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Standardization of Sampling for Isolation of Exosome-Like Small-Extracellular Vesicles from Peripheral Blood from Reproductive-Aged Women

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Abstract

Exosome-like small-extracellular vesicles (sEVs) are extracellular vesicles that act in intercellular communication and are involved in several biologic and pathologic processes. While sEVs increase the stability of their cargo molecules, there is still a need for standardization of sampling and isolation of these microvesicles. We aimed to determine the best sampling method for isolation of sEVs from peripheral blood from reproductive-aged women. **Material and Methods:** We included samples of plasma from our biobank collected in 2014 by venipuncture in heparin tubes and stored at −80˚C. We also included blood samples collected in heparin tubes and Ethylenediamine tetraacetic acid (EDTA) tubes and stored at −80˚C for one to two weeks prior processing. All blood samples were collected from the same nine reproductive-aged female volunteers. sEVs were isolated from plasma by ultracentrifugation and filtration and indirectly quantified using Pierce BCA Protein Assay kit. Transmission electron microscopy (TEM) and Nano Tracking Analysis (NTA) were performed to confirm the isolation of sEVs. **Results and Discussion:** TEM and NTA confirmed the isolation of sEVs. Protein concentration of short-time stored heparin samples was not statistically different from long-time stored heparin samples (1847.2 ± 651.4 vs. 2363.2 ± 1025.1, p = 0.14). There was no difference between heparin and EDTA plasma samples recently collected (2363.2 ± 1025.1 vs. 2044.8 ± 653.2, p = 0.44). In

conclusion, blood samples may be collected using heparin or EDTA for isolation of sEVs. Long-time stored plasma samples maintain sEVs integrity and may be used, especially in comparative studies.

Keywords

Exosome-Like Small-Extracellular Vesicles, Plasma, Reproductive-Aged Women

1. Introduction

Exosome-like small-extracellular vesicles (sEVs) are doubled-membrane vesicles with a diameter that varies between 30 to 150 nm that along with microvesicles and apoptotic bodies form the heterogeneous group of extracellular vesicles [1] [2]. These vesicles are classified according to their size, components and generation mechanisms and can be found in different biological fluids such as blood and cerebrospinal fluid [3].

sEVs are important regulators of immune response and act as crucial mediators of cellular communication in several physiologic and pathologic processes by serving as vehicles for transfer of different molecules between cells [4] [5]. They have been described to carry a variety of molecules such as membrane and cytosolic proteins, lipids and nucleic acids [6]. sEVs represent a rich source of prospective biomarkers studies in many fields as they confer stability to their cargo and can orientate their content to specific types of cells [3].

The best-characterized mechanism for exosome formation involves the recruitment of the endosomal sorting complex required for transport machinery to ubiquitinated proteins in the early endosome. An alternative pathway has been recently described and involves synthesis of ceramide as a mechanism to induce vesicle curvature and budding. A third biogenesis mechanism that has been proposed is tetraspanin-mediated organization of specific proteins such as the amyloidogenic protein pre-melanosome protein [7].

Several studies have analyzed the potential role of sEVs and their molecular content as biomarkers in different diseases including gestational pathologies such as pre-eclampsia (PE) and gestational diabetes. This type of investigation can be especially useful once it can be performed using fairly non-invasive samples such as blood. In a recent study Pillay *et al.* [8] have reported that total placental-derived exosomes was increased in pre-eclamptic patients when compared with normotensive women. Salomon *et al.* [9] have demonstrated that two types of microRNA (has-miR-486-1-5p and has-miR-486-2-5p) were upregulated in exosomes from peripheral blood of pregnant women with PE when compared to non complicated pregnancies. Another study from the same group identified that the exosome concentration was significantly higher in the gestational diabetes group than in normal pregnancies matched by gestational age [10].

Besides their role as prominent biomarkers, these nanovesicles also hold a promise for therapeutic use. They have the potential to be used as a drug delivery vehicle since they have the desirable features of an ideal drug delivery system such as long circulating half-life, the intrinsic ability to target tissues, biocompatibility and minimal toxicity issues. sEVs also offer a major advantage because there is no unwanted accumulation or homing in the liver and they avoid the first pass metabolic effect [11] [12]. These microvesicles have also shown the ability to induce tissue regeneration by delivering growth factors, proteins, miRNA, non-coding RNA, mRNA and lipids. In a myocardial infarction, for example, sEVs derived from stem cells have been able to regenerate cardiac tissue [13].

In this scenario, optimization of sampling and isolation of sEVs are important to obtain improved and reliable results. We aimed to determine the best sampling method for isolation of sEVs from peripheral blood of reproductive-aged women considering the storage length and the type of anticoagulant.

2. Material and Methods

2.1. Sampling

We included samples of plasma from our biobank collected in 2014 by venipuncture in heparin tubes and stored at −80˚C. We also included blood samples collected in heparin tubes and EDTA tubes and stored at −80˚C for one to two weeks prior processing. Inclusion criteria were reproductive aged women fasting for at least one hour prior blood collection. Sleep deprived women or those with coagulation disorders were excluded as these conditions may affect levels of sEVs. All blood samples were collected from the same nine reproductive aged female volunteers (convenience sample method) by venous puncture in sterile tubes and plasma was obtained by centrifugation at 1800 g for 10 minutes.

2.2. Isolation of Small-Extracellular Vesicles

sEVs were isolated using aliquots of 2 mL of plasma following the ultracentrifugation protocol adapted from Lässer *et al.* [14]. First, samples were centrifuged at 20,000 g for 30 minutes to remove cell debris. Then, the supernatant was transferred to a falcon tube and diluted with 4 mL of cold sterile PBS (phosphate buffered saline). This mix was filtered using a sterile syringe filter pore size 0.20 μm (Millipore) and ultracentrifuged at 120,000 g for 90 minutes. At this stage, the supernatant was discarded and the pellet containing the sEVs were resuspended in 200 μL of cold sterile PBS and stored in sterile tubes at −80˚C. All the centrifugation stages were performed at 4˚C.

2.3. Indirect Quantification of sEVs

Considering that sEVs are coated by proteins, we performed the protein quantification of the isolated sEVs using the Pierce BCA Protein Kit (Thermo Scientific) according to the instructions provided by the manufacturer.

2.4. Characterization of sEVs

Transmission electron microscopy (TEM) was performed to visually confirm the isolation of sEVs. Briefly, sEVs-PBS suspension were added to a nickel grid with formvar for 1 hour then washed three times with PBS and treated after every wash using paraformaldehyde 2%, glutaraldehyde 2.5% and uranyl acetate 2%. Then samples were finally fixed (metil celulose 0.13% and uranyl acetate 0.4%) and visualized by TEM (Tecnai Spirit).

Additionally, sEVs size was measured using a Nanosight NS300 Instrument (Malvern) and analyzed with NanoSight Nano Tracking Analysis (NTA) Software v3.1. Videos (5 × 30 s) were acquired after the manual introduction of EV samples (diluted 1:500 in PBS), at camera level 13, temperature (37˚C), and by reference to 50, 100, and 150-nm calibration beads (Malvern) to verify accuracy. Vesicle size and concentration were defined as mode and mean according to the acquired videos using a detection threshold of 3.

2.5. Statistical Analysis

Paired T test was used to compare protein quantification between long vs. short time stored and heparin vs. EDTA using the software GraphPad Prism 5.0. A p value of 0.05 was considered statistically significant.

3. Results

Median age of the participants was 28.6 years old (±3.1). All the participants were non-smokers and non-drug users. Their sociodemographic characteristics are shown in Table 1.

Successful isolation of sEVs was visually confirmed by TEM (Figure 1). NTA also confirmed the isolation of sEVs. The medium size of the particles was 96.6 ± 20.0 nm and medium concentration was $1.0 \times 10^{11} \pm 5.3 \times 10^{10}$ particles/mL, a size compatible with the diameter described for sEVs (Figure 2).

Regarding indirect quantification of sEVs, protein quantification of short time stored heparin samples was not statistically different from long time stored heparin samples (2363.2 ± 1025.1 μg vs. 1847.2 ± 651.4 μg, p = 0.14). Similarly, there was no difference in protein concentration between recently collected heparin and EDTA plasma samples (2363.2 ± 1025.1 μg vs. 2044.8 ± 653.2 μg, p = 0.44).

4. Discussion

Communication is critical for survival in the natural environment. On an intercellular level communication can be mediated by extracellular vesicles. All organisms, from bacteria [15] to animals [16] are known to produce these vesicles and they have been identified in several human body fluids, such as blood [17], breast milk [18] [19], ejaculate [20] and also in tissues including tumors [21]. Thus, understanding extracellular vesicles-mediated communication is important for both basic science and translational research.

Table 1. Sociodemographis characteristics of participants.

Variables	Participants (n = 9)
Age	28.6 ± 3.1
Self-reported ethnicity—white	88.9%
Marital Status—single	66.6%
Years of study > 12y	100%

Variables age presented as mean (±SD). Remaining variables presented as percentage.

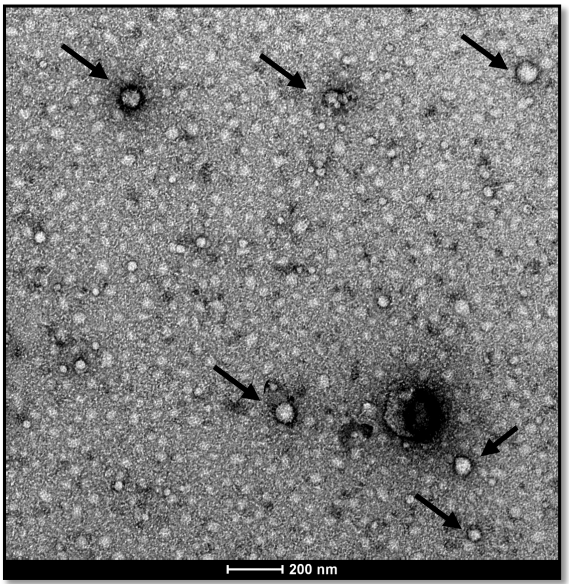

Figure 1. Electron micrograph of sEVs (arrows). The scale bar represents 200 nm (×23,000).

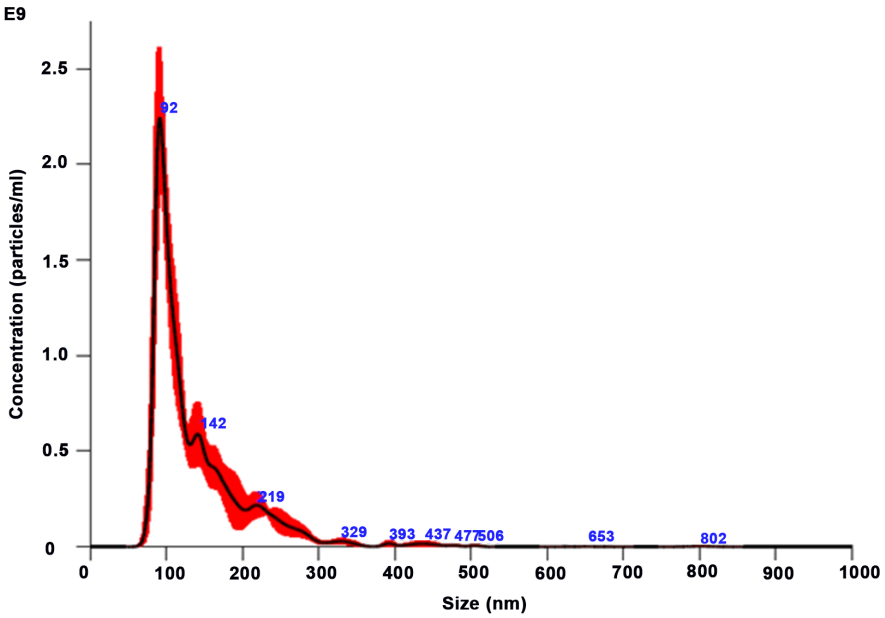

Figure 2. Concentration and size of particles by NTA. The y-axis represents concentration of particles per ml and the x-axis shows their size.

Purification methods and sample preparation techniques may indirectly select for some subpopulations of vesicles with specific biochemical or physical characteristics over others, possibly affecting the ultimate outcome of experiments [22]. Therefore, standardization of sEVs isolation is an essential step to studies that aim to characterize their cargo in biological and pathological processes and also for therapeutic purposes.

In the present study we did not observe statistical differences in sEVs concentration indirectly measured by protein quantification between two types of anticoagulants commonly used to collect blood samples, heparin and EDTA. This indicates that blood samples may be collected using either anticoagulant for isolation of sEVs.

Nevertheless, further investigation must be performed to evaluate downstream applications. A recent report by the International Society of Extracellular Vesicles recommends to avoid the use of heparin as anticoagulant when molecular techniques such as real time PCR is to be performed. According to this report, heparin competes with primers and/or enzymes for binding to acid nucleic and a prior heparinase treatment is recommended [23].

Regarding length of storage, we did not observe statistical differences between long-time stored plasma and recently collected samples. While collection of fresh material may result in more fidelity in absolute quantification of sEVs, our results demonstrate that long time stored plasma maintains sEVs integrity and may be used, especially in comparative studies.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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