analysis

Elemental pattern of extracellular vesicles isolated using PEG precipitation method showed percentage of different elements that included oxygen, sodium, aluminium, silica, chlorine, potassium, titanium and zinc. Semen is rich in different anthropogenic factors including mineral components such as Ca^{2+}, Zn^{2+} and Mg^{2+}, for maintenance of fertilisation capacity. Extracellular vesicles are found to act as ligands for these divalent cations. Vivacqua et al. (2004) reported seminal plasma extracellular vesicles as ligands of zinc through dialysis experiments which confirmed zinc-binding ability of prostasomes. Mogielnicka Brzozowska et al. (2015) found out that extracellular vesicles are involved in many fertility-related functions such as motility and on the membrane integrity of sperm cells. Zhao et al. (2016) has identified that decreased levels of Zn in seminal plasma resulted in infertility, is a result of multiple effects such as decreased sperm cell integrity which affected capacitation and acrosome function. Thus, the proposed PEG precipitation protocol resulted in the good isolation of EVs without disturbing its mineral pattern (Supplementary Fig. 2).

Total RNA extraction

Total RNA extracted from normospermia EVs analysed with nanodrop spectrophotometer showed presence of RNA content in varying concentrations obtained from different ratios of PEG precipitated extracellular vesicles in nanograms (Fig. 4). Total RNA content of EVs are a mixture of different RNA molecules and could be further analysed for specific mRNA, microRNA and snRNA candidates which play different roles in fertilisation processes. RNA packaging into EVs is found to be specific and is regulated by hnRNP (heterogeneous nuclear ribonucleoprotein) that are usually involved in RNA transport and function. Specific microRNA candidates are packaged by identification of EXOmotif present in them, whereas

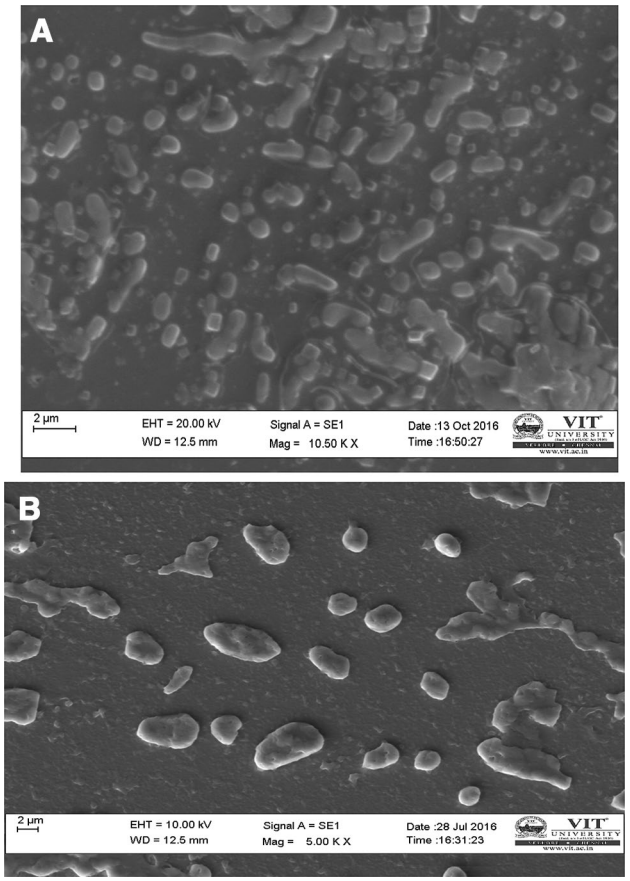

Fig. 3 **a** SEM image of PEG-isolated EVs. **b** SEM image of ultracentrifugation (UC) isolated EVs. EVs isolated from normospermia samples by PEG methods and structures were found to be similar with previous studies related to seminal plasma exosomes and prostasomes. The structures were seen with 100–1000 nm which is confirmed as extracellular vesicles. The isolated vesicles are very stable with Zeta value of − 64.1 mV. These structures were elegant in nature, this method of isolation will give a better result rather than UC

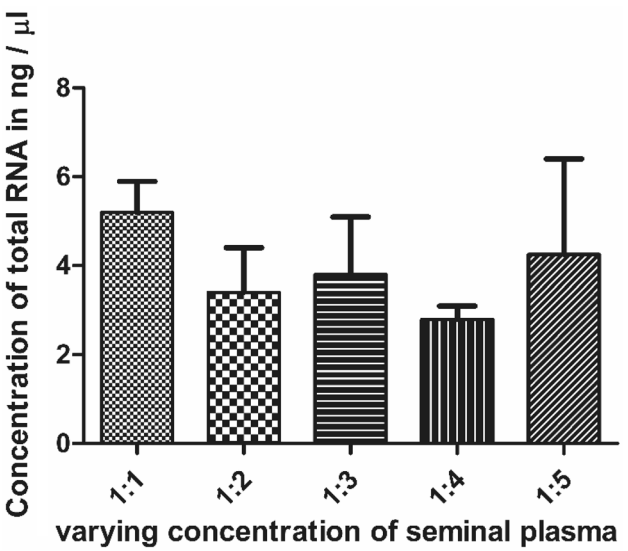

Fig. 4 Total RNA of the PEG-isolated EVs was done at varying concentration of seminal plasma by nanodrop spectrophotometer. RNA was found to be higher in case of 1:1. This shows stability of RNA from EVs isolated by PEG method

conserved sequences in 3′ UTR of mRNAs act as specific codes to get packed into EVs (Yanez-Mo et al. 2015). Vojtech et al. (2014) isolated RNA from seminal plasma exosomes which included different groups of noncoding RNAs such as microRNAs, YRNAs tRNAs and protein-coding mRNAs. Seminal plasma EV microRNAs are involved in regulation of immunomodulating mRNAs of female genital tract for successful fertilisation. Mihelich and Nonn (2012) found that overexpression of extracellular vesicle secreted microRNA-183 family members: microRNA 96, microRNA182 and microRNA183, inhibited protein hZIP1, involved in Zn transport. Lázaro Ibanez et al. (2017) identified presence of specific mRNAs in EVs secreted from prostate cell lines. So, detailed analysis on the different RNA candidates by studying its signature profile under various physiological conditions could serve as a noninvasive marker to identify different disease conditions such as infertility and cancer.

Conclusion

Based on the reports and results of the current study, isolation and analysis of seminal plasma EVs by PEG precipitation showed reliable results. EVs protein quantification, DLS size analysis, zeta potential for EVs integrity, SEM structural confirmation, edax mineral pattern along with extracted total RNA, isolation of EVs by PEG method is found to be a simple, economic and productive method under academic laboratory conditions, when compared to highly expensive standard ultracentrifugation (Table 1). Further analysis of EV encapsulated smallRNA signatures could serve as a noninvasive biomarker to identify many infertility disorders. Either a specific smallRNA candidate of EVs or the EVs as a whole could be potentially exploited as a therapy to overcome infertility.

Table 1 Comparison of different properties of EV's isolated with PEG and UC

No.	Properties	PEG	UC
1	Yield (mg/ml)	9.166	7.564
2	Equipment	Highly economical	Highly expensive
3	Reproducibility (calculated with coefficient of variation (COV): standard deviation/mean)	Good (COV = 6.12%)	Good (COV = 4.33%)
4	Time taken in hours	12	2–3

Acknowledgements Authors are thankful to the management of VIT University and Milann - The Fertility Center, for providing the best infrastructure to work.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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