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Research paper

Purified horse milk exosomes contain an unpredictable small number of major proteins

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Abstract

Exosomes are 40–100 nm nanovesicles containing RNA and different proteins. Exosomes containing proteins, lipids, mRNAs, and microRNAs are important in intracellular communication and immune function. Exosomes from different sources are usually obtained by combination of centrifugation and ultracentrifugation and according to published data can contain from a few dozens to thousands of different proteins. Crude exosome preparations from milk of eighteen horses were obtained for the first time using several standard centrifugations. Exosome preparations were additionally purified by FPLC gel filtration. Individual preparations demonstrated different profiles of gel filtration showing well or bad separation of exosome peaks and one or two peaks of co-isolating proteins and their complexes. According to the electron microscopy, well purified exosomes displayed a typical exosome-like size (30–100 nm) and morphology. It was shown that exosomes may have several different biological functions, but detection of their biological functions may vary significantly depending on the presence of exosome contaminating proteins and proteins directly into exosomes. Exosome proteins were identified before and after gel filtration by MALDI MS and MS/MS spectrometry of protein tryptic hydrolyzates derived by SDS PAGE and 2D electrophoresis. The results of protein identification were unexpected: one or two peaks co-isolating proteins after gel-filtration mainly contained kappa-, beta-, alpha-S1-caseins and its precursors, but these proteins were not found in well-purified exosomes. Well-purified exosomes contained from five to eight different major proteins: CD81, CD63 receptors, beta-lactoglobulin and lactadherin were common to all preparations, while actin, butyrophilin, lactoferrin, and xanthine dehydrogenase were found only in some of them.

The article describes the morphology and the protein content of major horse milk exosomes for the first time. Our results on the decrease of major protein number identified in exosomal preparations after gel filtration may be important to the studies of biological functions of pure exosomes.

Keywords: Horse milk; Exosomes; Proteins identification; MALDI mass spectrometry

1. Introduction

Mammalian breast milk is known as functionally active nutrient system. However, breast milk contains significant amounts of various factors: many bioactive proteins, peptides and antibodies. These compounds of breast milk promote neonatal

Abbreviations: EVs, extracellular vesicles; FPLC, fast protein liquid chromatography; MM, molecular mass; 2D-electrophoresis, two-dimensional electrophoresis (isoelectric focusing and SDS-PAGE); SDS-PAGE, SDS polyacrylamide gel electrophoresis.

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growth protecting newborns from viral and bacterial infections and they are usually considered as integral parts of the infant's intestinal physiology [1,2]. Milk is an essential source of a wide variety of proteins; during the last 16 years more than 200 publications examining the milk proteome were appeared in PubMed [2].

Secreted membrane-enclosed vesicles include microvesicles, microparticles, ectosomes, exosomes, apoptotic bodies as well as other extracellular subsets. All of them were collectively called extracellular vesicles (EVs) and currently belong to the rapidly growing field of biology and medicine [3].

Exosomes are 30–100 nm membrane vesicles. They are secreted into the extracellular liquids by different types of cells in various biological fluids: saliva, serum, amniotic fluid, breast milk, and urine [3–6]. Exosomes contain several major mRNAs, microRNAs, proteins, and lipids, and are considered as alternative pathway of secretion. It is shown that exosomes are involved in the development of pathological processes such as growth and spread of cancer, the transfer of infectious agents including the whole virus particles or viral RNA, and prion proteins (for review see Ref. [6]). In addition, recent findings suggest that the exosomes are specialized vesicles involved in intracellular communication in multicellular organisms, carrying on its surface various lipid and protein ligands, and passing the recipient cells are novel proteins and nucleic acid [7–11]. Exosomal components are usually transferred to the recipient cells and can change biological functions of the target cells [3–6]. It was suggested that exosomes may be involved in several neurodegenerative diseases [10,11].

Exosomes containing proteins and/or RNA were obtained from human [12–17], bovine [2,18,19] and porcine milk [20].

It has been suggested that preparations of exosomes may be used for clinical purposes as effective carriers of various drugs including proteins, lipids, and RNA and other compounds to mammalian cells [21]. It was reported that exosomes can deliver curcumin anti-inflammatory agent in vivo to activated myeloid cells and that this drug directing to the cells is associated with therapeutic, but not toxic, effects [22]. It was shown that catalase included into exosomes by special method is stable against degradation by proteases. In vitro exosomes effectively interacted with neuronal cells and after intranasal administration a significant amount of exosomes was detected in the brain of mice with Parkinson's disease [11]. Since catalase of exosomes leads in vivo to significant neuroprotective effects it was proposed that such approach may be useful for treat inflammatory and neurodegenerative disorders. One can assume that to deliver of different drugs to the human cells may be used exosomes of mammals. One of the main available sources of preparative amounts of exosomes is the milk of cows and horses.

To date, no data concerning horse milk exosomes have been published. At the same time, the only article devoted to horse exosomes evaluates these vesicles as markers of erythrocyte regeneration, as horses do not release reticulocytes into the peripheral blood [23]. The authors have proposed that transferrin receptor 1 (TfR1) expressed in exosomes of serum can provide a regeneration of new marker for anemic horses.

Many very different methods were used for isolation of exosomes from milk and other mammalian biological liquids including precipitation by centrifugation in special conditions, ultracentrifugation, ultracentrifugation in density gradients etc. [12–25]. The combination of sequential centrifugations with the increasing number of rpm (from 300 *g* up to 16,500 *g*) is universal and frequently used approach: the final step of ultracentrifugation is usually performed at 100,000 *g* [19,24–26]. Some protocols also contain ultrafiltration through 0.10–0.22 μm filters [25]. A protocol of exosome purification suitable for clinical use was proposed, in which ultracentrifugation into a 30% sucrose/deuterium oxide cushion was added [27]. A method of biological fluids incubation in the ice at acidic pH has also been proposed [28]: neutrally charged exosomes are precipitated and then may be separated by centrifugation. The authors believe that exosomes obtained by this method do not differ from those purified by ultracentrifugation. It should be mentioned that these methods basically allow obtaining samples enriched with exosomes, but not pure ones.

In some publications the analysis of some vesicular and exosomal proteins was carried out. Small membranous vesicles (25–75 nm in diameters) were purified from the ram cauda epididymal fluid by only high-speed centrifugation [29]. These vesicles according to SDS-PAGE protein pattern are specific and significantly differ from those of the seminal plasma, sperm extract and the caudal fluid. After 2D-electrophoresis more than 40 proteins were revealed. Using mass MALDI mass spectrometry analysis approximately 30 proteins were identified. These 30 proteins were grouped into a) vesicle-associated proteins including lactadherin (MFEG8-PAS6/7) and vacuolar ATPase; b) membrane-linked enzymes including neprilysin (NEP), dipeptidyl peptidase IV (DPP-IV), protein G-beta and phosphodiesterase-I (E-NPP3), c) several cytoskeleton-associated proteins including annexin and ezrin; and d) metabolic enzymes.

After 2D-electrophoresis using MALDI MS/MS sequencing there were identified 188 proteins spots of cell-derived exosomes obtained from mouse fibroblast NIH3T3 cells and Ras-transformed NIH3T3 cells (isolated using several centrifugations) many of which were previously revealed in exosomes from cell of other type [25]. However, some proteins, for example, Serpin B6, have been identified as novel for fibroblast exosomes. It was shown that more than 34 proteins including collagen alpha-1 (VI), guanine nucleotide-binding proteins (G proteins), milk fat globule EGF factor 8 (lactadherin), collagen alpha-1 (VI), 14-3-3 species, the eukaryotic translation initiation factors elF-3 gamma as well as elF-5A are accumulated in exosomes (>2-fold) upon Ras-induced oncogenic transformation.

Dendritic cell-derived exosomes were purified by three successive centrifugations at 300 *g*, then at 1200 *g*, additionally at 10,000 *g*, and finally at 110,000 *g* [30]. These preparations of exosomes according to SDS-PAGE analysis contained ~30 protein bands, 11 of which were major proteins corresponding to several cytosolic proteins such as heat shock cognate protein hsc73, annexin II, and heteromeric G protein

Gi2alpha. In addition, several different integral or peripherally associated membrane proteins were revealed: milk fat globule-EGF-factor VIII [MFG-E8], major histocompatibility complex class II, CD9, and hsc73, Mac-1 integrin, and a cytosolic heat shock proteins. In the next proteomic analysis of dendritic cell-derived exosomes the same authors [31] identified 21 additional new exosomal proteins including most of all cytoskeleton-related (elongation factor 1alpha and cofilin, profilin I) as well as intracellular signaling and membrane transport factors (such as rab 7 and 11, several annexins, rap1B and syntenin). They have also identified a novel category of exosomal proteins associated with apoptosis: Alix, 14-3-3, thioredoxin peroxidase II, and galectin-3.

Biochemical and proteomic analyses revealed that exosomes from mature comparing with exosomes from immature cells may be enriched in intercellular adhesion molecule 1 (ICAM-1), MHC class II, B7.2, and bear little milk-fat globule-epidermal growth factor–factor VIII (MFG-E8) [32]. Targeting of exosomes to dendritic cells is mediated via milk CD11a, CD54, phosphatidylserine, fat globule (MFG)-E8/lactadherin, as well as alpha(v)/beta(3) integrin, CD11a and CD54, and the tetraspanins CD9 and CD81 on the exosome surface.

Later exosomes from mature dendritic cells were separated by a centrifugation at 10^5 *g* using a linear 2.0–0.25 M sucrose gradient and pellets were analyzed by SDS-PAGE and Western blotting [32]. Tandem mass spectrometry extensive proteomic analysis led to identification of 93 new additional proteins that were not identified in exosomes before [32,33]. On overall, it turns out that dendritic cells exosomes can contain more than 150–200 different proteins.

Even more incredible results were obtained from the analysis of exosome proteins of cow's milk. The cow milk exosomes were purified by successive centrifugations at 100,000 *g* and 200,000 *g* and preparation of very crude exosomes was subjected for centrifugation of sucrose gradient [2]. Purified preparations were first trypsin digested, then were subjected for offline high pH reverse phase chromatography, and finally were fractionated on a nanoLC connected to tandem mass spectrometer. This led to revealing of 2107 proteins including several major exosome proteins, which were previously identified as typical exosome markers. The major fat globule membrane proteins of milk including adipophilin, lactadherin, butyrophilin, and xanthine oxidase were the principal proteins found in milk exosomes in this paper [2].

It has been developed a single-step protocol to size-exclusion chromatography (SEC) isolation of vesicles from human body fluids on Sepharose CL-2B columns. It was shown that SEC efficiently isolates exosomes and other extracellular vesicles having a diameter over than 70 nm from concentrates of supernatant free of platelet [34] and from the concentrated urine samples [35].

It should be noted that according to our experience all methods mentioned above usually obtain samples only enriched with exosomes, but not pure ones. Various biological fluids contain easily degradable high molecular weight aggregates of various proteins that can be coprecipitated with exosomes under centrifugation at different conditions. In addition, we have recently shown that human milk and placenta contain very stable high molecular mass (~1000 kDa) multiprotein complex having size to some extent comparable with the vesicles [36,37]. In addition, some free proteins can specifically or nonspecifically interact directly with the surface or receptors of exosomes. Taking this into account, we assume that the relative number of proteins in the composition of exosomes may be to some extent overestimated.

Here we have for the first time performed a proteomics analysis of horse milk exosomes. To obtain crude preparations of exosomes from horse milk we first used traditional protocol including several centrifugations and ultracentrifugation. We obtained preparations containing exosomes and according to SDS-PAGE many different proteins with molecular masses of 10–300 kDa. Then exosomes were separated from very stable multiprotein complexes and other associated proteins by FPLC gel filtration of crude preparations of exosomes on Ultrogel effectively separating proteins with a molecular masses (MMs) of 55–9000 kDa. The preparations at each stage of the purification and concentration were subjected to electron microscopic control. The proteins of crude and purified exosome preparations were compared using different methods including MALDI MS and MS/MS analysis of tryptic hydrolyzates of proteins separated by SDS-PAGE and by two-dimensional electrophoresis. We have obtained a much unexpected result; the well purified individual exosomes contain only 4–7 different major proteins.

2. Materials and methods

2.1. Materials

Sorbents and different reagents used in this work were purchased from Sigma, Ultrogel from Life Technologies (USA). Fresh milk samples were obtained from healthy horses of the Siberian breed from dairy farm Verkh-Irmen (Novosibirsk region, Russia).

2.2. Preparation of exosomes from horse milk

Milk was collected from eighteen horses 10–30 days after delivery. The milk samples were cooled to 4 °C and then centrifuged at 10^5 *g* for 40 min at 4 °C. The milk samples (500 ml) were centrifuged twice for 40 min at 12,000 rpm (Beckman Coulter Avanti J-E, rotor JA-14), after each centrifugation, lipid layer at the top and the cells and protein precipitates from the bottom were removed. The supernatants were subjected for three sequential ultracentrifugations at 30,000 rpm (Beckman Coulter Avanti J-30I, rotor JA-30.50 Ti). The first ultracentrifugation was performed in the beakers of 50 ml. After the first centrifugation the precipitate was resuspended using 10 ml of TBS (20 mM Tris–HCl buffer (pH 7.5) containing 50 mM NaCl), the mixture was re-centrifuged at 30,000 rpm (Beckman L8-M, rotor SW-40) in 14 ml tubes and then this operation repeated. The resulting pellet corresponded to crude preparation of horse milk exosomes. The

preparations were resuspended and filtered through a filter (0.1 μm) and then analyzed. For additional purification the preparations were applied on a column Ultrogel (volume 30 ml, length 370 mm, diameter 8 mm; chromatograph GE Akta Purifier), which efficiently separates proteins with MMs of 55–9000 kDa. The concentrated solution of exosomes (0.25 ml) was applied on the column with Ultrogel equilibrated in 20 mM Tris–HCl (pH 7.5) containing 0.5 M NaCl and fractions (1.5 ml) eluted by the same buffer were collected. The proteins were monitored by absorbance at 280 nm. Fractions were dialyzed against 20 mM Tris–HCl buffer (pH 7.5) for 16 h at 4 °C for removing of NaCl and then different types of analysis were performed. All experiments were carried out under sterile conditions.

2.3. SDS-PAGE assay

SDS-PAGE analysis of exosomal proteins was carried out according to Laemmli using a 4–18% gradient gel containing 0.1% SDS. Before SDS-PAGE preparations 20–40 μg of protein were incubated in a buffer containing 50 mM Tris–HCl buffer (pH 6.8), 10% glycerol, 1% SDS, 0.025% bromophenol blue, 10 mM EDTA. This buffer was with or without 10 mM DTT; the mixtures after incubation for 20 min at 100 °C were applied on the gel. After the gel filtration on Ultrogel, similar analysis was carried out for all protein fractions. Coomassie R-250 was used for protein dyeing.

2.4. Preparation of trypsin digests

Identification of proteins was performed using MS and MS/MS data of MALDI-TOF mass spectrometric analysis of tryptic hydrolyzates after standard SDS-PAGE described above or after 2D-electrophoresis. In the case of 2D-electrophoresis the preparations were first separated by isoelectric focusing on Protean IEF Cell (Bio-Rad, USA): exosome samples were mixed with solubilizing buffer containing 8 M urea, 2% Nonidet P-40, DTT 50 mM, 0.2% ampholytes pH 3–10 and 0.01% Bromophenol blue were added in the cell (0.3 ml). A special strip (17 cm) with linear pH 3–10 gradient was overlaid (Bio-Rad, USA), top of strip was layered with mineral oil. Strips were dehydrated passively for 1 h, then actively for 12 h. Isoelectric focusing was performed at 250 V for 15 min, then at 10,000 V for 7 h. After IEF the strips were incubated in 0.375 M Tris–HCI buffer (pH 8.8) containing 2% SDS, 2% DTT, 6 M urea, 20% glycerol, and 0.01% Bromophenol blue for 30 min. Then the strips were incubated in the same buffer, contained 100 mM iodoacetamide and not contained DTT. After incubation the strips were used for standard SDS-PAGE, protein spots on the gels were revealed by Coomassie R-250 staining.

The Coomassie stained gel fragments after SDS-PAGE were consequently washed twice with 100 μl of milliQ water by shaking for 15 min, and to remove the dye gel fragments were twice washed for 30 min with 50 mM NH_4HCO_3 (50 μl) containing 50% acetonitrile, finally, to remove the H_2O, gel fragments were washed with 100 μl of 100% CH_3CN for 20 min. Then gel fragments were dried for 10 min at 30 °C using Eppendorf Concentrator plus rotary vacuum evaporator. For the protein digestion 20 μl NH_4HCO_3 (25 mM) containing 12.5 μg/ml of trypsin (sequencing grade, Promega, USA) was added to the gel pieces for 45 min at 0 °C and the solution was removed after the incubation. The fragments of gel were incubated for 18 h at 37 °C with 20 μl of 25 mM NH_4HCO_3 and the solutions were removed. For additional extraction of the peptides the gel fragments were washed twice with 25 μl of 50 mM NH_4HCO_3 containing 50% acetonitrile with shaking for 15 min. Fractions obtained after three treatments of gel fragments were pooled and lyophilized for subsequent MALDI-TOF mass spectrometric analysis.

2.5. MALDI mass spectrometry analysis of proteins

All mass spectra were acquired with an Autoflex speed MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). The spectrometer was operated in the positive reflector mode (standard method RP 700-3500 Da.par) with the control of FlexControl software (Bruker Daltonics; version 3.4). Saturated solution α-cyano-4-hydroxycinnamic acid was used as matrix; the acid was solved in 0.1% trifluoroacetic acid and acetonitrile (1:2). To 2 μl of the reaction mixture containing analyzed component, 2 μl of a mixture of 0.2% trifluoroacetic acid and matrix were added; 1 μl of the final mixture was spotted on the MALDI standard steel plate, air-dried, and used for the analysis. The analysis was carried out using the automatic mode (AutoXecute – automatic Run). The spectra were externally calibrated using Calibrate Peptide Standards. FAMS method and the standard mixture for calibration (Protein Calibration Standard II) from Bruker Daltonics were used. For automated peak extraction, files with the data were transferred to the Flex analysis (Bruker Daltonics software, version 3.4). Assignment of the first monoisotopic signals of the spectra was automatically performed using the special signal detection algorithm SNAP (Bruker Daltonics). For MS and MS/MS analyses, we used the PMF:FAMS method and SNAP full process of FALIFT method, respectively. Each spectrum was obtained by averaging 1500–5000 laser shots (300 shot on a step); the minimum laser power, which is necessary for the ionization of the samples, was used. Data were analyzed using BioTools (Bruker Daltonics; version 3.2). The *m/z* spectra were searched against the SwissProt 2015_01 database; peptide and protein identifications were carried out based on MS and MS/MS data. Protein identifications were accepted if they were established at score significantly greater than 40 and contained at least 3 identified peptides, using the Mascot search engine. Further data were analyzed using 2016 SwissProt program and UniProt (http://www.uniprot.org/uniprot/).

2.6. Transmission electron microscopy

Samples containing exosomes were applied for 1 min for adsorption on a copper grid covered by formvar film stabilized with carbon and used for negative contrast. The grid was

placed on a drop of 0.5% solution of uranyl acetate or 2% solution of phospho-tungstic acid (5−10 s), excess of liquid was removed with filter paper. The grid was examined using a transmission electron microscope Jem1400 (Jeol, Japan), and images were obtained using a Veleta digital camera.

Gold-immunolabeling of horse milk vesicles obtained using several centrifugations, 0.1 μm ultrafiltration and gel filtration was implemented with mouse monoclonal antibodies against CD81 and CD63. Vesicles preparations were incubated with anti-CD81 or anti-CD63 antibodies at room temperature overnight. Immunolabeled preparations were adsorbed on the copper grid for 2 min as described above. Then the grid was washed with PBS buffer (10 mM Na_2HPO_4, 1.76 mM KH_2PO_4 (pH 7.4) containing 137 mM NaCl and 2.7 mM KCl) and incubated with Protein A conjugated with 10−12 nm gold nanoparticles conjugated with rabbit IgG antibodies for 2 h at 24 °C. The grids were washed with PBS to remove unbound antibody and negatively stained with 2% phosphomevalonate acid for 15 s. The samples were analyzed in transmission electron microscope Jem1400 with a Veleta digital camera.

3. Results

As mentioned above, exosomes obtained from different biological fluids using only different type of centrifugation and ultracentrifugation or even additional purification in sucrose gradient according to [2,29−33] can contain from a few dozen to several thousands of different proteins. We have hypothesized that this very large number of proteins may be due to these purification methods do not allow to separate exosomes effectively from co-precipitated very stable high molecular mass (~1000 kDa) multiprotein complex and probably some major and minor proteins, which can specifically or non-specifically interact with the exosomal surface or receptors. Therefore, first we obtained a crude exosome preparations from milk of eighteen horses by standard approach using several centrifugation and ultracentrifugation similar to [12−33] with some modifications. In order to remove large complexes and vesicles with size larger than 100 nm these preparations were filtered through a 0.1 μm filter similarly to [25]. Mixture of five crude individual exosome preparations was first subjected for isoelectric focusing and then for SDS-PAGE (Fig. 1). After Coomassie staining we revealed 46 major and moderate protein spots. Gel fragments corresponding to protein spots were cut and subjected for in-gel trypsinolysis. The tryptic hydrolyzates were used for proteins identification using MS and MS/MS MALDI mass spectrometry. Interestingly, it was found only one spot corresponding to: human serum albumin, lactoferrin, and lactadherin, while nine spots corresponded to different species of beta-lactoglobulin. All other protein spots corresponded to different species of milk casein (number of spots): kappa-casein precursor (1), beta-casein (2), alpha-S1-casein (7), kappa-casein (10), and alpha-S1-casein precursor (14). The data are summarized in Supplementary Table 1. Thus, the mixture of five relatively crude partially purified preparations of horse milk exosomes contain only nine different major and moderate proteins, while five of them consist of different of caseins and their precursors.

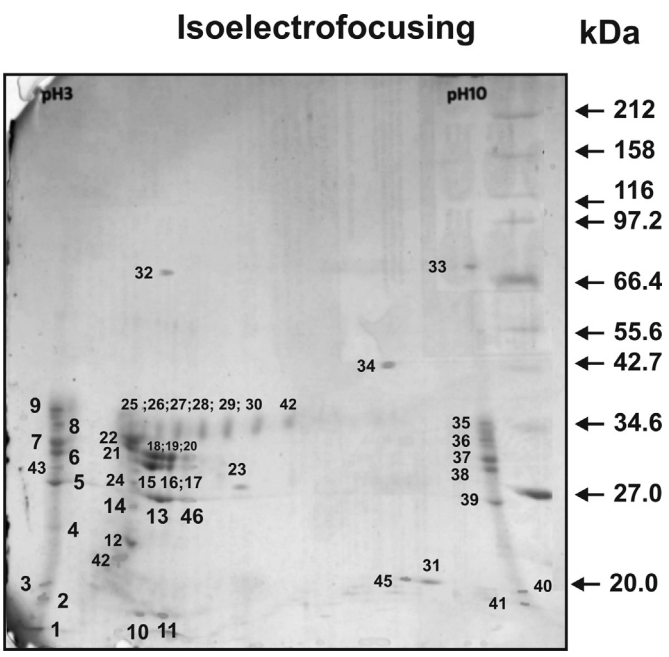

Fig. 1. Two-dimensional gel electrophoresis of the proteins of a mixture of five partially purified exosome preparations by different centrifugations and filtration through a filter 100 nm. The preparation was treated and separated first by isoelectrofocusing and then by SDS-PAGE in denaturing conditions. The spots were stained with Coomassie R-250, then cut, proteins we subjected to proteolysis for their identification using MALDI MS and MS/MS approaches.

It was previously shown that in buffer containing 0.5 M NaCl the main part of nonspecific proteins relatively weakly bound with specific stable complexes can be dissociated and stable complexes with high molecular masses may be separated from other different proteins by FPLC gel filtration [36,37]. Exosomes are quite stable structures, which after centrifugation and ultracentrifugation could contain specific and nonspecific proteins or their associates with lower MMs than that of exosomes. Therefore, for the additional purification of horse milk exosomes from co-precipitating proteins and their complexes we used FPLC gel filtration on Ultrogel sorbent effectively separating proteins with molecular masses (MMs) of 55−9000 kDa. Fig. 2 demonstrates four types of very different gel filtration profiles. First peak corresponds to exosomes (see below), from one to two next peaks correspond to the co-precipitating proteins. Supplementary Fig. 1 shows profiles of gel filtration for fourteen other individual exosome preparations. One can see that all crude exosome preparations purified by several centrifugations are characterized by significantly different profiles of the gel filtration. In several cases there are only two separated peaks. However, there are several preparations demonstrating three peaks, where first peak corresponding to exosomes is very poorly separated from the second protein peak. All preparations are characterized by a very specific ratio of A_{280} corresponding to peak of exosomes and one or two peaks of co-precipitated proteins (Fig. 2 and Supplementary Fig. 1).

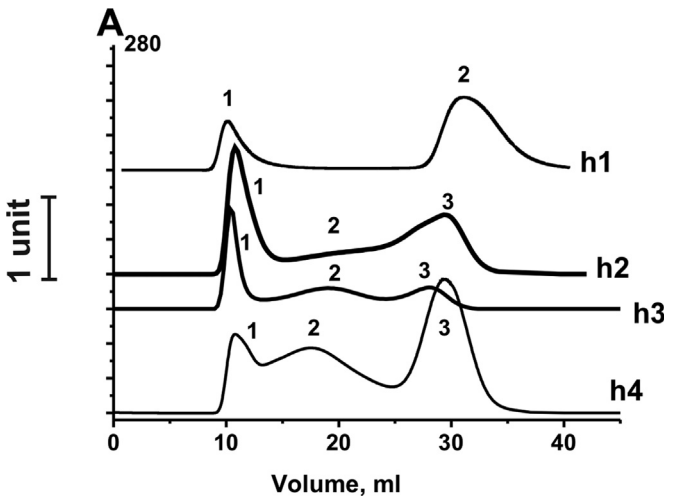

Fig. 2. Four typical types of isolation of horse milk exosomes by FPLC gel filtration on Ultrogel column from individual preparations of milk (numbers h1–h4) previously partially purified by several different centrifugations: (—), absorbance at 280 nm (A_{280}).

Several exosome preparations obtained using several centrifugations were analyzed by electron microscopy before and after their ultrafiltration through a 0.1 μm filter and after gel filtration. Before the ultrafiltration exosome preparations contain numerous membrane structures, the size of approximately 40–400 nm, which form large clusters of vesicles and also specific clusters of large protein associates. Fig. 3A and B demonstrates typical picture for preparation of horse 1 milk (h1) purified by several centrifugations. Among these structures there are vesicles (40–100 nm); some of them could be assigned to exosomes. After ultrafiltration of exosome preparations through a 0.1 μm filter they did not contain structures with the size higher 100 nm and relative content of amorphous material corresponding the impurity proteins significantly reduced, vesicles morphologically corresponding to exosomes, were observed (Fig. 3C and D).

After gel filtration the exosome preparations did not contain visible protein amorphous material (Fig. 3E and F). Exosomes contain some of transmembrane proteins, including CD81 or CD63, which are considered as specific markers of the exosomes [3]. We performed immuno-cytochemical study, and detected the CD81 (Fig. 3G–I) and CD63 (Fig. 3J–L) on exosomes isolated from horse milk using gel filtration. Thus, we purified vesicles corresponding to exosomal size containing CD81 and CD63 receptors. It should be mentioned that these preparations also contain microvesicles (<30–40 nm).

Using SDS-PAGE and MALDI mass spectrometry we analyzed the proteins of horse milk exosome preparations before and after FPLC gel filtration (For example, Fig. 4A). Crude exosome preparation h1 before gel filtration was incubated without (lane b-GF-DTT) and with DTT (lane b-GF + DTT), one can see many proteins with apparent molecular mass (MMs) from ~10 to >200 kDa (Fig. 4A) on the SDS PAGE; these proteins were identified using MS and MS/MS and the results summarized in Supplementary Table 2. After SDS-PAGE some protein band may contain two or even three proteins with comparable MMs, but all these proteins were reliably identified using MS or MS and MS/MS approaches. Interestingly, protein band 13 before protein treatment with DTT corresponded to polymeric form of beta-casein, alpha-S1-casein precursor, but this protein was not detected after incubation of protein samples with DTT (Fig. 4A, lanes b-GF-DTT and b-GF + DTT, respectively). Therefore, one cannot exclude that different species of caseins form large-size associates in which some proteins may be bound by S-S bridges. Totally, after SDS PAGE of crude exosome h1 preparation the stained gel contained 12–13 major protein bands corresponding to nine various milk proteins and their species or isoforms with different MMs (Supplementary Table 2).

After gel filtration of horse milk exosome preparations many proteins including major ones were not detected on SDS PAGE, comparing with not gel filtered preparations. Peak 1 after gel filtration corresponding to exosome preparation h1 (lane 1 of Fig. 2, effective separation of the peaks) incubated without (lane exo-DTT) and with DTT (lane exo + DTT) contains only four major proteins (1e-4e and 1e-d–4e-d protein bands, Fig. 4A) with MMs from ~15 to <60 kDa. This indicates that h1 exosome preparations contain only four major proteins: beta-lactoglobulin-1, lactadherin, actin cytoplasmic 1, and butyrophilin subfamily 1 (Table 1).

Supplementary Fig. 1 demonstrates gel filtration of exosome h5 preparation. Fig. 4B shows data on SDS PAGE protein analysis of exosome h5 preparation before and after FPLC gel filtration. Crude exosome preparation h5 before and after treatment with DTT also contains many proteins with apparent molecular mass (MMs) from ~10 to >200 kDa (Fig. 4B); results of protein identification by MS and MS/MS summarized in Supplementary Table 3. After SDS-PAGE some protein bands contain two proteins with comparable MMs, but all these proteins were precisely identified using MS and MS/MS approaches. Interestingly, protein bands 10 and 11 of lane b-GF-DTT as well as 11d and 12d of lane b-GF + DTT corresponded to polymeric forms of beta-casein, alpha-S1-casein and its precursors (Fig. 4B, Supplementary Table 3). Overall, after SDS-PAGE the crude exosome preparation h5 contains 11–12 major protein bands corresponding to seven various proteins and their species demonstrating different MMs (Supplementary Table 3). Crude preparations of h1 and h5 contain the same seven major proteins: beta-lactoglobulin-1, lactadherin, actin cytoplasmic 1, xanthine dehydrogenase, alpha-S1-casein precursor, beta-casein, kappa-casein, three of which are various species of different caseins including probably its partially hydrolyzed forms (Supplementary Tables 2 and 3). In addition, only crude preparation of h1 contains serum albumin, while preparation h5 – lactoferrin. In contrast to crude preparations purified by gel filtration h1 and h5 exosome samples do not contain any species of casein. Preparations h1 and h5 contain the same four proteins: beta-lactoglobulin-1, lactadherin, actin cytoplasmic 1, butyrophilin subfamily 1, while preparation h5 contains additionally lactoferrin and xanthine dehydrogenase (Table 1). Data on MS and MS/MS analysis of these six proteins are summarized in Supplementary Table 4.

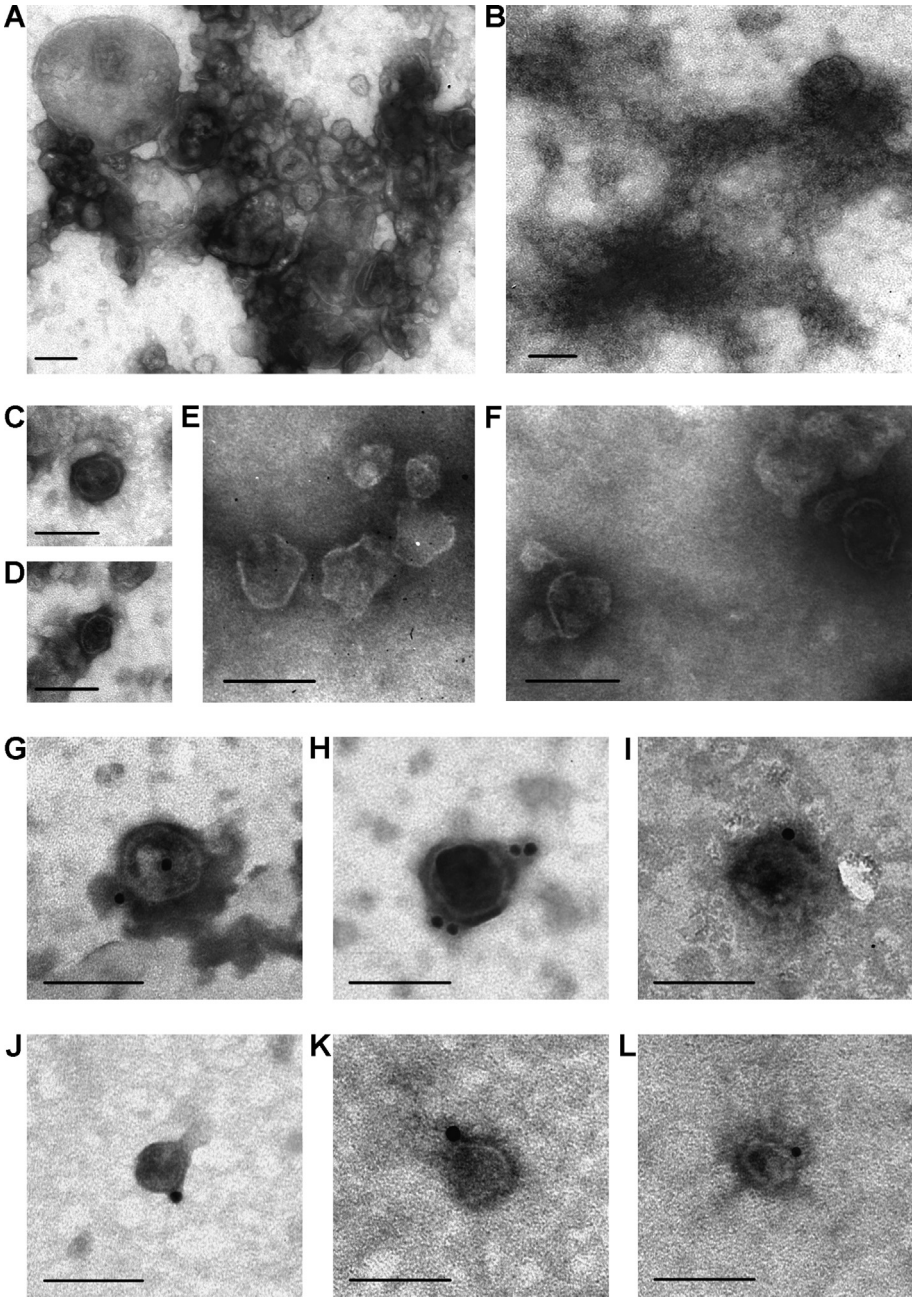

Fig. 3. Electron micrographs; analysis of the vesicle preparations after different steps of their purification. Typical data for vesicle preparation h1 purified by several centrifugations (A and B). Among visible structures there are bubbles the size of 40–100 and more 100 nm that could be assigned to different vesicles including exosomes. After filtration of exosome preparations through a 100 nm filter they did not contain structures with the size higher 100 nm and relative content of amorphous material corresponding the impurity proteins significantly reduced (C and D). After gel filtration the exosome preparations did not contain visible protein amorphous material (E and F). Exosomes isolated from horse milk using gel filtration were gold-immunolabeling by antibodies to the CD81 (G–I) and CD63 (J–L) receptors.

Then we processed proteins of exosome h8 preparation by 2D electrophoresis. In the case of this preparation we have observed after FPLC gel filtration very good separation of exosomes from co-isolating proteins (Supplementary Fig. 1). After the FPLC gel filtration we revealed on the 2D gel only four major protein spots (Fig. 5) corresponding to only three proteins: beta-lactoglobulin-1 (2 spots), lactadherin and fatty acid binding protein 1 (heart) (Table 1).

As one can see from Fig. 2 and Supplementary Fig. 1 gel filtration profiles differ significantly for various exosome preparations. A very bad separation of exosomes from impurity proteins was observed in the case of h4 exosome preparation (Fig. 2 and Supplementary Fig. 2A); in this case the second peak is practically not separated from the first exosomal peak. Therefore, it was interesting which proteins are included the second peak after FPLC gel filtration with relative

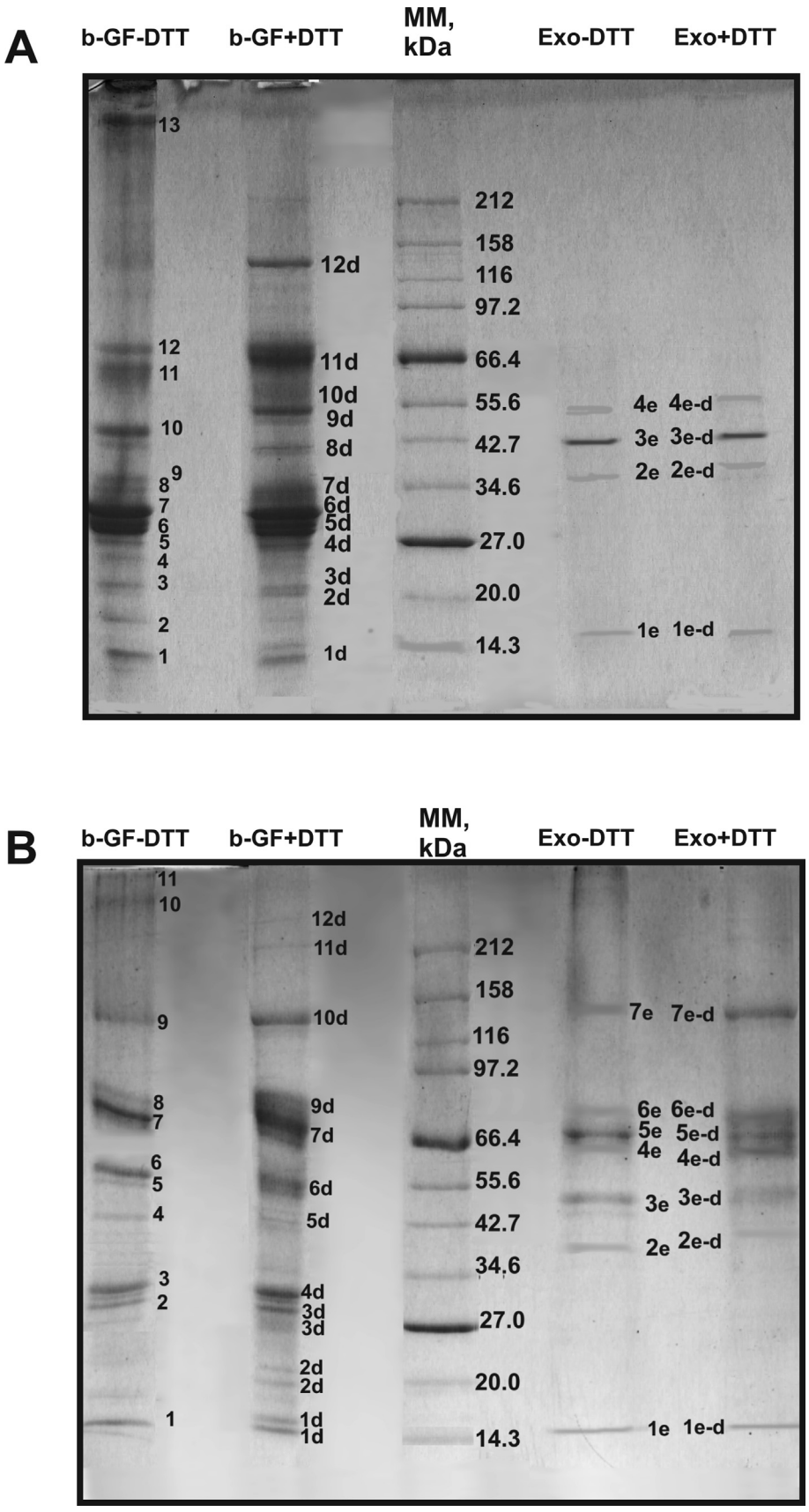

Fig. 4. SDS-PAGE separation of the proteins of exosome preparations h1 (A) and h5 (B) before (b-GF) and after (Exo) their gel filtration (10–20 μg) using 4–16% gradient gel. The preparations were used before (−DTT) and after their treatment with DTT (+DTT). Lanes MM correspond to control proteins with known MMs (A and B). Protein bands we cut and used for identification of proteins by MALDI mass analysis. See Section 2 for other details.

Table 1
Proteins of the first peak after gel filtration of exosome preparations h1, h5 and h8 (Fig. 2 and Supplementary Fig. 1) revealed by SDS-PAGE for h1 and h5 before (Fig. 4A and B, lanes Exo-DTT) and after their treatment with DTT (Fig. 4A and B, lanes exo + DTT) as well as after 2D-electrophoresis of h8 proteins.

Number of protein band − DTT	Number of protein band + DTT	Database MM,[a] Da	Identified protein[a]	Methods of identification[b]	
Horse 1 (SDS-PAGE)					
1e	1e-d	20,331[a]	Beta-lactoglobulin-1	MS[b]	MS/MS[b]
2e	2e-d	35,539	Lactadherin	MS	MS/MS
3e	3e-d	41,982	Actin, cytoplasmic 1	MS	MS/MS
4e	4e-d	58,634	Butyrophilin subfamily 1	MS	MS/MS
Horse 5 (SDS-PAGE)					
1e	1e-d	20,331	Beta-lactoglobulin-1	MS	MS/MS
2e	2e-d	35,539	Lactadherin	MS	MS/MS
3e	3e-d	41,982	Actin, cytoplasmic 1	MS	MS/MS
4e	4e-d	58,634	Butyrophilin subfamily 1	MS	MS/MS
5e	5e-d	78,132	Lactoferrin	MS	MS/MS
6e	6e-d	78,132	Lactoferrin	MS	MS/MS
7e	7e-d	146,468	Xanthine dehydrogenase	MS	MS/MS
Horse 8 (2D-electrophoresis)					
1	No	14,849 or 14,773	Fatty acid 1 binding protein (heart)	MS	MS/MS
2	No	20,331	Beta-lactoglobulin-1	MS	MS/MS
3	No	20,331	Beta-lactoglobulin-1	MS	MS/MS
4	No	35,539	Lactadherin	MS	MS/MS

[a] For identification of proteins and their molecular mass (MM) the 2016 SwissProt program was used.
[b] MS − identification on the basis of a set of different peptides of proteins tryptic hydrolyzates, MS/MS according to the sequences of the peptides (from three to seven).

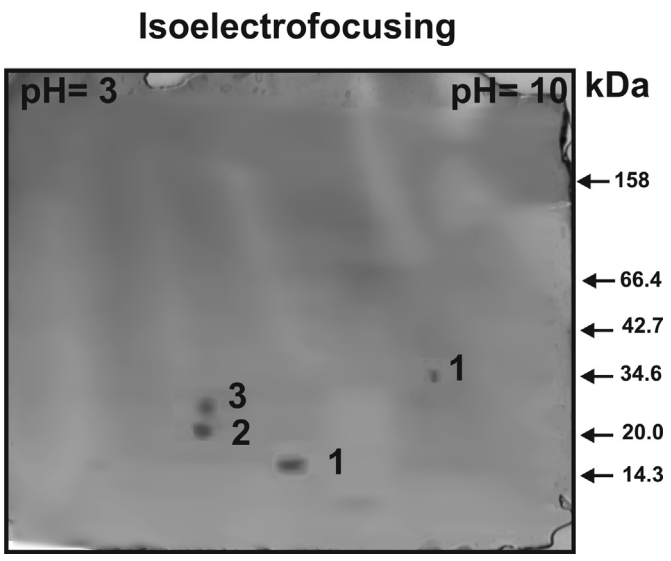

Fig. 5. Two-dimensional gel electrophoresis of the proteins of h8 exosome preparation after its isolation from impurity proteins by FPLC gel filtration. The proteins were first separated by isoelectrofocusing and then SDS-PAGE in denaturing conditions. The spots were stained with Coomassie R-250, then cut, proteins we subjected to proteolysis for their identification using MALDI mass spectrometry. Four protein spots correspond to: fatty acid 1 binding protein (heart) (1), beta-lactoglobulin-1 (2 and 3), lactadherin (4).

size comparable with that of exosomes. We analyzed proteins of the second peak of h4 preparation with 2D-electrophoresis (Supplementary Fig. 2B). Nine protein spots were revealed with Coomassie staining; two fragments of gel corresponded to beta-casein while other 7 fragments to different species of alfa-S1-casein and its precursors. Thus, especially large-size associates in horse milk are formed by different kinds of caseins and perhaps mainly by alpha-S1 casein and its precursor.

4. Discussion

We have obtained for the first time exosomes with characteristic morphology, size 40–100 nm, and shape from 18 horse milks. Exosome preparations obtained by previously described standard approaches of centrifugation and ultracentrifugation contained numerous structures with size of ~40–400 nm and also specific clusters of large protein associates (Fig. 3A and B). Ultrafiltration of crude exosome preparations through 0.1 μm filter led to the removal of >100 nm particles and the large protein associates (Fig. 3). The most efficient separation of exosomes from impurity proteins in the case of most of the preparations was achieved by using an additional purification step − FPLC gel filtration (Fig. 2, Supplementary Fig. 1, and Fig. 3E and F). Similar to other described typical exosomes [3], gel filtration purified horse milk exosomes bearing CD81 and CD63 receptors (Fig. 3G–L). Thus, purified vesicles satisfy the typical requirements for exosomal morphology, size and content on the surface CD81 and CD63 receptors.

As mentioned above, according to the literature data, exosomes from various body fluids and tissues incorporate from a few dozen to several thousand different proteins. Taking these into account, after additional purification of exosome preparation by passing through a 0.1 μm filter, one can be expected revealing as minimum several dozen different proteins. Using 2D-electrophoresis we have found 46 major and moderate protein spots, but they corresponded only to nine proteins: serum albumin, lactoferrin, lactadherin, beta-lactoglobulin, kappa-casein, kappa-casein precursor, beta-casein, alpha-S1-casein, and alpha-S1-casein precursor (Supplementary Table 1). In addition, various species of different caseins accounted for the bulk of these proteins. This apparently may be due

to the fact that the various caseins in the horse milk form large protein associates. Fig. 4 shows that exosome preparations h1 and h5 before DTT treatment after SDS PAGE contain protein with apparent MMs >200 kDa corresponding to polymeric forms of beta-casein, alpha-S1-casein, and its precursor, and these bands completely disappear after exosome incubation with DTT. Second peak of h4 milk proteins poorly separated from the first exosomal peak contained only various species of beta-casein and alpha-S1-casein (Supplementary Fig. 2). According to the literature data human [7–9] and cow [2] milk exosomes contain different caseins. Interestingly, after FPLC gel filtration we did not find any species of different caseins in purified exosomes. All exosome preparations contained beta-lactoglobulin and lactadherin (Table 1). At the same time, h1 and h5 preparations contained cytoplasmic actin 1 and butyrophilin; lactoferrin was specific for h5, while fatty acid 1 binding protein was specific for h8. Beta-lactoglobulin, lactadherin, actin 1, butyrophilin, lactoferrin, and xanthine dehydrogenase [2,12,13], fatty acid binding protein [13] were previously revealed in human and/or cow milk exosomes.

Cell-to-cell communication is important for proper coordination between cells of different types of various tissues. It is known that cells communicate using secreted and cell surface molecules, which are decrypted by the target cell after binding to receptors, or by direct intercellular contacts mediated by specialized molecules. According to recent data the exosomes may be specialized vesicles involved in communication between cells in multicellular organisms, carrying within and/or on their surfaces various mRNAs, microRNAs, proteins, and lipids and other components and transferring these compounds to different recipient cells [7–11]. Thus, exosomes may represent a new type of the long distance transport of biological molecules among different cells.

It was shown that the exosome phenotypes, content of the various components may be different for healthy and sick people, changed after their treatment with different drugs, and also can depend on age, condition, and lifestyle [6–11,17,32,38,39]. For example, it was revealed that proteins of plasma exosomes in patients with acute myeloid leukemia may be markers of therapeutic response [38]. It was shown that exosomes of mature dendritic cells treated *in vitro* with lipopolysaccharide are significantly more potent to induce activation of antigen specific T-cell comparing with exosomes from immature cells [32]. A higher content of exosomes and different content of various proteins was revealed in early milk compared with mature milk [39], as well as the phenotype of breast milk exosomes and their different proteins varies with maternal sensitization and lifestyle [17,39]. These data speak in favor that exosomes of healthy and diseased people as well as in other different human conditions can carry to various cells very different information. Therefore, the study of different intrinsic components of exosomes seems a very important for understanding of their possible application for exosome-based therapeutics. We have analyzed here protein content of exosomes of healthy horses, which may potentially be able in future to use in clinic application.

The biological role of the major proteins of milk exosomes observed by us directly in the exosomal preparations is not yet known, but all these proteins have different biological functions in mammalian organisms. Beta-lactoglobulin was reported as being implicated among different hydrophobic ligand transport and uptake, regulation enzymes, as well as the neonatal acquisition of passive immunity [40]. These functions however did not appear to be consistent between species. Beta-lactoglobulin is over-expressed in the lactating mammary glands of many species; it was proposed to be important source of amino acids for the offspring. Lactadherin (MFGE8) may function as a cell adhesion protein connecting smooth muscle to elastic fiber in arteries [41]. An amyloid fragment of MFGE8 is known as peptide, which accumulates in the aorta with aging [42]. In the vasculature of adults MFGE8 can induce recovery from ischemenia by facilitating angiogenesis [43]. MFGE8 forms complex with phosphatidylserine, which is exposed on the surface of apoptotic cells. Opsonization of the apoptotic cells together with binding to integrins on the phagocytic cells surfaces can mediate the engulfment of the dead cell. Actin usually participates in many important cellular processes including cell signaling, cell motility, muscle contraction, vesicle and organelle movement, cell division, and cytokinesis, as well as the establishment and maintenance of cell shapes and junctions. Many of these processes are mediated by actin specific interactions with cellular membranes [44].

Butyrophilin regulates secretion of milk-lipid droplets [45]. A number of LF functions have been established: immunomodulation and cell growth regulation, antibody-dependent cytotoxicity, cytokine production, protection from iron-induced lipid peroxidation, activation of natural killer cells, influences on granulopoiesis, and growth of some cells *in vitro*, as well as activation of transcription of specific DNA sequences (for review see Ref. [46]). In addition, LF possesses five different activities: phosphatase, DNase, RNase, ATPase, and protease. Xanthine dehydrogenase belongs to the group of molybdenum-containing hydroxylases; this enzyme involved in the oxidative metabolism of purines [47]. Defects in xanthine dehydrogenase cause xanthinuria, this enzyme may contribute to adult respiratory stress syndrome as well as it may potentiate influenza infection through an oxygen metabolite-dependent mechanism. This enzyme may produce very reactive oxygen forms which can be pathogenic. Although it is one of the longest known and most continuously studied enzymes, its exact biological function however remains unclear. The fatty-acid-binding proteins are known as the family of proteins of a transport of fatty acids and other lipophilic compounds including retinoids and eicosanoids [48]. These proteins are believed to carry out fatty acids transfer between extra- and intracellular membranes [49]. Some family members are also believed are responsible for transport of lipophilic compounds from outer cell membrane to certain intracellular receptors [50]. It has been shown that level of fatty-acid-binding proteins in the mouse brain decreases with aging, possibly contributing to age-associated decline in synaptic activity [51]. One cannot exclude that all

these functions of exosome proteins are important for the development of protective functions of the mother's organisms, as well as for stimulation the development of such functions in the newborns.

Taken together, we analyzed for the first time major proteins of horse milk exosomes and we found that well purified exosomes contain less than a dozen of major proteins. One cannot exclude that there may be several reasons for the significant difference in the number of proteins described by us in horse milk exosomes with previously published data on proteins of exosomes from human and cow milk, as well as from other biological sources. On the one hand it is possible that the number of horse milk exosomal proteins, in principle, can be smaller than of the human or bovine milk. In addition, some of our data suggest that proteomic analysis of milk exosomes made previously by other authors in some cases was performed on crude exosomal preparations, containing co-precipitating and co-isolating proteins and their complexes. It should be noted that we have analyzed only the major proteins. At the same time, the use of some approaches (like shotgun peptide LC-MS/MS analysis) by other authors can lead to the description a vast number of very minor proteins.

We have shown that well-purified exosomes contained from only five to eight different major proteins. The discovery of several dozens to several hundreds and thousands of major proteins in exosomal preparations seems to be to some extent overestimated. In addition, the detection of major and very minor proteins by other authors leads to the question whether minor proteins co-purifying or actually composing milk exosomes have any important biological role in the exosome functioning.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biopen.2017.02.004.

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