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Comparison of Methods for Concentration Assessment of Extracellular Vesicles Isolated from Different Biological Fluids

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Abstract—Accurate quantification of extracellular vesicles (EVs) remains a significant challenge in biomedical research. Although various analytical methods have been developed, their reliability is often limited by the presence of non-vesicular nanoparticles and biological contaminants, particularly in biological fluids. Moreover, for some sources of EVs, such as uterine aspirates and gastric juice, quantitative evaluation of EVs has not been investigated. The aim of the study is to perform comparative analysis of three EV quantification methods: total protein content measurement, nanoparticle tracking analysis (NTA), and esterase activity assessment using commercial FluoroCet exosome quantitation kit in EVs isolated from various biological fluids: blood plasma, ascitic fluid, uterine aspirates, gastric juice, and medium conditioned by ovarian and non-small cell lung cancer cells. All three methods demonstrated strong correlation for the EV samples derived from the conditioned medium, supporting their validity for *in vitro* EV quantification in highly purified samples. In contrast, blood plasma, ascitic fluid, and uterine aspirates exhibited discrepancies between the methods, likely attributable to the presence of non-vesicular nanoparticles. Notably, the EVs from gastric juice demonstrated strong correlation between the protein content and esterase activity, indicating prevalence of the vesicle-associated proteins and, potentially, unique EV composition in this fluid. The findings underscore the necessity for multifactorial approach to EV quantification, taking into account factors such as sample origin and limitations inherent to the specific method employed. These results may serve as a basis for the development of standardized protocols for EV quantification, which is particularly relevant for clinical sample analysis.

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Keywords: exosomes, extracellular vesicles, vesicle concentration, NTA, FluoroCet

INTRODUCTION

Extracellular vesicles (EVs) comprise a heterogeneous group of nanoparticles enclosed into a lipid-bilayer membrane, which are released by cells into environment. Exosomes (with sizes 30-150 nm) and microvesicles (sizes up to 1 μm) are the most investigated types of EVs, which differ in mechanisms of formation and molecular composition. EVs play a key role in cell–cell communications transporting proteins, lipids, RNAs and DNAs between the nearest and distant cells and tissues, thus affecting physiological and pathological processes in an organism. Tumor cells excrete larger numbers of EVs in comparison with the normal cells thus facilitating intercellular exchange of tumor-associated molecules; that is why their function have been investigated most thoroughly in the context of carcinogenesis, where EVs mediate malignization of normal cells, remodeling of stromal microenvironment, evasion of immune surveillance,

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acquiring drug resistance, formation of pre-metastatic niche, and other processes [1]. Mechanism of EV formation and selection of their molecular cargo is strictly controlled; hence, their composition could reflect molecular profile of the cells-producers; that is why EVs are often considered as promising biomarkers for non-invasive fluid-based diagnostics in oncology and other areas of medicine [2]. Moreover, due to the protective function of the membrane and unique composition of the receptors on its surface, EVs are subjects of intensive investigation in the area of targeted drug delivery [3].

Considering growing interest in EVs for diagnostics and therapy, the International Society for Extracellular Vesicles (ISEV) developed standards and recommendations for working with these structures. According to the MISEV recommendations (Minimal Information for Studies of Extracellular Vesicles), it is necessary prior to start of investigation to characterize the preparation using several independent methods [4]. These methods include morphological analysis, identification of molecular markers, as well as quantitative assessment including measuring concentration of EVs and their size distribution.

Assessment of EV concentration is an essential step of their characterization; however, at present there is no universal technique known that has sufficient accuracy, sensitivity, and specificity. The most often used approaches are the following: measuring concentration (total content) of protein in EV preparations, determination of concentration using nanoparticle tracking analysis (NTA), enzymatic methods (such as FluoroCet and ExoCet), as well as less often used but promising technologies such as tunable resistive pulse sensing (tRPS), flow cytometry, and fluorescent modification of NTA [5, 6].

Determination of total protein content in the samples with extracellular vesicles is performed using either classic colorimetric techniques (Bradford protein assay and bicinchoninic acid assay), or using highly sensitive fluorescent reagent kits such as Qubit and NanoOrange. The latter are especially useful for working with low-concentration samples, obtained, for example, from the conditioned cell culture medium (CM). The main advantages of the method are its simplicity and reproducibility, however, the key drawback of the method is lack of specificity: the obtained values include both proteins associated with vesicles, as well as non-vesicular proteins including from viruses, supramolecular attack particles (SMAPs), chylomicrons, exomeres, supermeres, as well as protein complexes and aggregates, lipoproteins of different density, and ribonucleic complexes such as vault-type ribonucleoprotein complexes, etc. [7, 8]. The EV preparations most contaminated with non-vesicular structures are preparations isolated from clinical samples, particularly from blood [9, 10].

The NTA method allows quantitative evaluation of size distribution and concentration of the particles based on analysis of their Brownian motion in a solution. Unlike in the method of dynamic light scattering (DLS), this method evaluates each particle in the solution separately, while DLS operates with ensemble of particles, it is demanding to the sample concentration, and is rarely used for assessment of concentrations. The NTA method exhibits high sensitivity and it provides information on heterogeneity of particles in the sample, however, similar to the DLS-based methods, it does not allow distinguishing vesicles from non-vesicular particles of the similar size such as lipoproteins, virus-like particles, and other structures mentioned above [11].

The enzymatic methods, such as the one based on using the FluoroCet reagent kit, are based on determination of activity of acetylcholine esterase (AChE) in the vesicles, membranes of which are, presumably, enriched with this enzyme. This approach requires minimal sample volume and exhibits high sensitivity, but it depends on the level of expression of AChE, which could vary depending on the cell origin and type of biological fluid. Other methods, in particular the tRPS method based on recording changes of electrical resistance, when individual particles pass through a nanopore, or flow cytometry, which uses antibodies against specific markers of EVs (CD9, CD63, CD81), as well as mass-spectrometry allow more accurate assessment of composition and origin of the vesicles, but so far they remain more labor-intensive and expensive [12, 13]. Many of the alternative methods, such as, for example, methods based on immunoprecipitation, allow assessment of only particular populations of EVs, but not the entire pool.

Hence, the issue with standardization of the methods for quantification of EVs and correct interpretation of the results, especially in the cases of clinical preparations of EVs with high levels of contamination (blood plasma, urine, etc.), has been unresolved yet. At the same time, selection of the most adequate and accessible method for quantification of EVs is one of the most important task of the research in this area. That is why, the goal of this study was comparison of three methods of quantification of EVs – evaluation of protein concentration in the EV preparations, analysis of trajectories of nanoparticles, and analysis of esterase activity with the help of commercial kit for quantitative analysis of exosomes FluoroCet – in the samples of EVs isolated from different biological fluids. The following biological fluids were selected as sources of EVs commonly used in the context of studying EVs (blood plasma, ascitic fluid), as well as less studied in the context of EVs biological fluids,

but which attract significant interest with regard to screening of oncomarkers and as drug targets, such as uterine aspirates and gastric juice.

MATERIALS AND METHODS

Clinical samples. Clinical samples were obtained from Blokhin National Medical Research Center of Oncology, Ministry of Health of the Russian Federation from the patients prior to surgery or any other treatment. All donors of biomaterials signed voluntary informed consent form to participate in the study. Study protocol was approved by the local ethics committee (Protocol no. 5, June 10, 2022, project no. 22-15-00375; and Protocol no. 1, January 25, 2024, project 24-25-00052). Samples of aspirate from uterus cavity (uterine aspirates, n = 14) were collected with the help of a Pipelle type C device from the donors without oncological anamnesis. Immediately after collection, the sample (volume varied from 0.2 to 1 ml) was diluted in 0.5 ml of cold phosphate buffer (PB; Gibco, USA). Samples of ascitic fluid (n = 10) were collected under sterile conditions in the process of laparocentesis from the female patients with histologically verified diagnosis of ovarian serous carcinoma. Sample volumes varied from 5 to 500 ml (more often 10-25 ml). Samples of uterine aspirates and ascites were provided by the department of oncogynecology; samples of gastric juice (GJ) – by the Endoscopic department. Gastric juice samples (n = 18) were collected during endoscopic examination of the individuals without oncological diseases using a video gastroscope GIF H-185 (OLYMPUS, Japan). Prior to procedure, the patients were fasting (12 h without food and 6 h without water). Volume of collected GJ was from 2 to 10 ml; after collection it was diluted in 5 ml of PB. GJ samples were collected from different regions of the stomach. Peripheral blood samples (n = 12) were provided by the department of thoracic oncology; they were collected from the patients with verified diagnosis of non-small cell lung cancer into vacuum tubes with EDTA. Blood plasma was produced with a standard centrifugation technique.

Cell cultures. Ovarian cancer cell lines (OVCAR-3, OVCAR-4, OVCAR-8, SKOV3) and non-small cell lung cancer cell lines (H460, H1299, A549) were cultivated at 37°C in atmosphere of 5% CO_2 in a RPMI-1640 and DMEM medium (PanEko, Russia) supplemented with 10% fetal bovine serum (FBS; HyClone, Austria), 100 U/ml penicillin and 100 mg/ml streptomycin (PanEko). To prepare exosome-free medium FBS was used, which was first purified from native vesicles with the help ultracentrifugation at 110,000g for 16 h. To collect conditioned medium, cells were seeded into 6 cell culture flasks with surface area of 175 cm^2, next day the medium was exchanged with the exosome-free medium. When the cells reached 90% confluency the medium was collected, combined, and used for isolation of small EVs.

Sample processing and isolation of EVs. Samples of conditioned medium after removal of cell debris (centrifugation at 2000g for 15 min at 4°C) was stored without freezing at 4°C up to 7 days. Combined supernatants were used for the following isolation of EVs according to the protocol described previously [14]. All clinical samples were processes no later than 1 h after collection and stored on ice for the duration of the entire process. Tubes with whole blood were centrifuged at 2000g for 15 min at 4°C to produce blood plasma, which next was centrifuged again at 10,000g (30 min) and stored at −80°C until isolation of EVs. Protocols for processing samples of uterine aspirates and ascites [15], as well as of gastric juice [16] were described in our previous studies. Isolation of EVs was carried out using the method of differential centrifugation according to the standard protocol [17]; all modifications of the technique for isolation of EVs for each biological fluid have been described in the respective abovementioned papers.

Nanoparticle tracking analysis. Size composition and concentration of extracellular vesicles were determined using NTA method using a NanoSight LM10 HS device equipped with a LM14 temperature-control system (Malvern Panalytical Ltd., United Kingdom), a laser module LM 14C (405 nm, 65 mW), and high-resolution CMOS camera with (C11440-50B; Hamamatsu Photonics, Japan). All measurements were carried out in accordance with the ASTM E2834-12(2018) standard. Prior to examination samples were diluted in a particle-free PB to final concentration ~1.5×10^8 particles/ml. For each sample 12 60-s videos were recorded. Processing and combining of the data were performed using the NTA 2.3 build 33 software (Malvern Panalytical Ltd.). Primary NTA data are presented in the Online Resource 1.

Transmission electron microscopy. Carbon coated grids (Ted Pella, USA) were first treated for 45 s in an Emitech K100X device (Quorum Technologies Ltd., United Kingdom) to increase hydrophilicity of the surface. Vesicle samples were diluted 5-40-fold in PB depending on concentration determined with NTA; next samples were applied onto grids and incubated for 30-60 s. Next, grids were stained twice for 45 s with a 1% solution of uranyl acetate and dried at room temperature. Images (no less than 10 for each sample) were acquired with a JEM-1400 electron microscope (JEOL, Japan) operating at acceleration voltage of 120 kV.

Analysis of protein and exosome concentration. Total protein content in the preparations of extracellular vesicles was determined using two methods

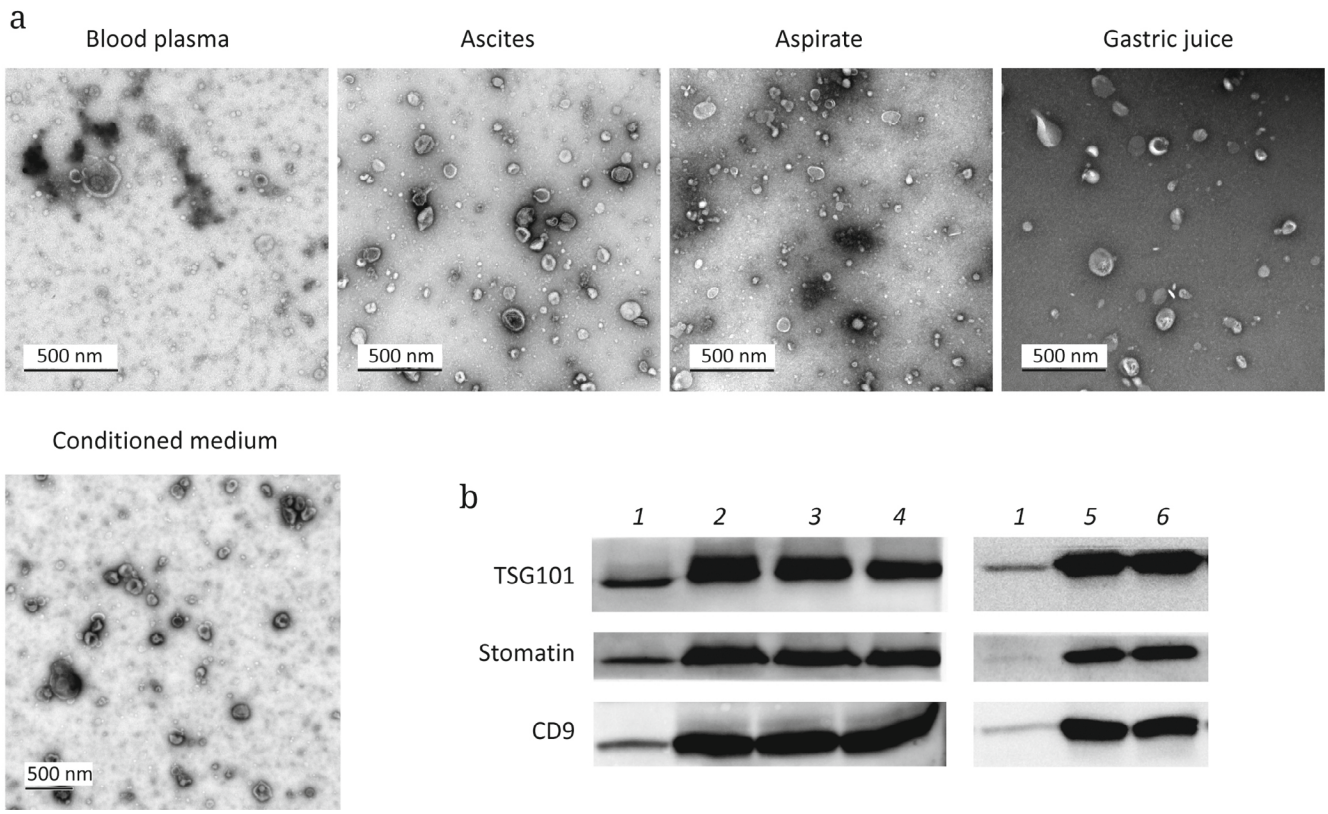

Fig. 1. Morphological and molecular characterization of extracellular vesicles (EVs) isolated from different biological fluids. a) Examples of microphotographs of EVs from the investigated biological fluids obtained with the help of transmission electron microscopy. Scale bar: 500 nm. b) Examples of Western blot analysis of exosomal proteins markers in the same preparations of EVs. Lanes: *1*) control, cell lysate of OVCAR-8 cell line; *2-6*) vesicles isolated from five different biological sources in the following order: uterine aspirates (*2*), ascitic fluid (*3*), gastric juice (*4*), blood plasma (*5*), conditioned medium from the culture of OVCAR-8 cells (*6*).

depending of protein concentration in the sample. The samples with low protein concentration (<1 μg/ml) were analyzed using a NanoOrange® Protein Quantitation Kit (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. Fluorescence was recorded with a SpectraMax M5e microplate reader (Molecular Devices, USA). The samples with high protein concentrations (such as ascites and plasma) were examined using the Bradford protein assay with reagents from Bio-Rad (#500-0006; Germany) according to the manufacturer's instructions. Measurements were carried out with a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, USA). A FluoroCet™ Exosome Quantitation Kit was used enzymatic activity of acetylcholine esterase (System Biosciences, USA). Analysis was carried out according to the manufacturer's protocol. Samples were incubated with a substrate at room temperature; next measurements were performed. Each sample was analyzed in at least two technical replicates.

Immunoblotting and antibodies. Protocol used for immunoblotting of the vesicle samples from different sources was described in our previous studies [14-16]. The following primary antibodies were used: anti-Flotillin-2 (1 : 1000; #3436S; CST, USA); anti-CD9 (1 : 2000; #13174; CST); anti-Stomatin (1 : 500; #sc-134554; Santa Cruz, USA). Goat-anti-rabbit antibodies were used as secondary antibodies (1 : 80 000; #29902; CST).

Statistical data processing. Spearman's Rank Correlation was used to calculate correlation coefficients between the methods for quantification of extracellular vesicles. Calculations were carried our using the GraphPad Prism 9 software (GraphPad Software, USA). p-values < 0.05 were considered statistically significant.

RESULTS

Characterization of vesicle of different origins. Morphology and size of EVs isolated from different biological fluids were evaluated with the help of transmission electron microscopy and nanoparticle tracking analysis. As can be seen in the presented microphotographs (Fig. 1a), particles of spherical

Table 1. Average values of size characteristics of extracellular vesicles isolated from different biological sources (based on NTA data)

Source of EVs	Number of samples, *n*	Average size, nm (SEM)	Modal size, nm (SEM)	Median size, nm (SEM)	10th percentile, nm (SEM)	90th percentile nm (SEM)
Uterine aspirate	14	139.0 (2.3)	102.9 (3.6)	122.8 (2.4)	59.4 (1.0)	235.9 (4.0)
Ascitic fluid	10	143.6 (4.7)	91.3 (3.9)	122.9 (4.5)	58.4 (1.3)	252.1 (8.4)
Gastric juice	18	145.1 (5.6)	88.4 (5.6)	130.1 (6.2)	54.3 (2.3)	261.1 (8.8)
Blood plasma	12	132.5 (4.3)	89.2 (4.1)	125.7 (4.4)	57.8 (2.4)	249.3 (6.9)
Conditioned medium	13	127.5 (2.5)	95.0 (4.9)	112.2 (2.8)	56.4 (1.0)	211.6 (3.8)

Note. Values are presented in nm with standard error of the mean (SEM) in brackets.

or cap-like shape typical of vesicles have been observed in all preparations.

In the next stage molecular verification of the vesicular nature of the isolated particles was conducted. In accordance with the ISEV recommendations the following proteins localized in different compartments of EVs were used as positive markers of exosomes: luminal component of the ESCRT complex TSG101; component of lipid rafts, stomatin, suggested in our previous study as an exosome marker [18]; and CD9 – component of tetraspanin-enriched microdomains. All investigated samples were enriched with these proteins in comparison with the cell lysates, which supports their vesicular nature (Fig. 1b).

Analysis of concentration and size-distribution of the particles conducted using NTA demonstrated that the average size of EVs varied from 127.5 nm (CM) to 145.1 nm (GJ), while the modal values were in the range 88.4-102.9 nm. Complete results on size characteristics are presented in Table 1.

To evaluate reliability of different approaches for quantification of extracellular vesicles the samples obtained from five different sources (conditioned medium of tumor cells (n = 13), blood plasma (n = 10), ascitic fluid (n = 12), uterine aspirates (n = 14), and gastric juice (n = 18)) were analyzed. In all cases vesicles were isolated using differential ultracentrifugation. Three independent methods were used for quantification: measurements of total protein content (commercial reagent kit NanoOrange or Bradford method depending on the sample concentration), NTA, and fluorescent analysis of acetylcholine esterase activity (further mentioned as FluoroCet).

Vesicles from the conditioned medium. The highest convergence of the results was obtained during analysis of EVs isolated from the conditioned media of different tumor cells lines. Results obtained for all samples demonstrated significant and pronounced correlation between the three used methods (Fig. 2).

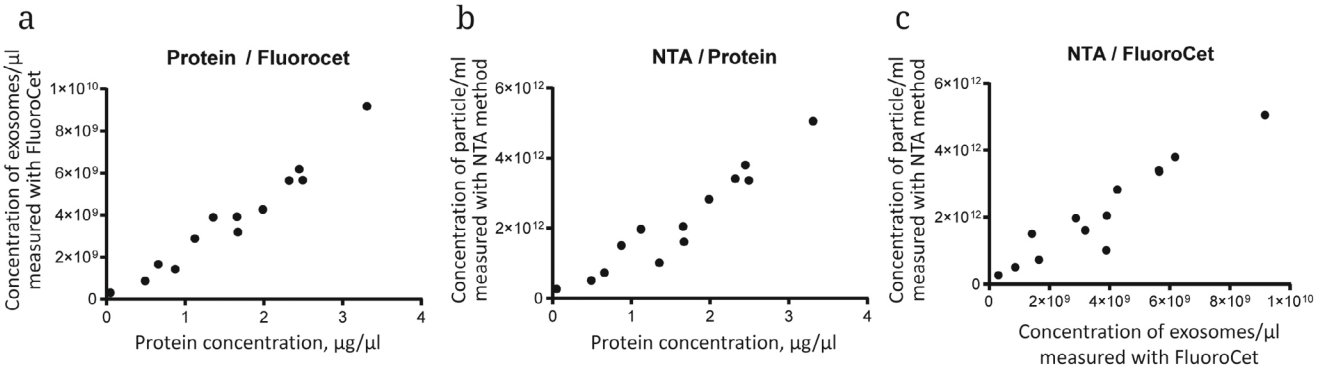

Fig. 2. Correlation analysis of the results of quantification of extracellular vesicles isolated from the media conditioned by ovarian cancer tumor cells (OVCAR-3, OVCAR-4, OVCAR-8, SKOV3) and by the non-small cell lung cancer cells (H460, H1299, A549) using three methods. a) Comparison of FluoroCet with NanoOrange; b) comparison of NTA with NanoOrange; c) comparison of NTA with FluoroCet. Each dot on the graph corresponds to the individual sample of extracellular vesicles isolated from the indicated biological source.

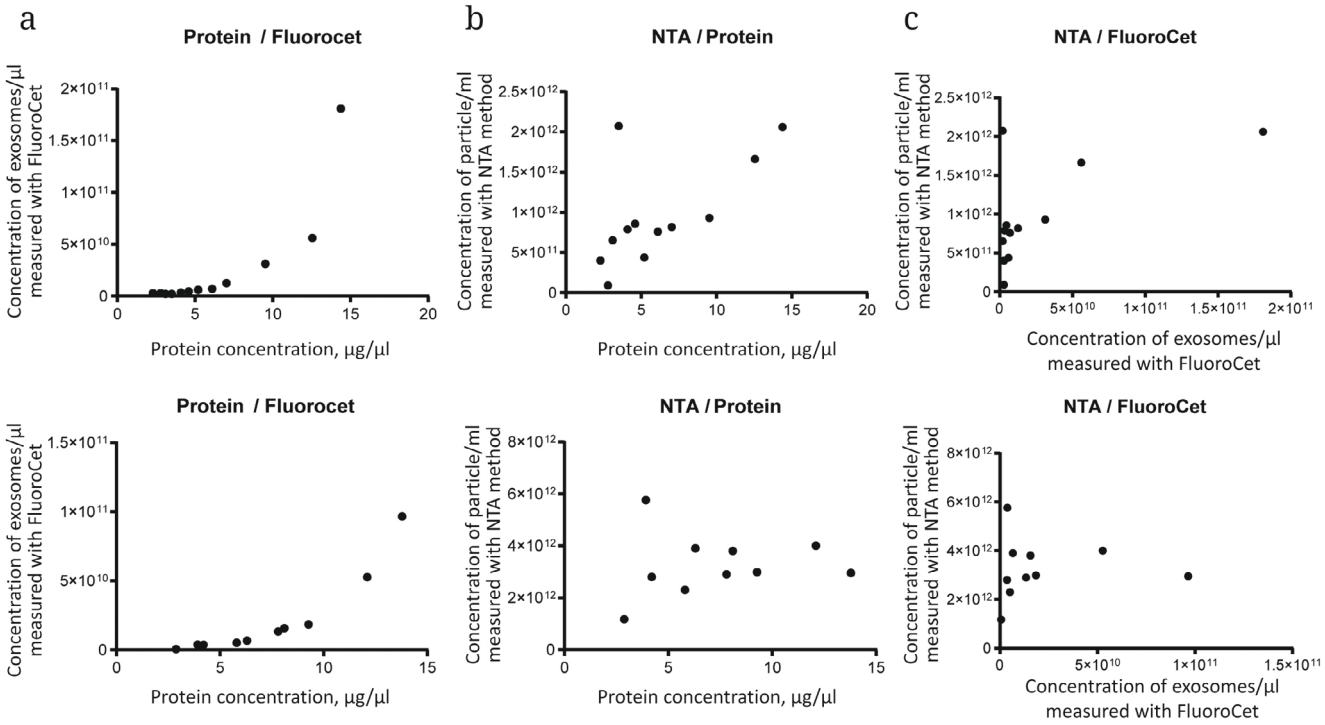

Fig. 3. Correlation analysis of the results of quantification of extracellular vesicles isolated from blood plasma (upper panel) and ascitic fluid (lower panel) using three methods. a) Comparison of FluoroCet with Bradford assay; b) comparison of NTA with Bradford assay; c) comparison of NTA with FluoroCet. Each dot on the graph corresponds to the individual sample of extracellular vesicles isolated from the indicated biological source.

Spearman's Rank Correlation coefficients (r) were 0.95 for the pairs protein/NTA and NTA/FluoroCet, and for the pair protein/FluoroCet – 0.97 ($p < 0.01$). With high purity of the isolated vesicles and absence of significant contribution of external proteins or non-vesicular nanoparticles, the results of these methods show similar patterns and allow comparing concentrations of EVs in different samples. Hence, in the case of using standardized protocol of isolation, all three methods can be used for quantification of relative concentration of EVs.

Vesicles isolated from blood plasma and ascitic fluid. Unlike in the case of CM, in the samples isolated from blood plasma significant differences were revealed between the results of different methods. The weakest correlation was observed between NTA and FluoroCet (r = 0.63), as well as between NTA and protein content measured with the Bradford method (r = 0.63), which indicates presence in the plasma of a large number of non-vesicular particles with sizes comparable with EVs (Fig. 3, upper panel). At the same time, the correlation dependence in the pair protein/FluoroCet was shown to be non-linear, which was most pronounced in the samples with high protein concentration (>8 µg/µl). This could be explained by the possibility of presence of AChE-positive lipoprotein complexes in the high-concentration samples. Although AChE is not typical for the main lipoproteins, this enzyme has been found in the specialized complexes, for example, being associated with the lipid membranes of erythrocytes via the GPI-anchors stabilized by phospholipids (including cardiolipin) [19]. The enzyme could be released during hemolysis from the membranes in composition of complexes with lipids, which could ensure non-proportional contribution to the signal at high protein concentrations. These results highlight limitations for the use of both NTA and FluoroCet in the native biological fluids with high concentrations of proteins and lipoproteins. At the same time measurements of total protein content also do not ensure sufficient specificity for the reliable quantification of vesicles, because significant fraction of protein could be from the soluble components of plasma not associated with EVs.

Patterns of the results obtained in analysis of vesicles from ascitic fluid were similar to those observed in the case of blood plasma, but exhibited even more pronounced divergence between the methods. The lowest correlation was observed between NTA and FluoroCet (r = 0.42) and between NTA and Bradford assay (r = 0.33), which indicates higher level of foreign particles and protein complexes in the samples (Fig. 3, lower panel). Same as in the case of blood plasma, quantitative dependence between the total protein content and activity of acetylcholine esterase (FluoroCet) in the samples of ascitic fluid was non-linear.

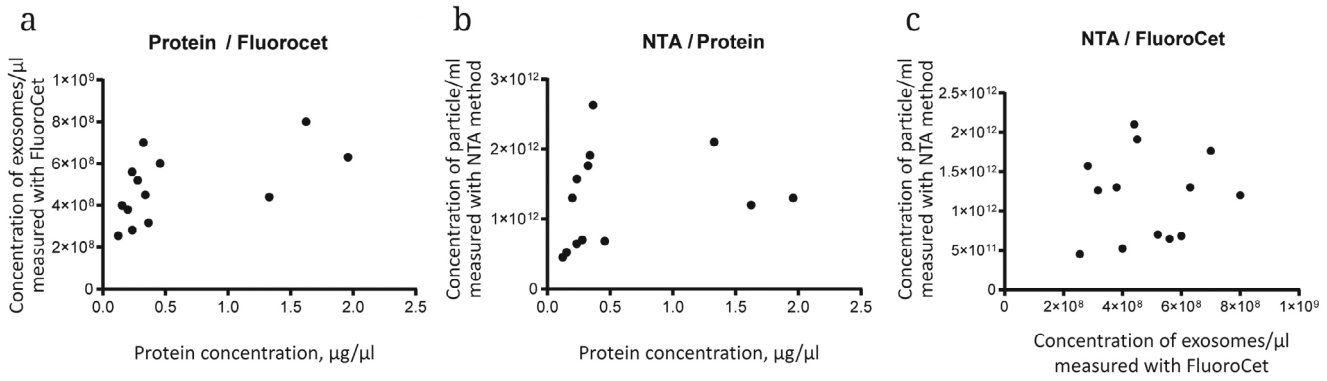

Fig. 4. Correlation analysis of the results of quantification of extracellular vesicles isolated from uterine aspirates using three methods. a) Comparison of FluoroCet with NanoOrange; b) comparison of NTA with NanoOrange; c) comparison of NTA with FluoroCet. Each dot on the graph corresponds to the individual sample of extracellular vesicles isolated from the indicated biological source.

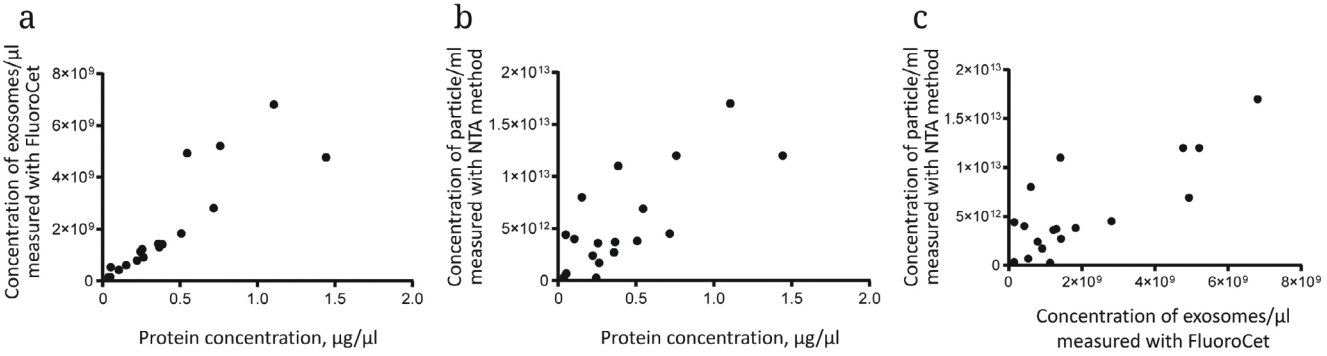

Fig. 5. Correlation analysis of the result of quantification of extracellular vesicles isolated from gastric juice using three methods. a) Comparison of FluoroCet with NanoOrange; b) comparison of NTA with NanoOrange; c) comparison of NTA with FluoroCet. Each dot on the graph corresponds to the individual sample of extracellular vesicles isolated from the indicated biological source.

Considering that ascites often contain admixtures of blood, this effect could be explained by the presence of specific AChE-positive lipoprotein complexes similar to those observed in plasma. At the same time, contribution of variable expression of AChE on its content in the extracellular vesicles obtained from different types of cells cannot be ruled out. Hence, similar to the case of blood plasma, EVs from ascitic fluid require more stringent purification method, use of several quantification techniques, and accurate interpretation of the results.

Vesicles from uterine aspirates. The samples obtained from uterine aspirates typically have low total protein content (not exceeding 2 μg/μl), which allowed avoiding saturation of the signal in enzymatic reactions. Nevertheless, the degree of correlation between the methods was moderate (r = 0.62 – for the protein/FluoroCet pair; r = 0.52 – for the protein/NTA pair), while no significant correlation between the results of FluoroCet and NTA was observed (Fig. 4).

Overall, the data indicate that quantification of EVs from uterine aspirates requires using of several approaches and, if possible, following normalization based on marker proteins.

Vesicles from gastric juice. Unexpectedly, the results obtained during analysis of EVs isolated from gastric juice differed significantly from the results observed during investigation of EVs from other biological fluids. Unlike in other cases, there was very high correlation between the protein content and activity of acetylcholine esterase in the EVs samples isolated from GJ (r = 0.97; $p < 0.0001$), which implied presence of high proportion of vesicular proteins in the total protein composition (Fig. 5).

Moreover, significant, although moderate, correlation between the results of NTA and two other methods was observed: r = 0.63 – for the NTA/FluoroCet pair and r = 0.67 – for the NTA/NanoOrange pair; which indicated that contribution of non-vesicular particles to the NTA signal was still present. It is important to note that total protein concentrations in the samples of GJ were significantly lower (<0.5 μg/μl) in comparison with other fluids, which could facilitate more accurate evaluation of vesicles and decrease

Table 2. Spearman's correlation coefficients between three methods of quantification of extracellular vesicles derived from different types of biological fluids

Pair of compared methods	Spearman's correlation coefficient				
	CM	Plasma	Ascites	Aspirate	GJ
Protein/FluoroCet	**0.97**	n/d*	n/d*	**0.62**	**0.97**
NTA/Protein	**0.95**	**0.63**	0.33	0.52	**0.63**
NTA/FluoroCet	**0.95**	**0.63**	0.42	0.07	**0.67**

Note. Statistically significant values are shown in bold. * Correlation dependence is non-linear.

of the background signal. The obtained data allow suggesting that the EV samples obtained from GJ are characterized with high purity and absence of contaminants, majority of which have protein components. Hence, one of the significant results of this study is characterization of gastric juice as a promising, previously not investigated in detail biological source of EVs, which could potentially be important for the search of markers and development of non-invasive diagnostic approaches based on molecular composition of EVs.

Summarizing the obtained results of comparative analysis, it could be concluded that consistency of the results between the used methods of quantification of extracellular vesicles depends significantly on the type of biological fluid from which the EVs were produced (Table 2).

DISCUSSION

Heterogeneity of vesicles in size, composition, and origin in combination with difficulties of distinguishing them from other interfering particles, such as lipoproteins, protein complexes, viruses, and a whole number of other particles, complicates standardization of approaches for biochemical and physical characterization of EVs. Despite the existence of a wide variety of approaches developed and used in investigation of EVs, they all have their peculiarities and limitations in the detection range, accuracy, processivity, and applicability for analysis of particular parameters of EVs. At present, there are no methods, which could be considered universal for the reliable quantification of EVs.

The results obtained in our study confirm that selection of the method for assessment of concentration of EVs is critically dependent on the type of analyzed biological fluid and degree of purity of the sample. In the case of cell culture CM, where content of 'foreign proteins' and nanoparticles is minimal, all three methods (NTA, measurement of total protein, and FluoroCet), demonstrated high degree of consistency. This allows considering any of them as suitable for relative quantitative assessment of vesicles in the highly purified samples, which is achieved by the second round of ultracentrifugation. The data obtained in this study are in agreement with the previously published results demonstrating significant effect of the sample purity on the results of EVs quantification; in particular, it was shown in the study by Escudero-Cernuda et al. [20] that variations between the replicates decreased from 43 to 15% with the decrease of the level of admixtures present in the CM.

NTA is one of the most popular methods for quantification of EVs, which uses the principle of Brownian motion for determination of both size and concentration of particles. However, its application for analysis of complex biological fluids, such as blood plasma and ascitic fluid, was shown to be severely limited due to impossibility to distinguish true EVs from lipoproteins and protein aggregates. These data are in full agreement with the results of previous studies showing that up to 70% of the particles detected by the NTA method in blood plasma could be not EVs [9].

Determination of total protein concentration remains the most affordable and widely used method for quantification of EVs. However, as was shown in this study, high concentration of soluble proteins typical for blood plasma and ascitic fluid, decreases significantly reliability of this approach. Similar limitation has been reported previously: albumin and other serum proteins could be responsible for up to 60% of the total protein signal in the EV preparations from blood plasma [21].

The enzymatic FluoroCet method based on measuring activity of AChE demonstrated high sensitivity and linearity in the samples with low degree of contaminations, such as conditioned cell culture medium and gastric juice. However, in the EV samples from blood plasma and ascites non-linear dependence between the esterase activity and protein concentration was observed, which could be associated with

both presence in the protein-enriched samples of AChE-positive lipoprotein complexes, as well as with uneven distribution of AChE between the populations of vesicles [22]. In this context, the results reported by Grigor'eva et al. seem to be of great interest [23]. It was shown that the preparations of exosomes isolated from different biological fluids including blood plasma inevitably contain a large number of lipoproteins ('non-vesicles') comparable in size with exosomes. Fraction of these structures could reach 40% in plasma, which significantly distorts the results of molecular analysis of exosomes. These data confirm that the observed in our study non-linear dependence between the AChE activity and protein concentration in the EVs from protein-rich biological fluids could be explained primarily by contamination with lipoproteins emphasizing the necessity of a complex approach for quantification of EVs, which should include control of purity of the vesicle preparations.

The most interesting results were obtained during analysis of EVs from gastric juice, which revealed extremely high correlation between the protein content and activity of acetylcholine esterase ($r = 0.97$). This phenomenon has not been observed for other biological fluids, and it allows suggesting that the aggressive medium in stomach facilitates selective preservation of vesicular proteins and degradation of proteins not associated with vesicles. In comparison with blood plasma and ascites, GJ contains lower amounts of proteins and have relative low protein load, which facilitates reduction of artefacts during measurements. In our previous study we for the first time isolated and characterized EVs from gastric juice in accordance with the ISEV recommendation including description of their morphology, size characteristics, and composition of marker proteins [16]. Furthermore, we demonstrated that the vesicles from GJ collected from the patients with stomach cancer differ in size, distribution, and level of expression of tetraspanin CD9 from the vesicles collected from the GJ of the healthy donors [24]. These data confirm potential of EVs in GJ as a promising object for search of diagnostically significant molecular signs of stomach cancer. This study supplements these observations: high degree of correlation between the independent methods of quantification demonstrates applicability of GJ as a stable and representative source of EVs for further molecule studies devoted to search of potential biomarkers.

Use of different techniques at the same time seems as the most effective strategy, which helps to overcome limitations of individual approaches, when used simultaneously. In particular, combination of enzymatic assay with the methods assessing size and concentration (NTA or tRPS) seems reasonable to use for examination of complex fluids (plasma, ascites) supplementing this with determination of protein content and calculation of the degree of purity (particles/protein ratio). This approach allows simultaneous evaluation of the yield of EVs and quality of the preparation [25]. Lack of standardized methods for quantification of EVs is a serious problem in the studying of vesicles. It was shown both in our study and in the studies published worldwide that the use of different approaches often produces significantly different results for the identical samples, which complicates comparison of the results of different studies and their clinical interpretation [26, 27].

In the context of standardization, it is necessary to mention that at present there is no commonly accepted 'gold standard' for the method for isolation of EVs from biological fluids, and, obviously, selection of the method affects purity of preparations and amount of contaminating particles of one or another origin. The available techniques such as different variants of ultracentrifugation including the ones using density gradients, methods based of capture of nanoparticles by biopolymers, chromatographic methods, methods of microfiltration and ultrafiltration, methods based on isolation of EVs based on binding of one or another vesicular molecules have their advantages and drawbacks with regards the balance between the yield of EVs and purity of the preparations (absence of non-vesicular contaminations). In this study the method based on differential centrifugation/ultracentrifugation was used for isolation of EVs from different biological sources, which is used most often and is recommended by ISEV. The revealed in this study correlations between the results of different methods for quantification of EVs indirectly support the notion that this technique is ideally suitable for quantification of EVs from the samples of cell culture medium, which are characterized with minimal contaminations by non-vesicular particles with sizes and density similar to EVs, as well as for quantification of EVs from GJ, which, presumably, also have low level of contaminations, at least of those of protein nature. At the same time, as has been mentioned above, absence of correlation between the results of analysis of EVs from other biological fluids indicates presence of a large number of contaminating particles in the EV preparations, which, undoubtedly, is associated with the selection of the isolation technique. This problem could be resolved to a large extent by using other techniques (such as, for example, exclusion chromatography, micro- and ultrafiltration, immunoaffinity methods, and others); however, for many of these methods loss of the total amount of isolated EVs or of individual populations of vesicles is typical. Nevertheless, it could be assumed that in the case of using these techniques, selection of the method for quantification of EVs would affect the results of analysis to a lesser degree.

Quantification of EVs is essential for comparison of their concentrations in the clinical samples, especially considering the actively discussed at present hypothesis on the increase of concentration of EVs in the biological fluids (primarily in circulating fluids) of oncology patients, which has been suggested for diagnostic purposes and for monitoring relapses [28-30]. Moreover, majority of the studies investigating functional significance of EVs and their *in vitro* and *in vivo* molecular composition in carcinogenesis and tumor progression (as well as in pathogenesis of other diseases) require very accurate enumeration of EVs. Hence, the results obtained in our study are important for further progress in this area of research.

From the practical point of view, the obtained data could be used for optimization of protocols for quantification of EVs in clinical samples, as well as the basis for selection of the method depending on the type of biological fluid. In addition, our results allow recommending gastric juice as a promising object for the search of vesicular biomarkers with high specificity and low background levels in the case of using protein and enzymatic assays for quantification.

CONCLUSIONS

The conducted study clearly demonstrates that none of the investigated methods of quantification of extracellular vesicles is universally applicable for all types of biological samples. The obtained results confirm the necessity of differential approach for the selection of the methods depending on the origin and degree of purity of the analyzed preparations of EVs. All three considered methods (NTA, total protein content, and analysis of esterase activity) could be successfully used for the EV preparations isolated from the standardized cell-conditioned medium, while examination of biological fluids derived from an organism requires combination of several approaches that also should take into consideration degree of purity of the samples. Unique feature of the EVs from gastric juice, which demonstrate exceptionally high correlation between the protein content and activity of acetylcholine esterase revealed in this study, is especially interesting. This indicates minimal level of contamination of the samples with non-vesicular protein particles. The obtained data could be used by the researchers for selection of the technique for quantification of EVs of different origin, which especially important in examination of clinical samples.

Abbreviations

AChE	acetylcholine esterase
CM	culture medium
EV	extracellular vesicles
GJ	gastric juice
NTA	nanoparticle tracking analysis
PB	phosphate buffer

Supplementary information

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Contributions

G. O. Skryabin, E. M. Tchevkina – concept and supervision of the study; G. O. Skryabin, A. D. Enikeev, A. A. Beliaeva, S. A. Galetsky, D. V. Bagrov – conducting experiments; K. I. Zhordania, O. T. Imaraliev, I. A. Karasev – preparation of clinical materials; G. O. Skryabin, A. D. Enikeev, D. V. Bagrov, E. M. Tchevkina – discussion of the study results; G. O. Skryabin – writing text of the paper; G. O. Skryabin, E. M. Tchevkina – editing text of the paper.

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Ethics approval and consent to participate

All procedures involving samples derived from humans were carried out in accordance with institutional, national and international standards. Voluntary informed consent form was signed by all participants of the study.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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