Postprint: Isolation and Characterization of PEG6000-Enriched Semen-Derived Exosomes

Authors: Yang Cheng, Guo Wenbin, Zhang Wansong, Bian Jun, Yang Jiankun, Qi Tao, Wang Chunyan, Liu Cundong

Date: 2017-12-21T00:00:00+00:00

Abstract

Objective To introduce the extraction and identification methods for PEG6000-enriched semen-derived exosomes, laying a foundation for research on semen exosomes. Methods Semen from 6 normal volunteers was selected from the Laboratory Center of Nanfang Hospital, Southern Medical University, and extracts were obtained through enrichment with 8% PEG6000 and multi-step centrifugation combined with ultracentrifugation. The morphology of the extracts was then observed using transmission electron microscopy, their size was analyzed using nanoparticle tracking analyzer, and their protein composition was detected using Western blotting. Results The extracts exhibited round or oval vesicles with intact membranes, measuring approximately 30-150 nm, containing low electron-density material internally, all positively expressing CD63, ALIX, and TSG101 proteins, and lacking the sperm endoplasmic reticulum protein Calnexin. Conclusion Analysis of the morphology, size, and protein composition of the extracts confirmed that the extracted material was semen-derived exosomes, providing a methodological basis for subsequent clinical and basic research on semen exosomes.

Full Text

Extraction and Identification of Semen-Derived Exosomes Using PEG6000

YANG Cheng[1], GUO Wenbin[1], ZHANG Wansong[1], BIAN Jun[1], YANG Jiankun[1], QI Tao[2], WANG Chunyan[2], LIU Cundong[1]

[1]Department of Urology, Third Affiliated Hospital of Southern Medical University, Guangzhou 510630, China;
[2]Department of Laboratory Medicine, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China

Abstract

Objective To establish a method for extracting and identifying semen-derived exosomes using PEG6000 enrichment, providing a methodological foundation for future research on seminal exosomes. **Methods** Semen samples were collected from six healthy volunteers at the Laboratory Medicine Center of Nanfang Hospital, Southern Medical University. Exosomes were enriched using 8% PEG6000 combined with multi-step differential centrifugation and ultracentrifugation. The extracted vesicles were characterized by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and Western blotting to examine their morphology, size distribution, and protein composition. **Results** The extracted vesicles appeared as round or elliptical membrane-bound structures with intact membranes, measuring approximately 30–150 nm in diameter and containing low electron-density material. They positively expressed the exosomal marker proteins CD63, ALIX, and TSG101, while lacking calnexin, an endoplasmic reticulum protein abundant in sperm cells. **Conclusion** Based on morphological, size, and protein composition analyses, the extracted material was confirmed to be semen-derived exosomes. This PEG6000-based method provides a reliable methodological basis for subsequent clinical and basic research on seminal exosomes.

Key words: semen; exosomes; PEG6000; centrifugation; isolation

Introduction

Exosomes are membrane vesicles approximately 30–150 nm in diameter secreted by various cell types into the extracellular matrix [1-2]. They originate from late endosomes known as multivesicular bodies (MVBs), which form through inward budding of the MVB membrane into the endosomal lumen and are subsequently released into the extracellular space upon fusion with the plasma membrane. First discovered by Johnstone in 1987 during reticulocyte differentiation, exosomes were initially considered a mechanism for red blood cells to eliminate waste proteins [3]. However, recent studies have revealed that exosomes contain diverse bioactive molecules, including proteins, mRNA, and microRNA [4]. They can readily fuse with neighboring cell membranes, selectively delivering their cargo to recipient cells and thereby serving as crucial mediators of intercellular communication involved in various biological functions [5]. Exosomes are present not only in culture supernatants of hematopoietic cells such as erythrocytes, platelets, and lymphocytes [6], but also in numerous biological fluids including plasma, serum, milk, saliva, urine, and semen [4, 7].

Human semen contains heterogeneous nanoscale vesicles primarily secreted by the male urogenital system, including the testes, epididymis, prostate, and seminal vesicles, traditionally referred to as prostasomes [8]. In 2014, Madison et

al. [9] first proposed that semen-derived vesicles are more appropriately termed seminal exosomes and reported their potent anti-HIV-1 activity, sparking growing interest in this field. Exosome research holds promising prospects for early diagnosis and treatment of urogenital diseases and offers new insights into disease pathogenesis [10-11].

Current research on semen-derived exosomes remains in its infancy, with their functional roles and mechanisms in critical reproductive processes such as sperm maturation, acrosome reaction, and sperm-egg fusion still poorly understood [12]. Before clinical applications can be realized, numerous challenges must be addressed, particularly the difficulty of obtaining high-yield, high-concentration, and high-purity exosomes from complex biological fluids while preserving their biological properties. This challenge is intimately linked to isolation and extraction methodologies. Current exosome extraction methods include ultracentrifugation (differential centrifugation), sucrose density gradient centrifugation, chromatography, ultrafiltration, magnetic bead immunoprecipitation, and commercial extraction kits. The most commonly used approach combines differential centrifugation with sucrose density gradient ultracentrifugation; however, this method requires large sample volumes, is time-consuming, and is susceptible to centrifugation-related artifacts [13-14]. Therefore, there is an urgent need to identify an ideal exosome extraction method that achieves optimal yield, purity, and biological activity.

Polyethylene glycol (PEG) has been used for virus concentration and purification for over fifty years [15]. Given the similar biophysical properties between exosomes and viruses, recent studies have demonstrated that PEG-based methods can successfully enrich exosomes from cell culture supernatants for in-depth proteomic and sequencing analyses [16-17]. Domestic research has also reported successful extraction of placenta-derived exosomes from serum using PEG6000, highlighting the method' s simplicity, speed, and efficiency [18]. However, this approach has not yet been applied to seminal exosome extraction. In this study, we employed 8% PEG6000 to enrich semen-derived exosomes and validated the extracts through TEM, NTA, and Western blotting for protein markers (CD63, ALIX, TSG101). This work establishes a simple and efficient method for seminal exosome enrichment and characterization, laying the groundwork for future functional and mechanistic studies.

Materials and Methods

1.1.1 Study Subjects From March to June 2016, semen samples were collected from six healthy volunteers at the Laboratory Medicine Center of Nanfang Hospital, Southern Medical University, according to the WHO 5th edition diagnostic criteria [19]. Participants abstained from ejaculation for 3–7 days and collected semen samples via masturbation into dry, sterile wide-mouth containers, allowing liquefaction at room temperature. All samples were excluded if

they showed abnormalities in liquefaction time, pH, sperm morphology, seminal fructose, acid phosphatase, or -glucosidase parameters. This study was approved by the Ethics Committees of the Third Affiliated Hospital and Nanfang Hospital of Southern Medical University, and all volunteers provided informed consent after receiving detailed information about the study.

1.1.2 Instruments and Reagents The following instruments and reagents were used: SCA (Sperm Class Analyzer) sperm quality analyzer (MicroPtic, Spain); Optima XPN-100 intelligent ultracentrifuge (Beckman, USA); NanoSight nanoparticle tracking analyzer (NanoSight, UK); H-7650 transmission electron microscope (Hitachi, Japan); PEG6000 (Sangon, Shanghai); 0.45 m and 0.22 m filters (Millipore, USA); electrophoresis and electrotransfer apparatus (Bio-Rad, USA); total protein extraction kit (Beyotime, Shanghai); protein concentration assay kit (Beyotime, Shanghai); rabbit anti-human CD63, TSG101, and ALIX polyclonal antibodies (Abconal, USA); rabbit anti-human calnexin polyclonal antibody (Bioworld, USA); HRP-labeled goat anti-rabbit IgG (Ray Antibody, Beijing); and PVDF membrane (BioTrace, Mexico).

1.2.1 Isolation and Extraction of Seminal Exosomes Semen samples were liquefied at room temperature for 20 minutes, transferred to sterile EP tubes, and centrifuged at 1,000 g for 10 minutes at 4 °C to remove sperm cells. The supernatant was then centrifuged at 12,000 g for 30 minutes at 4 °C to remove smaller cells and debris. The resulting seminal plasma was mixed 1:1 with PBS, filtered through 0.45 m and 0.22 m membranes to remove larger microvesicles, and then mixed 1:1 with PEG6000 solution (2× stock: 16 g PEG6000 and 5.844 g NaCl dissolved in 100 mL ddH O) for overnight incubation at 4 °C (final concentration 8% PEG6000). The following day, the mixture was centrifuged at 12,000 g for 30 minutes at 4 °C. The pellet was resuspended in PBS and ultracentrifuged at 100,000 g for 90 minutes at 4 °C. The final pellet was resuspended in PBS, filtered through a 0.22 m membrane for sterilization, and stored at -80 °C until use. Total protein content was quantified using the BCA method.

1.2.2 Western Blotting Exosome suspensions were mixed with loading buffer, denatured at 95 °C for 10 minutes, and 40 g of protein per lane was separated by 10% SDS-PAGE (80 V for 30 minutes, then 100 V for 60 minutes). Proteins were transferred to PVDF membranes via wet electrotransfer (200 mA for 120 minutes). Membranes were blocked with 5% bovine serum albumin in TBS for 1 hour at room temperature, then incubated overnight at 4 °C with primary antibodies: rabbit anti-human CD63, ALIX, and TSG101 polyclonal antibodies (1:1000) and rabbit anti-human calnexin polyclonal antibody (1:1000). After washing five times with TBST (5 minutes each), membranes were incubated with HRP-labeled goat anti-rabbit IgG (1:10,000) for 1 hour at room temperature, washed again five times with TBST, and visualized using an ECL chemiluminescence kit. Images were captured using a Bio-Rad gel

imaging system.

1.2.3 Transmission Electron Microscopy For TEM analysis, 20–40 L of seminal exosome suspension was applied to a sample grid and allowed to stand at room temperature for 5 minutes. Liquid was absorbed from the side with filter paper, and approximately 10 L of 4% phosphotungstic acid solution was added for negative staining at room temperature for 5 minutes. After removing the staining solution and drying under an incandescent lamp for 10 minutes, the grid was examined under a transmission electron microscope to observe exosome morphology and capture images.

1.2.4 Nanoparticle Tracking Analysis Nanoparticle tracking analysis (NTA) visualizes nanoparticles by passing a concentrated laser beam through a glass prism to illuminate the sample (seminal exosome suspension). A microscope-mounted camera captures video footage of Brownian motion, which is then analyzed to calculate the hydrodynamic radius and concentration of nanoparticles in the sample [20]. Following the manufacturer' s instructions, parameters were set with a measurement time of 60 seconds. Seminal exosomes were diluted 1:2500 in water to a final volume of 1 mL and analyzed by NTA to determine particle size and concentration.

Results

2.1 Basic Characteristics According to the inclusion and exclusion criteria, semen samples were collected from six normal volunteers. SCA sperm quality analyzer was used to evaluate basic semen parameters including age, semen volume, pH, progressive motility, sperm concentration, and normal morphology rate (Table 1).

2.2 Western Blot Validation of Exosomal Markers Western blotting was performed to detect exosomal marker proteins and assess purity using calnexin, a highly expressed endoplasmic reticulum protein in sperm cells. The results demonstrated that PEG6000-enriched seminal exosomes positively expressed CD63, ALIX, and TSG101 proteins, while corresponding sperm cells and exosome-depleted seminal fluid were negative. Conversely, calnexin was expressed only in sperm cells but not in seminal exosomes or exosome-depleted fluid (Figure 1 [Figure 1: see original paper]).

2.3 Transmission Electron Microscopy TEM observation revealed that seminal exosomes were surrounded by intact double membranes, appearing as round or elliptical vesicles approximately 100 nm in diameter containing low electron-density material (Figure 2 [Figure 2: see original paper]).

2.4 Nanoparticle Tracking Analysis After 2500-fold dilution in PBS, the extracted seminal exosomes showed a concentration of approximately 2.98×10^{12} particles/mL. The mode curve was smooth, with a peak particle size of 115 nm (Figure 3 [Figure 3: see original paper]A). Scatter plot analysis indicated that exosome diameters were relatively concentrated, mostly between 30–150 nm (Figure 3B). Cumulative percentage data showed that particles sized 0–30 nm, 30–150 nm, and >150 nm accounted for 0.189%, 82.253%, and 17.558% of the total particle count, respectively (Table 2).

Discussion

Interest in exosome research has grown substantially in recent years. Despite significant progress, the biological functions and mechanisms of exosomes remain poorly understood. Recent studies have reported that seminal exosomes carry small non-coding RNAs with potential regulatory functions [7]. Another study comparing infertile patients with oligoasthenozoospermia to normal individuals identified differential microRNA expression profiles in seminal extracellular vesicles, with miR-765 and miR-1275 upregulated and miR-15a downregulated in infertile patients, opening new perspectives for investigating male infertility mechanisms [21]. Further research on seminal exosomes promises to bring new directions to male urogenital health, enabling discovery of non-invasive early biomarkers and novel therapeutic approaches with broad clinical applications [10-11].

As research on the origins and biological functions of exosomes advances, developing robust isolation and identification methods has become a critical foundation for further exploration. Currently, there is no standardized method for exosome isolation, with differential centrifugation, sucrose density gradient ultracentrifugation, and commercial kits being the main approaches. However, ultracentrifugation is time-consuming, and commercial kits are costly, limiting their utility for large-scale studies. Each method has distinct advantages and disadvantages, but the key considerations for exosome isolation are minimizing contaminating proteins, ensuring high purity, and confirming expression of established exosomal markers. Only high-purity, high-yield, and high-concentration exosomes can guarantee the quality of subsequent investigations [14].

PEG6000 is a water-soluble, non-ionic polymer with strong hydrophilic properties that can induce dehydration and precipitation of biological macromolecules or form complexes via hydrogen bonding. Since exosomes are nanoscale membrane vesicles with hydrophobic lipid bilayers, PEG6000 can precipitate exosomes by interacting with hydrophobic proteins and lipid molecules. In 2016, Rider et al. [16] used seven different PEG6000 concentrations (5–10% and 12%) to extract exosomes from cell culture supernatants, finding that 8% PEG6000 yielded purity comparable to traditional ultracentrifugation and superior to commercial kits. The extracted total protein and RNA were sufficient for proteomic

and RNA sequencing analyses, and confocal microscopy confirmed that exosome biological function remained intact.

In this study, we successfully enriched seminal exosomes using 8% PEG6000. TEM and NTA analyses revealed minimal background contamination and the presence of heterogeneous nanovesicles exhibiting round, elliptical, cup-shaped, or irregular morphologies approximately 100 nm in diameter with electron-dense contents—characteristic features of exosomes. Compared to conventional dynamic light scattering and flow cytometry, NTA offers higher resolution, direct visualization, and the ability to calculate nanoparticle concentration [20-22]. NTA results showed high particle numbers (2.98×10^{12}/mL), high purity, and a concentrated size distribution, with 82.253% of particles measuring 30–150 nm, consistent with theoretical exosome size ranges and TEM observations. The small proportion of particles outside this range likely represents aggregated vesicles, as previously reported.

Vojtech et al. [7] isolated seminal exosomes from 12 volunteers using ultracentrifugation with a double sucrose cushion, reporting an average diameter of 93 nm, with >75% of particles distributed between 50–200 nm and an average concentration of 1.37×10^{13}/mL (range: 4.7×10^{11}–3.12×10^{13}/mL). Madison et al. [9, 23] used ExoQuick (SBI) kits and sucrose density gradient ultracentrifugation, finding that <20%, >40%, and >20% of seminal exosomes were distributed in ranges of 28–50 nm, 50–100 nm, and 100–150 nm, respectively. Despite size overlap among different vesicle types, our results indicate that PEG6000 enrichment yields predominantly semen-derived exosomes. Furthermore, exosomes stably express proteins involved in antigen presentation, such as tetraspanins (CD63, CD81, CD9), heat shock proteins (HSP70, HSP90), and biogenesis-related proteins (TSG101, ALIX) [24]. Previous studies have used transmembrane proteins like CD63, CD9, and HSP70 as seminal exosome markers [7, 9, 21, 23]. Our Western blot analysis confirmed positive expression of CD63, ALIX, and TSG101 in our extracts, with negative expression in sperm cells and exosome-depleted seminal fluid, demonstrating successful enrichment of semen-derived exosomes. Additionally, calnexin, an endoplasmic reticulum protein highly expressed in sperm cells but absent in seminal exosomes, served as a purity control, further validating the reliability of our PEG6000 enrichment method.

In summary, the PEG6000 method enables rapid, efficient, and convenient enrichment of semen-derived exosomes, providing a solid foundation for investigating their biological functions and mechanisms in male reproduction and establishing a theoretical basis for clinical applications.

References

[1] Lotvall J, Hill AF, Hochberg F, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles[J]. J Extracell Vesicles,

2014, 22(3): 26913.

[2] Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication[J]. J Proteomics, 2010, 73(10): 1907-20.

[3] Johnstone RM, Adam M, Hammond JR, et al. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes)[J]. J Biol Chem, 1987, 262(19): 9412-20.

[4] Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends[J]. J Cell Biol, 2013, 200(4): 373-83.

[5] Sun DM, Zhuang XY, Zhang SQ, et al. Exosomes are endogenous nanoparticles that can deliver biological information between cells[J]. Adv Drug Deliv Rev, 2013, 65(3): 342-7.

[6] Altevogt P, Bretz NP, Ridinger J, et al. Novel insights into exosome-induced, tumor-associated inflammation and immunomodulation[J]. Semin Cancer Biol, 2014, 28(7): 51-7.

[7] Vojtech L, Woo S, Hughes S, et al. Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions[J]. Nucleic Acids Res, 2014, 42(11): 7290-304.

[8] Ronquist G, Brody I. The prostasome: its secretion and function in man[J]. Biochim Biophys Acta, 1985, 822(2): 203-18.

[9] Madison MN, Roller RJ, Okeoma CM. Human semen contains exosomes with potent anti-HIV-1 activity[J]. Retrovirology, 2014, 11(1): 102.

[10] Machtinger R, Laurent LC, Baccarelli AA. Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation[J]. Hum Reprod Update, 2016, 22(2): 182-93.

[11] Foster BP, Balassa T, Benen TD, et al. Extracellular vesicles in blood, milk and body fluids of the female and male urogenital tract and with special regard to reproduction[J]. Crit Rev Clin Lab Sci, 2016, 53(6): 379-95.

[12] Poliakov A, Spilman M, Dokland T, et al. Structural heterogeneity and protein composition of exosome-like vesicles (prostasomes) in human semen[J]. Prostate, 2009, 69(2): 159-67.

[13] Lamparski HG, Metha-Damani A, Yao JY, et al. Production and characterization of clinical grade exosomes derived from dendritic cells[J]. J Immunol Methods, 2002, 270(2): 211-26.

[14] Zeringer E, Barta T, Li M, et al. Strategies for isolation of exosomes[J]. Cold Spring Harb Protoc, 2015(4): 319-23.

[15] Albertsson P A, Frick G. Partition of virus particles in a liquid two-phase system[J]. Biochim Biophys Acta, 1960, 37(13): 230-7.

[16] Rider MA, Hurwitz SN, Meckes J. ExtraPEG: A polyethylene Glycol-Based method for enrichment of extracellular vesicles[J]. Sci Rep, 2016, 12(6): 23978.

[17] Weng YJ, Sui ZG, Shan YC, et al. Effective isolation of exosomes with polyethylene glycol from cell culture supernatant for in-depth proteome profiling[J]. Analyst, 2016, 141(15): 4640-6.

[18] Li YJ, Diao ZY, Xue PP, et al. Isolation and identification of placenta-derived exosomes from serum[J]. J Med Postgrad, 2015(6): 632-6.

[19] Lu WH, Gu YQ. Insights into semen analysis: a Chinese perspective on the fifth edition of the WHO laboratory manual examination and processing of human semen[J]. Asian J Androl, 2010, 12(4): 605-6.

[20] Dragovic RA, Gardiner C, Brooks AS, et al. Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis[J]. Nanomedicine, 2011, 7(6): 780-8.

[21] Abu-Halima M, Ludwig N, Hart M, et al. Altered micro-ribonucleic acid expression profiles of extracellular microvesicles in the seminal plasma of patients with oligoasthenozoospermia[J]. Fertil Steril, 2016, 106(5): 1061-9.

[22] Filipe V, Hawe A, Jiskoot W. Critical evaluation of nanoparticle tracking analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates[J]. Pharm Res, 2010, 27(5): 796-810.

[23] Madison MN, Jones PH, Okeoma CM. Exosomes in human semen restrict HIV-1 transmission by vaginal cells and block intravaginal replication of LP-BM5 murine AIDS virus complex[J]. Virology, 2015, 482(8): 189-201.

[24] Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles[J]. Annu Rev Cell Dev Biol, 2014, 30(7): 255-89.

Edited by: Sun Changpeng

Note: Figure translations are in progress. See original paper for figures.