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Chapter

Milk Exosomes: Isolation, Biochemistry, Morphology, and Perspectives of Use

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Abstract

Cells of the multicellular organisms communicate with each other in many different ways, among which extracellular vesicles play a unique role. Almost all cell types secrete vesicles into the extracellular space and deliver their contents to recipient cells. Today, one of the groups of extracellular vesicles that is of particular interest for studying is exosomes—membrane vesicles with a diameter of 40–100 nm. Exosomes are secreted by cells and found in various biological fluids—blood, tears, saliva, urine, cerebrospinal fluid, and milk. Exosomes provide not only targeted delivery of molecular signals to recipient cells but also carry unique markers, which makes them a promising substrate in diagnostic studies, primarily due to their small RNA and protein contents. The milk of cows, horses, humans, and other mammals is a unique source of exosomes since these organisms can produce liters of milk per day, which is much higher than the volume of exosomes produced in cell culture fluid or blood plasma. Unfortunately, milk exosomes are currently much less studied than exosomes of blood or culture fluid. This review examines the methods of the isolation, biochemical analysis (composition of proteins, lipids, and nucleic acids), morphology, and prospects for the use of milk exosomes.

Keywords: milk, exosomes, isolation, milk exosomes, proteins, lipids, nucleic acids, miRNA, morphology, electron microscopy

1. Introduction

Exosomes are membrane vesicles with a diameter of 40–100 nm, secreted by cells and found in various biological fluids—blood, tears, saliva, urine, cerebrospinal fluid, and milk [1]. Exosomes deliver molecular signals to recipient cells and also carry unique markers of the parental cell, which makes them a promising substrate for noninvasive diagnostics (liquid biopsy) and targeted drug delivery. The microRNA and protein contents of exosomes are of particular interest [2, 3].

Milk contains proteins, lipids, nucleic acids, and its complexes and is not a simple source of nutrients. Since vesicles of different size and shapes were described in milk and since milking animals can produce liters of milk per day, which is much

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higher than the volume of culture fluid or blood plasma, milk is a unique source of exosomes. Unfortunately, milk exosomes are currently much less studied than exosomes of blood or culture fluid.

Milk exosomes may be used as carriers of drugs and therapeutic nucleic acids for delivery to cells. The basis of biological functions of milk exosomes is due to their components: lipids, proteins, and nucleic acids. The prospects for the further practical use of milk exosomes in medicine and biotechnology largely depend on investigations of the fine structure and biological functions of exosomes free of various contaminating impurities.

A clear classification of extracellular vesicles is difficult due to their considerable variability and, particularly, overlapping sizes. The term "extracellular vesicles" was introduced to designate all the vesicles secreted to the biological fluids, and the use of the term "exosomes" requires the fulfillment of some conditions [4, 5]. The distinctive features of exosomes are the size (40–100 nm), floating density in a sucrose gradient (1.1–1.19 g/ml), a particular cup-shaped form under electron microscope examination, and specific biochemical composition.

Exosomes isolated from human [6], cow [7], horse [8], pig [9], rat [10], and camel [11] milk have been described so far. However, it has been shown that the molecular composition of human milk exosomes largely depends on the mother's lifestyle, lactation stage, and mother's contact with allergens [12]. It is known that a change in the composition of proteins [13] and nucleic acids [14] of cow milk exosomes occurs during the development of inflammation of the mammary gland.

2. Methods of exosome isolation, purification, and analysis

According to the international nomenclature, exosomes include vesicles of 40–100 nm in size, which are formed by invagination of the membrane of the multivesicular bodies and carry a number of specific markers, such as CD9, CD63, and CD81. Exosomes are round or cup-shaped.

Most investigations of exosomes confirm their presence using transmission electron microscopy (TEM) without a detailed analysis of the preparation composition. TEM is the best method of analysis of size, morphology, and integrity of exosomes, as well as of evaluating sample composition. TEM shows the presence of impurities in the preparations of exosomes, which can distort the results and lead to a false interpretation. For example, researchers describe samples of cow milk exosomes and present images that clearly show vesicles larger than 100 nm in size and other particles that do not have an outer membrane. However, when analyzing the results, this fact is not discussed [15].

To confirm that the isolated vesicles are exosomes, the International Society for Extracellular Vesicles recommends identification of specific exosomal membrane proteins—tetraspanins CD9, CD63, and CD81—using Western blotting, flow cytometry, or immunoelectron microscopy [4, 16]. However, the first two methods fix all particles in a solution that have tetraspanins; therefore, there is no selectivity in analyzing membrane and non-membrane structures. The advantage of immunoelectron microscopy is the ability to detect exosomal markers directly on the surface of the vesicles.

When analyzing the literature data on exosomes obtained from the milk of human donor or other sources, one should take into account by which methods the exosome preparation was isolated and analyzed and what is the potential of these approaches in a generation of reliable data. Exosomes from different biological liquids including milk may be isolated with various methods such as centrifugation using special conditions, ultracentrifugation, ultracentrifugation in density

gradients, and salting out and with other approaches [17–19]. It should be noted that these methods allow obtaining preparations only enriched with exosomes, but not pure ones. Different biological fluids including milk contain various proteins and their highly stable molecular weight associates, which can be co-isolated with various vesicles including exosomes during centrifugations. For example, it was recently shown that extract of the placenta and human milk contains very stable high-molecular-mass (~1000 kDa) multiprotein complexes, in which the size is comparable to some extent with various vesicles [20, 21]. Moreover, some free proteins and their complexes can nonspecifically or even specifically interact either with vesicle surface or their receptors and coprecipitate with the vesicles during centrifugations.

Reliability of literature data is questioned in [22] where more than 200 exosome preparations were isolated from various sources using sequential centrifugations; this method has been used in many papers. Exosomes' preparations contained many structures with low electron density and having no membranes; these structures were named "non-vesicles." Using TEM, two main types of "non-vesicles" were described: of 20–40 nm (up 10–40% of all structures of the preparations) and of 40–100 nm. The morphology of the "non-vesicles" allowed referring them to lipoproteins of intermediate and low density (20–40 nm) and very low density (40–100 nm) [22]. Also, crude exosome preparations after different centrifugations without additional purification steps contained impurities of various components, including proteins and their complexes. In such types of exosome preparations, up to several hundred or thousand different contaminating co-isolating proteins and their complexes may be detected.

Recently our data confirmed the findings of [22]: using TEM we have shown that crude exosome preparations from human placenta and horse milk after centrifugation and ultracentrifugation (at 10,000 × g, 16,500 × g, and twice at 100,000 × g) contained many large >100 nm membrane vesicles, smaller <100 nm vesicles, and 20–100 nm non-vesicles [23]. Non-vesicles possessed medium electron density, distinct outer membrane, and spherical shape (**Figure 1**).

We observed a similar situation in the case of crude exosome preparations obtained from horse milk. All the same, components were identified in such preparations except ring-shaped structures of ferritin (**Figure 2**).

After preparations isolated from placenta [23] and horse milk [8] were enriched with exosomes by sequential centrifugation and ultracentrifugation, they were separated from co-isolating impurities using gel filtration on Sepharose 4B or Ultrogel (**Figure 3A** and **B**). The first peak corresponding to exosomes was successfully separated from contaminating proteins and other impurities of the second peak.

One can see from **Figure 3** that gel filtration can noticeably differ in the A_{280} ratio of the first and second peaks. The first peak, corresponding to exosomes, can be significantly lower than the second peak, corresponding to coprecipitating impurities. As it was shown in [8, 23], the second peak may contain various proteins. Vesicle preparations of human placenta and horse milk obtained after gel filtration contained exosomes of various sizes (30–100 nm) but did not contain any visible amorphous protein material (**Figures 1** and **2**). In contrast to horse milk exosomes, preparations of human placenta exosomes even after gel filtration contain some supramolecular ring-shaped associates of ferritin (10–14 nm). One of the criteria for belonging vesicles to exosomes is the content of CD81 or CD63 on the surface [4]. Using TEM with anti-CD63 and anti-CD81 gold-labeled antibodies, we analyzed the vesicle preparation obtained with gel filtration (**Figure 4C–G**). Extra-purified exosomes obtained after gel filtration correspond to the exosomes in terms of morphology, size, and content of tetraspanins CD81 and CD63 on their surface (**Figure 4**).

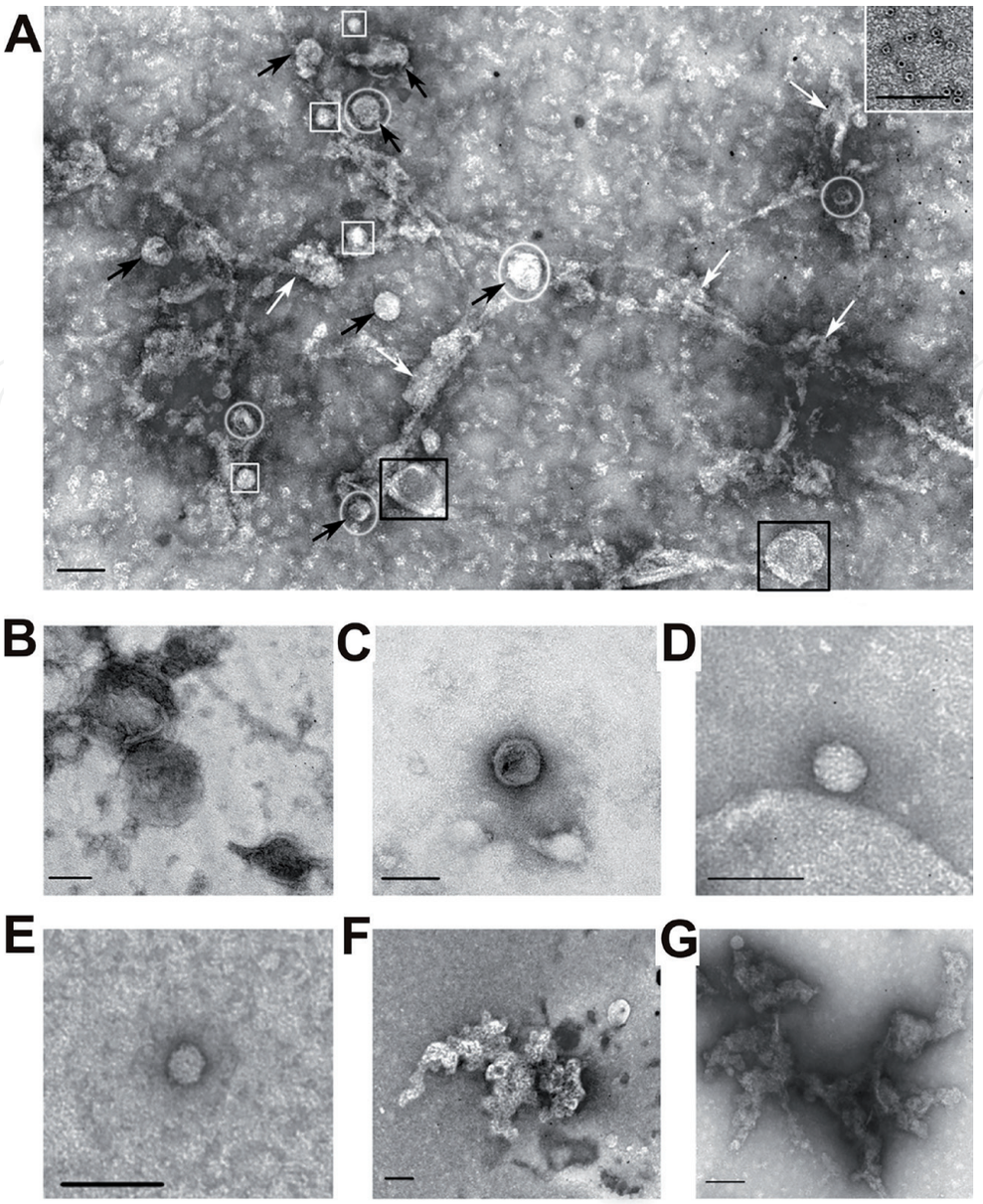


Figure 1.
Exosomes of human placenta after ultracentrifugation. (A) Vesicles <100 nm (black arrows), vesicles >100 nm (black squares), non-vesicles (white squares), amorphous aggregates of low electron density (white arrows), and particles associated with protein aggregates (white ovals). Individual structures in exosome preparations: Clumps of large vesicles (>100 nm) (B), vesicles <100 nm (C), non-vesicles (D, E), ring-shaped structures of ferritin (insert to A), clusters of aggregated proteins (F), and shapeless aggregations with low electron density (G). Images were obtained using TEM with negative staining. Scale bar corresponds to 100 nm.

Thus, various kinds of centrifugation and ultracentrifugation are not enough for a full purification of exosome preparations from coprecipitating impurities. Also, for improved exosome purification, it is necessarily what kind of ultracentrifugation was performed and how many times it was performed. The relative number of vesicles containing CD9 and CD81 tetraspanins was estimated using flow cytometry after the first and second ultracentrifugation and after gel filtration (**Figure 5**). It was shown that vesicle preparations after first 100,000 × g ultracentrifugation contain only ~16% of CD9- and CD81-positive vesicles (**Figure 5A** and **B**). The second 100,000 × g ultracentrifugation led to the increase of these markers in ~fivefold (up to 80–87% for CD81 and CD9, respectively; see **Figure 5C** and **D**). Thus, the number of impurities in the preparations of exosomes not subjected to the second 100,000 × g ultracentrifugation can be approximately 4–6 times higher.

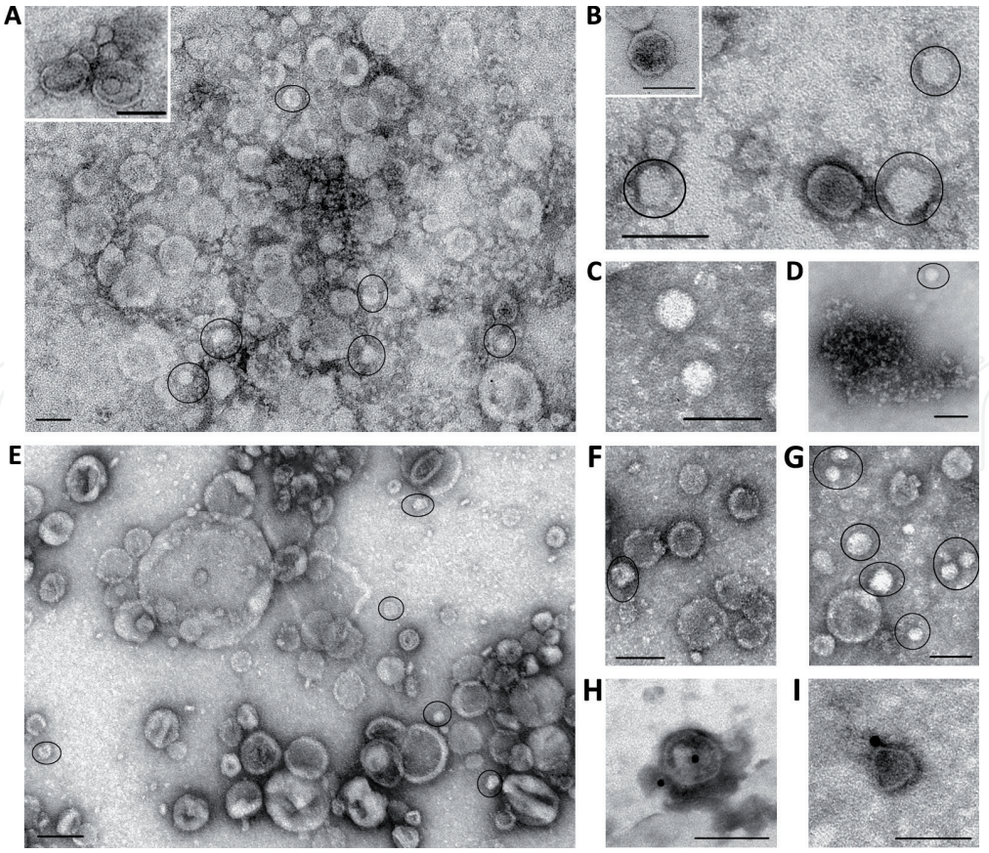


Figure 2.
Exosome preparations of horse milk after different steps of purification: Ultracentrifugation (A–D) and gel filtration (E–I). (A) Aggregates of vesicles and non-vesicles, (B) exosomes and non-vesicles (oval), (C) non-vesicles, (D) macromolecular aggregates, (E) aggregates of large and small vesicles, (F) exosomes, (G) exosomes and non-vesicles, (H) exosomes labeled with anti-CD81 antibody conjugates with gold spheres, and (I) exosomes labeled with anti-CD63 antibody conjugates with gold spheres. The oval shapes denote non-vesicles; squares in (A and B)—Exosomes. Scale bar corresponds to 100 nm.

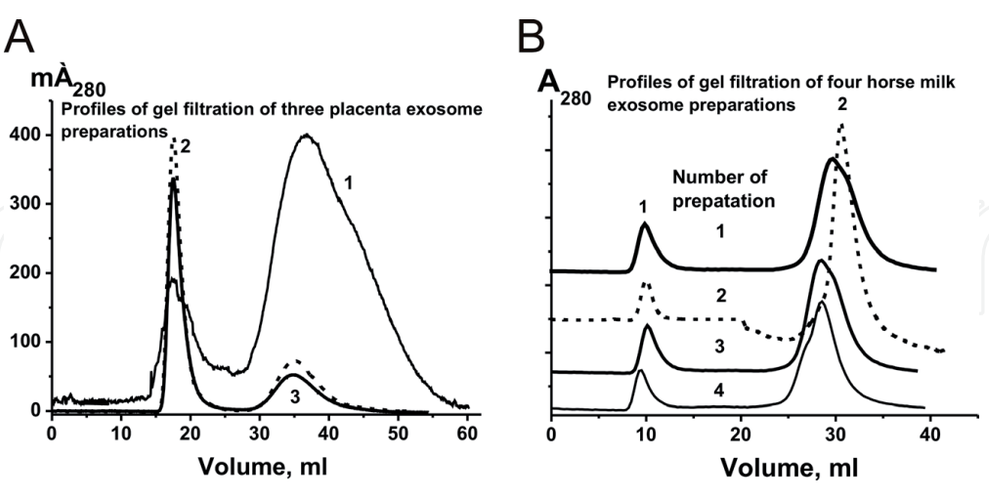


Figure 3.
Gel filtration of crude exosome preparations on Sepharose 4B (A) and Ultrogel (B). Crude exosome preparations from human placenta (A) and horse milk (B) preparations were obtained by sequential centrifugation and ultrafiltration through filter 0.1 μm (—), absorbance at 280 nm (A_{280}).

Exosomes of different origins according to literature data may contain varying amounts of proteins. For example, crude preparations of exosomes of dendritic cells can include more than 150–200 different proteins [24, 25]. An even more improbable result was obtained when analyzing proteins of the milk's exosomes of cows, whose preparations were isolated by centrifugation and ultracentrifugation in a

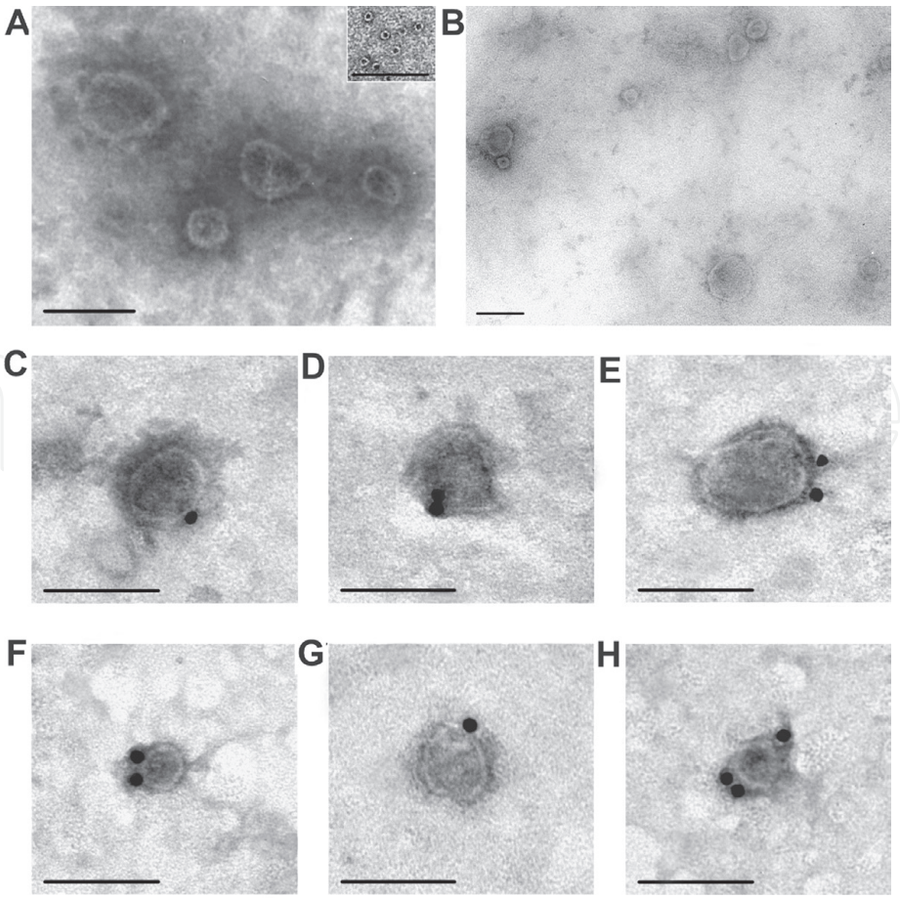


Figure 4.
Preparations of human placenta exosomes after filtration through 100 nm filter and gel filtration on Sepharose 4B. Vesicles (A) and ferritin ring structures (10–14 nm; inset in A). Exosomes purified by gel-filtration (B), labeled with conjugates of gold nanoparticles with monoclonal antibodies against tetraspanin CD81 (C–E) and CD63 (F–H). Transmission electron microscopy with negative contrast. Scale bar corresponds to 100 nm.

sucrose gradient [26]. As a result, 2107 proteins have been identified, which include all major protein markers of exosomes previously detected.

We have analyzed a possible number of proteins in extra-purified exosomes after gel filtration. Exosome proteins were identified before and after gel filtration by MALDI MS and MS/MS spectrometry of protein tryptic hydrolysates after SDS-PAGE and 2D electrophoresis (**Figure 6**).

Only 46 major and moderate protein spots were revealed on the gel after staining. Interestingly, only one of the spots corresponded to human serum albumin, lactoferrin, and lactadherin, while nine spots corresponded to different forms of beta-lactoglobulin. All other protein spots corresponded to various 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). Thus, the mixture of five relatively crude partially purified preparations of horse milk exosomes contains only nine different major and moderate proteins, while five of them consist of different caseins and their precursors.

It could be assumed that during gel filtration, the loss of a large part of the exosomes may occur, and as a consequence, the number of proteins analyzed in these exosomes may be underestimated. However, human placenta vesicles after gel filtration (fraction of the first peak) contain ~78% of CD9- and 74% of CD81-positive vesicles (**Figure 5E** and **F**). Thus, the yield of particles after gel filtration was relatively high up to ~90%. A similar result was obtained for horse milk exosomes. Consequently, the loss of the main part of the exosomes during gel filtration does not occur.

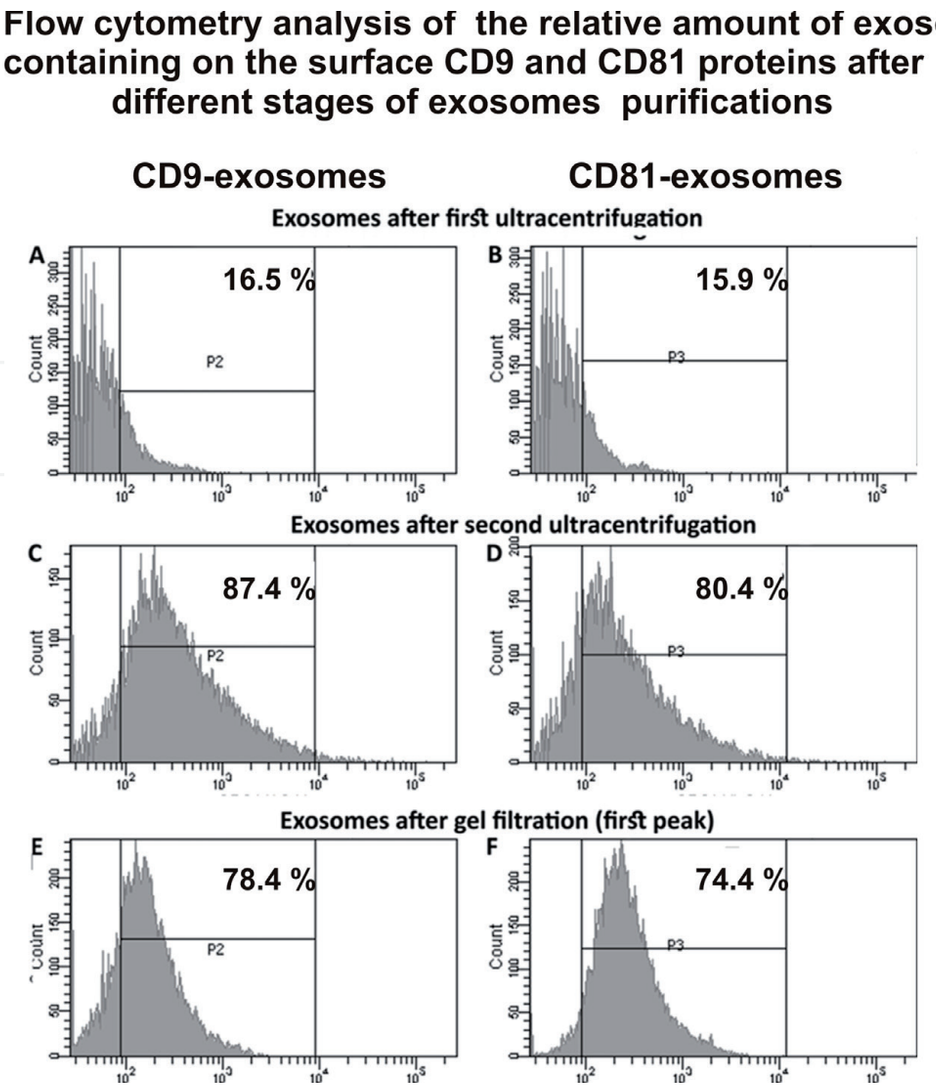


Figure 5.
Flow cytometry analysis of exosome preparations after two stages of ultracentrifugation (A–D) and gel-filtration (E, F). The relative amount of vesicles containing CD9 (left) and CD81 (right) are shown.

After gel filtration, extra-purified exosomes isolated from horse milk contained only eight different major proteins, CD81, CD63, beta-lactoglobulin, and lactadherin, which were common to all preparations, and actin, butyrophilin, lactoferrin, and xanthine dehydrogenase which were found only in some of them [8]. Exosome preparations from human placenta contain only ten major proteins: CD81, CD63, hemoglobin subunits, interleukin-1 receptor, annexin A1, annexin A2, annexin A5, cytoplasmic actin, alkaline phosphatase, and serotransferrin [23].

After 2D electrophoresis, only 28 protein spots were found in human placenta exosomes, which corresponded to just nine different proteins and their isoforms. Overall, using 2 methods of electrophoretic analysis (1D and 2D electrophoresis), 12 proteins were identified in 4 exosome preparations: CD81, CD63, hemoglobin subunits, annexin A1, annexin A2, annexin A5, cytoplasmic actin, alpha-actin-4, alkaline phosphatase, serotransferrin, human serum albumin, and immunoglobulins. Ferritin completely disappears after gel filtration and exosome treatment with proteolytic enzymes. After treatment of exosome preparations with trypsin and chymotrypsin, the protein bands corresponding to human serum albumin and immunoglobulins according to SDS-PAGE data almost wholly disappeared. Therefore, it cannot be excluded that human serum albumin and immunoglobulins form relatively stable complexes with exosome membrane proteins (i.e., tetraspanins) or interact directly with the surface of exosomes.

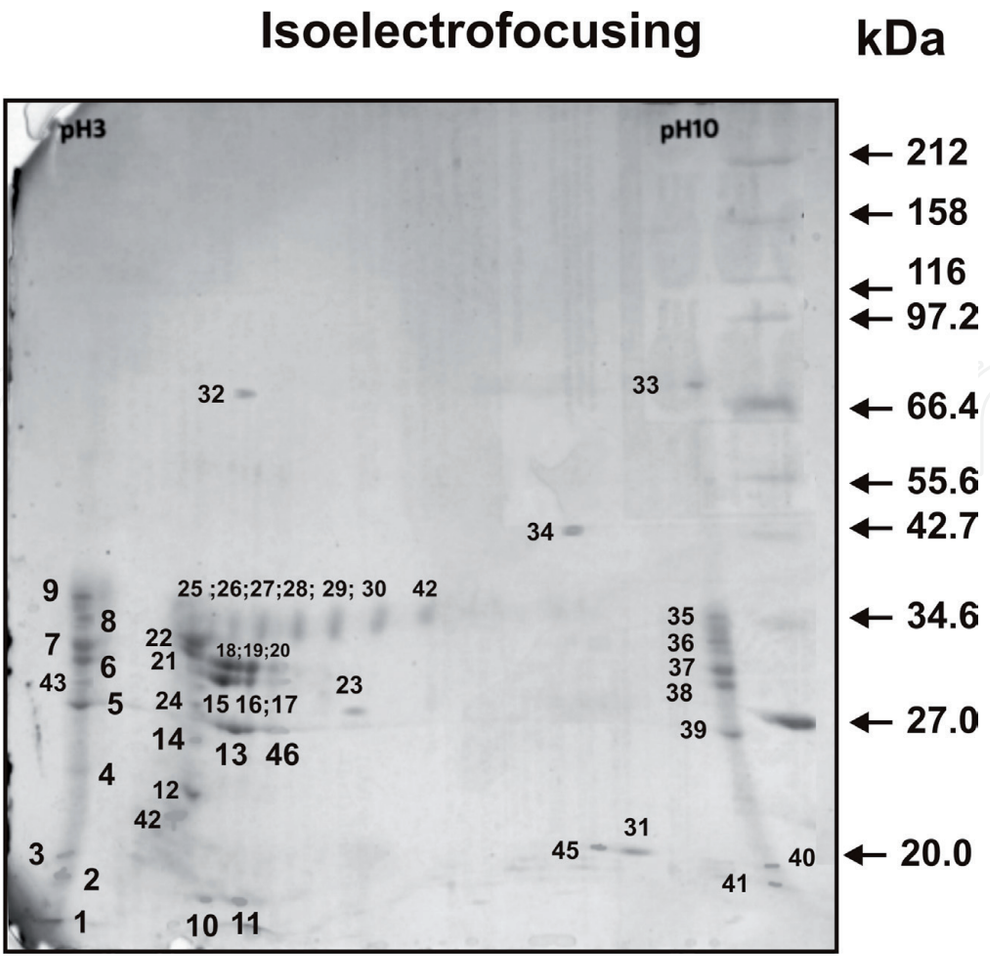


Figure 6.
2D gel electrophoresis of horse milk exosome proteins. A mixture of five crude horse milk exosome preparations was obtained by centrifugation and ultrafiltration through 100 nm. The sample was separated by isoelectrofocusing and then by SDS-PAGE in denaturing conditions. The spots were stained with Coomassie R-250 and then cut; proteins were subjected to trypsinolysis for their identification using MALDI MS and MS/MS spectrometry.

Authors of [27, 28] discovered proteins of oxidative phosphorylation in the urinary exosomes. It is known that the formation of exosomes occurs in the cell cytoplasm. However, oxidative phosphorylation is a typical function of the mitochondria. Mitochondrial enzymes of the oxidative phosphorylation and Krebs cycle are absent in the cytoplasm. The question may arise how these enzymes could get into the cytoplasm and then to exosomes. Mechanism of such a process has not been described anywhere. These results may be explained if the authors destroyed cells and the mitochondria and then co-isolated proteins of oxidative phosphorylation (or fragments of the mitochondria) with exosomes.

Several articles describe casein in milk exosome preparations. Precursors of caseins undergo posttranslational processing in the Golgi complex. The cytoplasm of mammary gland cells never contains any casein that may get inside the multivesicular bodies and exosomes. As one can see from **Figure 6**, crude horse milk exosome preparations include 46 major and moderate protein spots (number of spots) including some spots of casein isoforms: kappa-casein precursor (1), beta-casein (2), alpha-S1-casein (7), kappa-casein (10), and alpha-S1-casein precursor. Casein isomers were found only in crude exosome preparations, obtained by centrifugation. All forms of caseins have disappeared after gel filtration, and they were found just in the second peak containing contaminating proteins. Thus, it is possible that hundreds to thousands of proteins described in crude milk exosome preparations, in fact, are not intrinsic components of exosomes and are co-isolating impurities.

In this regard, we could note a critical review by academician Sverdlov, who believes that in the case of exosomes, there is an incorrect overestimated quantitative assessment of their internal molecular components, which, in his figurative expression, "would certainly make Amedeo Avogadro cry" [29]. We are sure that the real number of exosome proteins described previously using crude preparations of milk exosomes may be very much overestimated.

It is necessary to note another potential method leading to overestimation of the protein content in exosomes. The technique identifies proteins of exosome crude preparations by bulk trypsinolysis of proteins with subsequent separation of peptides using various HPLC. For example, cow milk exosome preparations were isolated by centrifugation and ultracentrifugation [29]. Purified exosomes after trypsin treatment were subjected to reverse-phase chromatography and then fractionated on a nanoLC column connected to the tandem mass spectrometer. This approach generated near 2100 proteins detected in milk exosomes. The question is what possible errors in the estimation of protein number might be using this approach?

After SDS-PAGE all proteins and peptides with molecular mass 10 kDa and less usually leave the gel during the gel staining with Coomassie blue. Exosomal peptides and small proteins are still practically not investigated. It was shown that peptides and small proteins are easily detected using cyano-hydroxycinnamic acid as a matrix for MALDI-TOF MS and MS/MS; these substances may be directly detected even in native cells [30–32]. Other low-molecular-mass components (lipids, sugars, oligonucleotides) will appear in the MALDI spectra in these conditions only if its content is 100–1000-fold higher than of peptides. Vesicles, eluted from anti-CD81-Sepharose with 0.15 M NaCl (**Figure 7**), contained a mixture of peptides and its complexes.

Small proteins and peptides of the fraction, eluted from anti-CD81-Sepharose with 0.15 M NaCl, were analyzed by MALDI mass spectrometry in 2–12 kDa range.

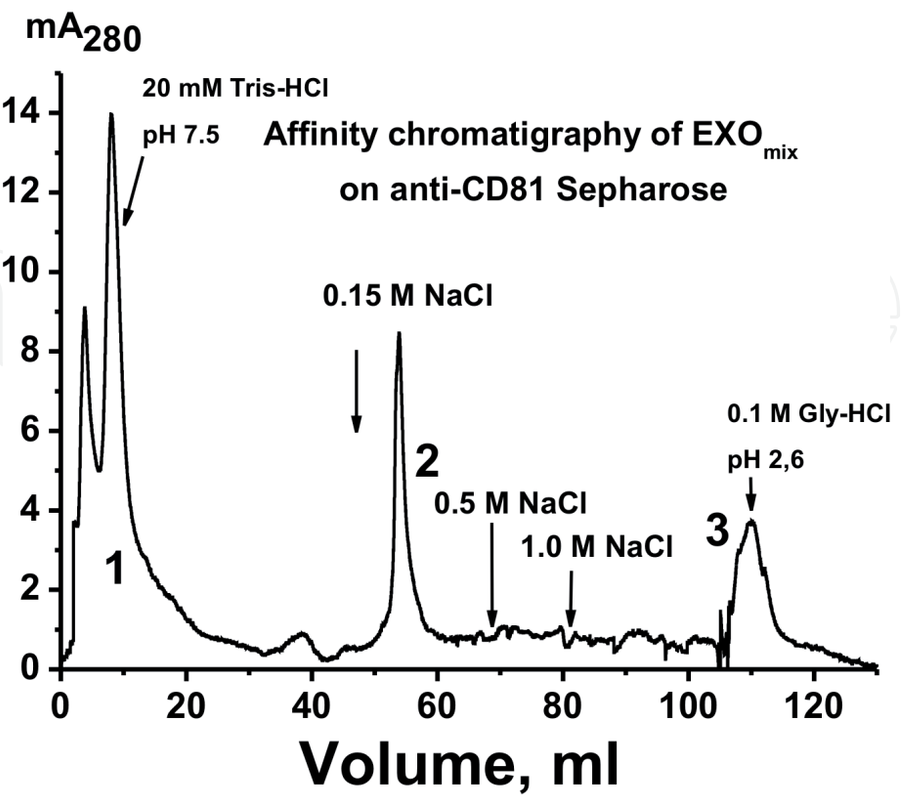


Figure 7.
Affinity chromatography of a mixture of five exosome preparations on anti-CD81-Sepharose: (—), absorbance at 280 nm (A_{280}). Peak numbers and elution conditions are indicated in the picture.

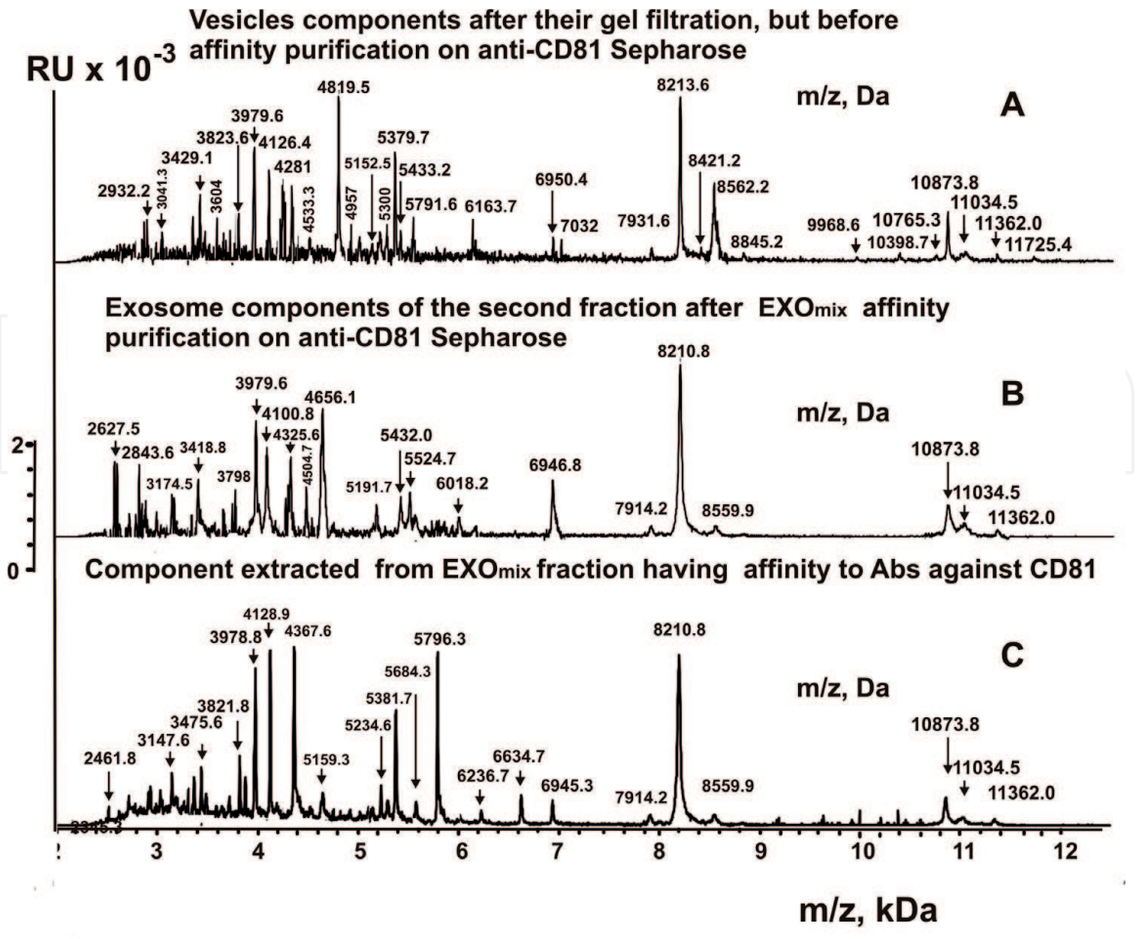


Figure 8.
MALDI mass spectra of human placenta exosomes obtained with gel filtration (A) and anti-CD81-Sepharose before (B) and after (C) trifluoroacetic acid and acetonitrile treatment. The analysis was performed using 2–12 kDa range of MALDI-TOF mass spectrometer.

The samples were examined before and after destruction of exosomes with trifluoroacetic acid (**Figure 8**).

Similar results were obtained for horse milk exosomes before and after gel filtration and after affinity chromatography on anti-CD81-Sepharose. MALDI mass spectra demonstrate many various small proteins and peptides with molecular masses in the range 2–9 kDa. The fractions were treated with proteases. **Figure 9A** shows MALDI MS spectra of peptides before the protease treatment. Spectra after the incubation with trypsin (**Figure 9B**), chymotrypsin (**Figure 9C**), and proteinase K (**Figure 9D**) doesn't contain any peaks >3 kDa but include peaks corresponding to the shorter products after hydrolysis. Masses of these shorter products do not coincide with the ones in **Figure 9A**.

Exosomes of the horse milk and placenta in addition to proteins >10 kDa contain small proteins and peptides. These peptides may be analyzed with highly sensitive methods of shotgun ESI-MS/MS analysis and lead to the incorrect number of large proteins in the exosome preparations. The structure of these small proteins and peptides is not yet established; one cannot exclude that initially they may be fragments of larger proteins. In that case, the identification of large proteins from the results of the shotgun analysis of peptides will lead to an incorrect determination of many proteins.

Some literature data on the analysis of the various components of milk exosomes, including lipids, mRNA, microRNA, and proteins, are described below. In most of the published papers, milk vesicles were isolated using only different centrifugation, and analysis was done only on crude preparations of exosomes.

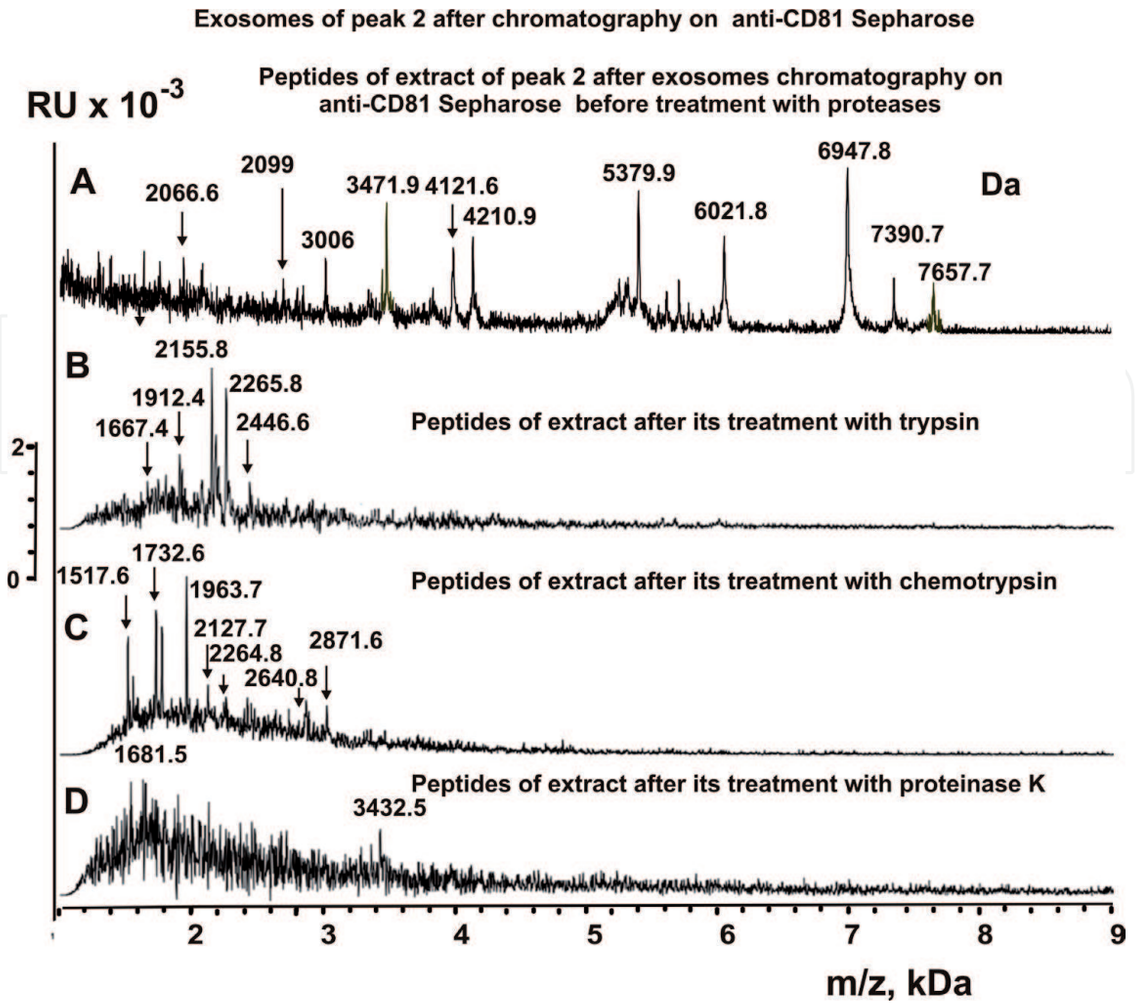


Figure 9.
MALDI mass spectra of <10 kDa extract of horse milk exosomes isolated on anti-CD81-Sepharose before (A) and after their treatment with trypsin (B), chymotrypsin (C), and proteinase K (D).

According to our data, the second peaks after gel filtration of exosomes (**Figure 3**) contain many different milk proteins as well as RNA, lipids, and oligosaccharides. Thus, the analysis of crude preparations of exosomes without additional purification can lead to the overestimation of proteins and other biochemical components of exosomes. Therefore, the literature data on milk exosomes described below must be analyzed very gingerly and critically, taking into account the above data.

3. Biochemistry of milk exosomes

There is no doubt that the biological functions of milk exosomes are due to the proteins, lipids, and nucleic acids that make up their composition. There are different estimations of the number of protein and nucleic acid molecules, which may be located in exosomes. Up to 16–20 thousands of different mRNA [33, 34] and up to 2 thousands of proteins [35] are described in cow and human exomes in various works. Prof. Sverdlov in [29] shows that no more than 1,6 thousand of mRNA molecules may fit in a single exosome, since it sounds very doubtful that such amount of different mRNA molecules may be transferred with exosomes. We believe that the papers describing thousands of proteins and nucleic acids in the preparations of exosomes may be due to the analysis of insufficiently purified preparations of exosomes, co-isolating with milk proteins and nucleic acids (of fat milk globules or other vesicular or non-vesicular particles). This assumption is well supported by the data, described

in [8, 23]. As mentioned above, most papers dedicated to the study of exosomes analyze very crude preparations. Below we describe the structure and possible biological functions of major components of milk exosomes following the data given in various papers without critical analysis. However, taking into account the data provided above, it is necessary to examine the given information critically.

3.1 Proteins of milk exosomes

Data on the protein composition of milk exosomes significantly varies between papers. There is no doubt that proteins play a crucial role in the physiology of milk exosomes; the other obvious thing is that most of the proteins, secreting in milk, cannot be a natural part of exosomes, due to the mechanism of exosome generation in the cell [8]. It was shown that the exosome proteome changes depending on the physical activity, nutrition, or various infections, suggesting that protein content of milk is an excellent biomarker [13]. Also, the data indicate that the concentration of exosomal proteins and the exosomes in milk is significantly lower in mature milk than the early stages of lactation [12].

Depending on the method of proteome analysis used, researchers found hundreds of proteins in milk obtained from different mammals: 100 [36], 200 [37], 400 [38], 600 [33], and even 1900 [35] or 2100 [26] proteins in exosome preparations. Such a significant difference may be due both to different levels of purification of the exosome preparations and to a substantial difference in the protein content in vesicles obtained from various sources. However, it is evident that exosomes cannot contain several thousand proteins.

According to literature data, exosomes can contain proteins that control the cytoskeleton dynamics and membrane fusion: Rab proteins (GTPase family) [39]; Alix, TSG101, and other proteins of the endosomal sorting complex [40]; and proteins that participate in the binding and transport of miRNA, target cell recognition, and fusion (tetraspanins CD9, CD63, CD81). Members of the tetraspanins family mediate the adhesion of exosomes on the surface of the recipient cell and are essential structural components of exosomal membranes [41, 42]. Also, one cannot exclude that exosomes may contain various enzymes: proteases and their activators, peroxidases, lipid kinases, and other proteins that exhibit catalytic activity [43]. Exosomes may be rich in cytoskeleton proteins (actin, tubulin, cofilin), proteins of membrane transport and heat shock (HSP60, HSP70, HSP90), and proteins involved in intracellular signal transduction like Wnt proteins, which activate the Wnt signaling pathway [44, 45]. The presence of proteins involved in the formation of vesicles (ADP-ribosylation factor) [46] and membrane fusion EHD1 (testilin) [47] confirms the endosomal origin of exosomes. The presence of integrins in milk exosomes is an essential marker of the internalization and biological activity of extracellular vesicles, which can also be used to predict their possible direction of delivery [48]. In general, exosomes include proteins that make up the endosome, plasma membrane, or cytosol, while proteins from the nucleus, mitochondria, endoplasmic reticulum, and Golgi must be absent in exosomes. These observations emphasize the specificity of the formation of these vesicles and demonstrate that exosomes are a special subcellular compartment but not random cell fragments [49].

The proteins described above are common to all types of exosomes, including milk exosomes, which in addition to these proteins contain specific milk proteins. Several milk proteins were characterized in milk exosomes: caseins, lactoglobulin, lactoferrin, CD36, and polymeric immunoglobulin receptor precursor [6]. The CD36 protein in cells mediates phagocytosis and cell adhesion and binds low-density oxidized lipoproteins [50], but its role in milk exosomes requires further establishment. It is worth to say that not all the proteins described in the milk exosomes

in literature may be really exosome components. For example, in highly purified preparations from horse milk, the caseins were not defined as exosomal components but co-isolated with exosomes during the ultrafiltration and ultracentrifugation and were separated from exosomal fraction by gel filtration [8]. In this regard, additional studies of milk exosomes that do not contain impurities of co-isolating proteins are required. Improvement of methods of exosome purification will make it possible to determine the protein composition of milk exosomes more accurately and to understand the role of specific proteins in their structure and functions.

Since the formation of the exosomal membrane occurs from the endosomal membrane, the exosomes carry similar protein markers as the cells secreting them. An example is the peripheral membrane protein MFG-E8 (lactadherin), a glycoprotein containing several domains, some of which are necessary for binding to the membrane, while some others carry integrin binding sites. Expression of MFG-E8 in the cells increases the secretion of vesicles since MFG-E8 may play a unique role in the secretion of membrane vesicles by budding or splitting the plasma membrane or by exocytosis of multivesicular bodies [51]. The expression of this protein in mammary gland increases during lactation. Human milk exosomes contain TGFb2 protein, the increased expression level of which is associated with the development of breast cancer [52].

Compared to cow milk fat globule membrane, the same proteins were the most abundant in cow milk exosomes: butyrophilin, xanthine oxidase, adipophilin, and lactadherin [26]. During the mastitis caused by *Staphylococcus aureus*, relative concentrations of several proteins were increased 9–20 times in cow milk exosomes: protein S100-A12, cathelicidin-2, annexin A3, myeloperoxidase, haptoglobin, histone H2A, and H4 [13].

Flow cytometry has shown that human B-cell exosomes, unlike human milk ones, give a positive signal to tetraspanins CD40, CD54, and CD80 in addition to CD63 and CD81, which are detected in milk exosomes. On the contrary, MUC-1 protein is present in milk exosomes but absent in B-cell-derived exosomes [6].

In the sediments obtained by the centrifuging of cow's milk at 35,000 × g and 100,000 × g, CD9, CD63, and CD81 tetraspanins were found mainly in the sediment after 100,000 × g, indicating the presence of exosomes. Also, sediment obtained by 100,000 × g centrifugation contained a higher number of complement proteins C2, C6, and C7 than the 35,000 × g one, which indicates the presence of multiple types of extracellular vesicles of this sediment. Complement C8 beta chain, C1GALT1-specific chaperone 1, cartilage-associated protein, α-mannosidase 2, and procollagen-lysine 2-oxoglutarate 5-dioxygenase 3 were found only in the 100,000 × g fraction. Functional analysis of these proteins revealed three functions: galactosidase, glycosyltransferase, and peptidase activities. It cannot be excluded that proteins found in the milk exosome fraction are involved in the regulation of translation, protein maturation, and maintenance of the cellular structure. Also, analysis of human cells after fusion with milk exosomes has shown some exciting changes in expression of proteins responsible for translation (ribosomal subunits, initiation, and elongation), innate immunity, vesicular transport, and cell migration [48].

Some data indicate that human milk exosomes have a unique composition of proteins, distinct from other milk components. Transmembrane (CD9, CD63, CD81, lactadherin, guanine nucleotide-binding protein), cytosolic (annexins A2, A4–7, and A11, Ras-related proteins Rab, syntenin), and also intracellular proteins (endoplasmin, calnexin) are presented in human milk exosomes. The unique combination of proteins with different biological roles, cell growth, inflammation, and others indicates that milk exosomes may play a key role in the infant's intestinal immune system. The list of the most represented exosomal proteins according to the literature data is combined in **Table 1**.

Source of milk exosomes	Most represented proteins	Number of proteins described	Method of protein analysis	Protein content compared in
Cow [26]	Butyrophilin Xanthine oxidase Adipophilin Lactadherin	2107	Trypsinolysis, LC-MS/MS	Exosomes and milk fat globule membrane
Human [36]	Lactoferrin Tenascin Serum albumin β-Casein Xanthine dehydrogenase Polymeric Ig receptor	115	Trypsinolysis, LC-MS/MS	During 12 months of lactation
Cow [13]	Butyrophilin Xanthine dehydrogenase Lactadherin Fatty acid synthase	2299	Trypsinolysis, LC-MS/MS with iTRAQ	Norm and mastitis
Human [35]	CD9, CD63, CD81 Flotilin Lactadherin Annexins G-protein subunits Ras-related proteins Rab syntenin	2698	SDS-PAGE, trypsinolysis, LC-MS/MS	Extracellular vesicles and high-density complexes
Horse [8]	β-Lactoglobulin Lactadherin Actin Butyrophilin Lactoferrin	8	SDS-PAGE, 2D-electrophoresis, trypsinolysis, MALDI-TOF-MS/MS	Before and after gel filtration
Swine [33]	Fibronectin Thrombospondin Albumin Lactotransferrin Ceruloplasmin Complement C4 α-Glucosidase	571	SDS-PAGE, trypsinolysis, LC-MS/MS	

Table 1.
Most represented proteins of milk exosomes.

The data on the protein composition of milk exosomes gives new information on the structure and biological significance of these vesicles and reveals the potential role of exosomes in the physiology of the mammary gland. Investigation of the proteome of highly purified milk exosomes compared to milk proteome can shed light on the real protein composition of exosomes; these data may be translated to the exosomes obtained from other biological liquids. Results of milk exosome proteome analysis will possibly lead to their use in medicine as biocompatible carriers of drugs or personal therapy tools.

However, as shown above, not all proteins found in crude vesicle preparations are proper exosome proteins. At the same time, it is possible that some proteins associated with the surface of the vesicles may also play some unique role in the exosomes' functioning. The identification of hundreds and thousands of proteins

in the composition of the exosomes seems to us enormously overestimated. Also, the diversity of proteins found in exosomes raises questions about whether proteins that coprecipitated with these vesicles, as well as possible intrinsic minor proteins of exosomes, have an or have no important role in the biological functions of exosomes.

3.2 Lipids of exosomes

The exosome membrane is enriched with specific lipids (phosphatidylcholine, cholesterol, sphingomyelin, ceramides) and has a unique protein composition that characterizes them as independent compartments [53, 54]. The minimum size of exosomes depends on the structure of the lipid bilayer, which is about 5 nm thick and has sufficient rigidity to form vesicles of 40 nm in size [55]. The lipid composition of exosomes greatly varies, due to differences in cell types, conditions of cell growth and development, as well as the use of different methods for isolating exosomes and analyzing them.

The first works on the lipid composition of exosomes were carried out using a thin layer and gas-liquid chromatography. The results obtained using these methods cannot be considered fully quantitative, since the phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and phosphatidic acids migrate together and are presented in the single band after separation. Similarly, sphingomyelin and ganglioside GM3 are not separated and, therefore, are taken into account together in the quantitative analysis. Today, these methods are considered obsolete for the analysis of lipids of exosomes, and most studies use modern mass spectrometry technology [56].

It was found that different classes of lipids are asymmetrically distributed in the membrane of exosomes, so sphingomyelin and other sphingolipids, as well as phosphatidylcholines, are mainly located in the outer layer of the membrane, while the different classes of lipids are located in the inner layer [57]. However, the established asymmetry of the membrane bilayer can be altered by the action of particular enzymes, such as flippases, floppases, and scramblases [58, 59].

Phosphatidylserine in exosomes, as in the plasma membrane, is located in the inner lipid layer [57], but several studies have shown its presence also in the outer layer together with annexin 5. It is known that the presence of phosphatidylserine in the outer lipid layer activates blood cells and acts as a signal to macrophages to capture [60].

The composition of certain classes of lipids in the exosomal membranes secreted by different cell types may be similar or not to the parental cells. Exosomes contain up to 2–3 times more cholesterol, sphingomyelin, glycosphingolipids, serine, and saturated fatty acids. In addition, GM3 ganglioside [56, 61], ceramides, and their derivatives [57, 62, 63] are also present in exosomes in significant amounts. In most cases, exosomes contain fewer phosphatidylcholines than their parent cells, and no noticeable differences in the content of phosphatidylserine in exosomes and parent cells were found [64].

The bis(monoacylglycero)phosphates (BMP) are present in the membranes of the intraluminal vesicles of multivesicular bodies and, as stated in [57], can also be contained in the exosomes. It was shown that BMP are not transferred to exosomes but, with high probability, are included in the intraluminal vesicles of those multivesicular bodies that are associated with lysosomes. In this regard, it is believed that the primary purpose of the BMP is to promote the stability and integrity of the lysosomes. Also, it is a necessary cofactor in the process of catabolism of sphingolipids in lysosomes [65].

The stiffness of exosomal membranes increases with the transition from acidic to neutral pH values, suggesting that during the secretion of the exosomes from the multivesicular bodies, some reorganization of the membrane occurs. At neutral pH, the packaging of lipids on the surface of exosomes is dense, but the transmembrane movement of lipids increases. Such a flip-flop effect (the transition of an individual molecule from one layer to another) disrupts the asymmetric distribution of lipids between the membrane layers. A uniform distribution of phosphatidylethanolamine between the two layers of the exosomal membrane [66] was shown; on the contrary, in the plasma membrane, it is located mainly in the inner lipid layer [67].

The lipids of the exosomal membranes are not inert molecules but participate in the biogenesis of vesicles and affect their biological activity. Besides, exosomes carry carbohydrate groups on the outer surface; the presence of mannose, polylactosamine, α-2,6 sialic acid, and complex N-linked glycans was shown [68].

Lipids form the basis of the exosome membrane; the composition of exosomal lipids significantly differs from the composition of lipids of non-vesicular structures.

The above data reflect the content of lipids and oligosaccharides in the composition of mainly crude preparations of exosomes. However, as noted above, the second peak after gel filtration of exosome preparations also contains various lipids and oligosaccharides. Therefore, it cannot be excluded that part of the lipids and polysaccharides found in the vesicles will be attributed in the future to the high-molecular complexes co-isolating with exosomes during different centrifugations.

3.3 Nucleic acids of milk exosomes

Several works show that milk exosomes contain different types of nucleic acids, including functional mRNA with a poly(A) tract at the 3′-end [33, 34, 41] and microRNA [34, 69, 70]. Currently the composition of nucleic acids in milk exosomes is described in the case of human [71, 72], cow [73, 74], porcine [33, 75], and rat [10] milk.

As we have noted above, the number of individual RNA molecules in some papers may be overestimated due to various reasons. For example, it has been shown that exosomes of porcine milk contain up to 16,304 mRNA. Most of these molecules may be involved in the development of the immune system, cell proliferation, and intercellular signal transduction. Protein products of identified mRNAs might be involved in the regulation of metabolism and the growth of the piglet's intestines [33].

Among the 19,230 mRNAs found in cow milk exosomes, the most common mRNA molecules are various isoforms of the mRNA of milk and ribosomal proteins: inositol 1,4,5-triphosphate receptor type 1; α-lactalbumin; β-lactoglobulin; caseins β, κ, and α; ribosomal proteins S28, S3, P0, L32, L21, and H1 histone; and many others. Since the expression level of most of the 50 most represented transcripts in exosomes exceeds the standard in supernatants obtained by ultracentrifugation, the authors of the [34] conclude that the mRNAs are concentrated in the exosomes of milk. Unfortunately, it is difficult to agree with this statement, since the process of enrichment of exosomes with mRNA transcript molecules during their biogenesis is entirely unclear, as well as it is difficult to suggest a mechanism by which this unimaginable number of mRNA molecules can fit into an exosome with a diameter of 40–100 nm. At the same time, human and porcine milk exosomes contain little or no 18S and 28S rRNA [41, 70], which correlate with the mechanism of exosome biogenesis.

It is shown that miRNA is ubiquitous in tissues and biological fluids and was previously isolated from and associated with exosomes formed from various biological fluids (serum, saliva, urine) and homogenates of body tissues, including the mammary gland [76]. Some studies have shown that the exclusion of milk exosomes and their contents, including exosomal miRNAs, from nutrition in newborns leads to impaired purine metabolism, as well as impaired spatial learning and memory in humans and mice [72, 77]. These data indicate the undesirability of feeding newborns with infant formulas since their content of microRNAs is absent or much lower than breast milk [78].

Analysis of nucleic acids isolated from human milk exosomes revealed about 452 pre-miRNAs, which is approximately 32% of 1424 miRNAs described for humans. These 452 pre-miRNAs lead to the generation of 639 mature miRNAs, many of which are involved in the regulation of immune responses. At the same time, the distribution of different miRNAs in human milk exosomes is irregular; some miRNAs are represented in a million copies and others in single molecules. Ten miRNAs compose up to 62% of the total number of miRNA; the most represented miRNAs are miR-30b-5p, miR-141-3p, miR-148a-3p, miR-182-5p, miRs let-7a-5p and let-7f-5p, miR-29a-3p, miR-146b-5p, miR-182-5p, miR-200a-3p, and miR-378a-3p [79]. Interestingly miR-148a-3p may comprise up to 35% of the total number of miRNAs of human milk exosomes. miR-148a specifically controls the expression of several genes, including the TGIF2, which encodes a transcription factor, inducing the expression of various transporters and drug-metabolizing enzymes [80], and the DNMT3B gene, which encodes DNA methyltransferase [81].

Transcriptome of cow milk exosomes contain various miRNAs, the most common of which are bta-miR-320a-1, bta-miR-193a, bta-miR-2284x, bta-mir-181b-1, bta -miR-19b-2, bta-miR-135a-1, bta-miR-200c, bta-miR-142, bta-miR-2887-1, bta-miR-30b, bta-miR-let7i, and bta-miR-6522 [72]. It was shown that cow milk exosomes penetrate intestinal and choroidal epithelial cells [82, 83] and macrophages [34], accumulate in peripheral tissues [84, 85], and transfer miRNA to the recipient cells [86]. Analysis of exosome bioavailability between species showed that microRNA of cow milk exosomes, after oral delivery to other organisms, is protected under the low pH, RNase, and other factors of the gastrointestinal tract [78, 87, 88].

Analysis of miRNA content in porcine milk exosomes revealed 366 pre-miRNAs, which can give rise to 315 mature miRNAs, and 176 of them were described in other sources. Functional analysis of porcine milk miRNA indicates their role in immune responses, and 14 of 20 of most represented miRNAs may be involved in the regulation of milk IgA production [69]. Also, it was shown that miR-148a, widely represented in the exosomes of human [71] and cow [89] milk, is also highly expressed during lactation in exosomes of porcine milk [70]. Other highly expressed miRNAs of porcine milk are miR-181 family (181a/181b/181c/181d), miR-30 family (b/c/d/e), let-7 family (a/b/d/f), and miR-98 family. Thus, miRNAs included in these families can participate in the development of the digestive tract in piglets [69].

Nucleic acids play an essential role in biological functions of milk exosomes. The further investigations of milk exosome miRNA and mRNA variety will significantly expand the prospects of their practical use. However, when analyzing different RNA in exosomes, it should not be forgotten that exosomal preparations used in most of the papers described above were crude. Therefore, some of the detected RNA may be in the fraction with co-isolating proteins and their complexes, which may be separated from exosomes with gel filtration.

4. Milk exosomes: perspectives of use

The first paper describing human milk exosomes was published in 2007 [6] and dedicated to the interaction of exosomes with blood cells in cell cultures. Preparations of human milk vesicles inhibited interleukin-2, γ-interferon, and tumor necrosis factor α production by peripheral blood mononuclear cells and stimulated the increase of $Foxp3^+$ cell proportion in vitro.

Exosomes are natural vesicles with very promising perspectives for drug therapeutic nucleic acid delivery into the cells. One of the unresolved problems so far is the development of universal sources for isolation of preparative amounts of exosomes. The use of milk allows researchers to obtain exosome preparation several liters of milk at once that makes milk a cheap and unique source of exosomes.

Milk exosomes can be used for targeted drug delivery to cells. It is shown that exosomes of cow [83] and human [88] milk penetrate intestinal crypt-like cells. The possibility of milk exosomes to be used as agents for delivery of proteins, nucleic acids, and drugs makes the subject of its investigation extremely relevant. Since the components of cow milk can be used for therapy with significant limitations, since in this case the transmission of prion diseases cannot be excluded [90], analysis of exosomes isolated from other milk sources is very actual.

Encapsulation of curcumin in buffalo milk exosomes increased its stability in salivary, gastric, pancreatic secrets as well as in bile juice. Also, curcumin encapsulated in milk exosomes was successfully uptaken and trans-epithelial transported in Caco-2 cells [91]. Since curcumin is a hydrophobic and water-insoluble molecule, it binds to the exosomes and probably incorporates in exosomal membranes. Hydrophobic drug molecules (paclitaxel [92], doxorubicin [93], and others) can also be delivered via milk exosomes with the same mechanism. Similar results were obtained in the case of chemically synthesized siRNA [86]. Transfection of siRNA in cow milk exosomes protects it from the activity of digestive juices and increases delivery to Caco-2 cells compared to the control samples.

According to several works, milk exosomes contain antibody molecules on the surface. Transport of IgG molecules in the intestine occurs as a result of binding to neonatal Fc receptor (FcRn). Cow milk-derived exosomes were successfully delivered to the mice liver, heart, spleen, lungs, and kidneys after the oral administration [94].

Auspicious work shows the possibility of transfer of bovine leukemia virus proteins Env (gp51) and Gag (p24), but not viral DNA with milk exosomes obtained from infected cattle [95]. This route of viral protein delivery doesn't require viral infection since that may have the potential of use for immunization and/or vaccination.

Incubation of human milk exosomes, but not the blood plasma exosomes with monocyte-derived dendritic cells, results in DC-SIGN inhibition of HIV infection of dendritic cells and protects from HIV transfer to $CD4^+$ T lymphocytes [96]. These results may partially explain why some breastfeed infants do not get infected from HIV-infected mothers.

Yak milk exosomes facilitate survival of intestine cells in hypoxic conditions in vitro and have significantly higher activity than cow milk exosomes. Yak milk-derived exosomes also decrease the expression of p53, increase the expression of oxygen-sensitive prolyl hydroxylase, and decrease the expression of vascular endothelial growth factor via the hypoxia-inducible factor-α in cell cultures [97]. Human milk exosomes protect intestinal epithelium cells in vitro from oxidative stress and probably defend newborns from enterocolitis [98].

It should be noted that the correct use of vesicles for medicine still requires extra-purified exosome preparation and analysis of the functioning of such exosomes. Also, it cannot be excluded that any proteins and other biologically active molecules firmly connected with the surface of vesicles can also be necessary for the manifestation of exosome biological functions. However, this issue also requires further research.

5. Conclusions

Milk is more than a source of nutrients and vitamins for newborn [99]; it contains different proteins and protein complexes [100] with very diverse functions. Milk is a biological liquid containing vesicles of different size and shapes. Exosomes are natural vesicles with a diameter of 40–100 nm and are found in milk obtained from human, cow, horse, camel, mice, rat, swine, and some other mammalian species. There is no doubt that milk obtained from any source contains such structures. Many biological effects are attributed to exosomes, and researchers are particularly interested in milk exosomes, since they can be used as carriers of drugs and therapeutic nucleic acids for delivery to cells. The physicochemical properties and biological functions of exosomes are primarily determined by their biochemical composition—the structure of lipids, proteins, and nucleic acids. The prospects for the further practical use of milk exosomes in medicine and biotechnology largely depend on investigations of the fine structure and biological functions of exosomes free of various contaminating impurities.

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Conflict of interest

The authors declare no conflict of interest.

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