Bioactive Compounds of Milk Exosomes

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Exosomes are biological nanovesicles that participate in intercellular communication by transferring biologically active chemical compounds (proteins, microRNA, mRNA, DNA, and others). Milk is the only exosome-containing biological fluid that is commercially available. In this regard, milk exosomes are unique and promising candidates for new therapeutic approaches to treating various diseases, including cancer. The biochemical components of milk exosomes—proteins, lipids, and nucleic acids—can significantly affect therapeutic molecule delivery. In this regard, a detailed analysis of the content of these molecules in milk exosomes, also called “exosomics” (by analogy with genomics, proteomics, and other -omics technologies), is required.

milk exosomes milk exosomes drug delivery cancer

1. Milk Exosome Proteins

The articles devoted to proteomic analysis of milk exosomes using modern highly sensitive methods describe dozens, hundreds, and even thousands of proteins and peptides, such as 115 [1], 571 [2], 2107 [3] or 2698 [4] individual proteins, and isoforms and 719 peptides [5]. According to the critical article [6], these numbers are greatly exaggerated, most likely, due to the attribution of co-isolating milk proteins with exosomes [7][8].

The milk exosomes contain several proteins that participate in their formation: Testilin controls membrane fusion [9], Rab GTPase interacts with cytoskeleton proteins [10], and Alix and Tsg101 are involved in endocytosis [11]. Moreover, milk exosomes contain proteins that determine their biological functions, namely, transport of microRNA and adhesion to the target cells (tetraspanins CD9, CD63, CD81) [12][13]. Functional activity of milk exosomal enzymes—fatty acid synthase, xanthine dehydrogenase, proteases, ADP-ribosylation factor—is not yet evident [7]. Milk exosomes also contain cytoskeleton proteins—actin, tubulin, cofilin, heat shock proteins, and molecules—involved in signal transduction, for example, in the Wnt signaling pathway [14][15]. Nuclear, mitochondrial, reticulum, and Golgi proteins cannot be an intrinsic part of exosomes as they are not transported to endosomes [16]. In any case, because the exosomes originate from an independent cellular compartment, their protein composition is not accidental.

Horse milk exosomes contain mainly actin, butyrophilin, β-lactoglobulin, lactadherin, lactoferrin, and xanthine dehydrogenase, as well as numerous peptides (see **Table 1**).

Table 1. Highly represented proteins of milk exosomes.

Highly Presented Proteins	Number of Proteins Described in a Paper	Source of Milk Exosomes, Ref	Method of Analysis
Butyrophilin, κ-casein, lactadherin, xanthine dehydrogenase	94	Bovine [17]	LC-MS/MS of tryptic hydrolysates
Angiogenin-1, lactoferrin, lactoperoxidase sulfhydryl oxidase	920	Bovine [18]	LC-MS/MS of tryptic hydrolysates with iTRAQ
Butyrophilin, CD36, complement component 3, fatty acid synthase, lactadherin, lactotransferrin, low-density lipoprotein receptor-related protein 2, polymeric immunoglobulin receptor, xanthine dehydrogenase	1372	Bovine [19]	LC-MS/MS of tryptic hydrolysates
α-casein, butyrophilin, fatty acid-binding protein, lactadherin, α-lactalbumin, β-lactoglobulin, xanthine dehydrogenase	1879	Bovine [20]	LC-MS/MS of tryptic hydrolysates
Adipophilin, butyrophilin, lactadherin, xanthine oxidase	2107	Bovine [3]	LC-MS/MS of tryptic hydrolysates
Butyrophilin, lactadherin, fatty acid synthase, xanthine dehydrogenase	2299	Bovine [21]	LC-MS/MS of tryptic hydrolysates with iTRAQ
Actin, butyrophilin, lactadherin, lactoferrin, β-lactoglobulin	8	Horse [22]	MALDI-TOF-MS/MS of tryptic hydrolysates after 2D-Electrophoresis
CD36, α-enolase, fatty acid synthase, lactadherin, lactotransferrin, polymeric-Ig-receptor, Rab GDP dissociation inhibitor, syntenin-1, xanthine dehydrogenase	73	Human [9]	LC-MS/MS of tryptic hydrolysates
β-Casein, lactoferrin, serum albumin polymeric Ig receptor, tenascin, xanthine dehydrogenase	115	Human [1]	LC-MS/MS of tryptic hydrolysates
Annexins, CD9 CD63, CD81, flotillin, G-protein subunits, lactadherin, Rab, Ras-related proteins, syntenin	2698	Human [4]	LC-MS/MS of tryptic hydrolysates
Albumin, ceruloplasmin, complement C, α-glucosidase, fibronectin, lactotransferrin, thrombospondin	571	Porcine [2]	LC-MS/MS of tryptic hydrolysates after SDS PAGE

These results correspond to the literature data obtained using highly sensitive proteomic methods [7], according to which butyrophilin, lactadhedrin, and xanthine dehydrogenase are the specific markers of milk exosomes. Because α-, β-, and κ-caseins and ribosomal proteins cannot be present in exosome preparations according to incompatible secretion mechanisms [23][24], these proteins are not intrinsic components and may be considered as “negative

markers" of milk exosomes (fractions containing these protein markers are not exosomes or contain co-isolated protein impurities).

2. Nucleic Acids of Milk Exosomes

Studies of the past decade have shown the content of various noncoding RNA—microRNA, long noncoding RNA, circular RNA [25]—in bovine [26], human [27], panda [28], porcine [29], and rat [30] milk. Exosomal RNAs are stable at low intestinal pH values and in the presence of RNases [26][31]. Milk mRNAs are mainly concentrated in exosomes, while microRNAs are concentrated in exosomes and the supernatant [32].

The microRNA contents of milk exosomes are analyzed by high throughput sequencing (NGS) and microarray technology. Analysis of global expression profiles using microarrays revealed 79 different microRNAs (miRNAs) in the exosomal fraction and 91 in the supernatant after ultracentrifugation of bovine milk, and 39 miRNAs were common for both fractions. Further studies have shown that the expression level of these microRNAs is significantly higher in the exosomal portion compared to the supernatant [32]. Some 491 miRNAs were described in porcine milk exosomes, including 176 known miRNAs and 315 new mature miRNAs. Analysis of the gene ontology of these microRNAs showed that most of them are targeting genes related to transcriptional, immune, and metabolic processes [33]. In a study of the microRNA profile in exosomes of human milk during type I diabetes mellitus, 631 exosomal microRNAs were identified, including immune-related ones such as hsa-let-7c, hsa-miR-21, hsa-miR-34a, hsa-miR- 146b, and hsa-miR-200b. Differential expression was described for nine miRNAs in healthy and diabetic groups [34]. MircroRNAs, highly represented in milk exosomes, are reviewed in **Table 2**.

Table 2. Highly represented microRNA of milk exosomes.

Highly Presented microRNA	Number of microRNAs Described in a Paper	Source of Milk Exosomes, Ref	Method of Analysis
2478, 1777b, 1777a, let-7b, 1224, 2412, 2305, let-7a, 200c, 141	79	Bovine [32]	Microarray
148a, let-7c, let-7a-5p, 26a, let-7f, 30a-5p, 30d	372	Buffalo [35]	RNA seq
30d-5p, let-7b-5p, let-7a-5p, 125a-5p, 21-5p, 423-5p, let-7g-5p, let-7f-5p, 30a-5p, 146b-5p	219	Human [36]	RNA seq
22-3p, 148a-3p, 141-3p, 181a-5p, 320a, 378a-3p, 30d-5p, 30a-5p, 26a-5p, 191-5p	308	Human [1] [33]	RNA seq
let-7a-5p, 148a-3p, 146b-5p, let-7f-5p, let-7g-5p, 21-5p, 26a-5p, 30d-5p	631	Human [37]	RNA seq
148a-3p, 30b-5p, let-7f-5p, 146b-5p, 29a-3p, let-7a-5p, 141-3p, 182-5p, 200a-3p,	602	Human [34]	RNA seq

Highly Presented microRNA	Number of microRNAs Described in a Paper	Source of Milk Exosomes, Ref	Method of Analysis
378-3p			
let-7b-5p, 92a-3p, 148a-3p, 30a-5p, let-7a-5p, 181a-5p, let-7i-5p, let-7f-1/2-5p, let-7g-5, 200a-3p	1191	Panda [28]	RNA seq
148a-3p, 182-5p, 200c-3p, 25-3p, 30a-5p, 30d-5p, 574-3p	234	Porcine [29]	RNA seq
148a, let-7b, let-7a, 21, let-7c, let-7i, 26a, let-7f, 125b, 143	84	Sheep [38]	RNA seq

portant in processes of fetal development, cell proliferation, pregnancy, immune system development, inflammation, sugar metabolism, insulin resistance [39][40], and many others (**Table 3**).

[1] Preterm delivery.

Table 3. Genes regulated by a highly abundant microRNA of milk exosomes.

microRNA(s)	Gene(s) Targeting with microRNA	Biological Function(s) of the microRNA Targets, Ref
22-3p	Transcription factor 7 of Wnt pathway	Regulation of gluconeogenesis, insulin resistance [41]
25-3p	KLF4 (Krüppel-like factor 4)	Development of the immune system [29]
30a-5p	P53, DRP1 (dynamin-related protein 1), GALNT7 (GalNAc transferase 7)	Mitochondrial fission, cellular invasion, immunosuppression, synthesis of interleukin (IL)-10 [29]
30d-5p	GalNAc transferases	Inflammatory processes [42]
148-3p	NF-κB (transcription factor)	Decrease of the immune response [43]
148a [1]	DNMT1	Epigenetic regulation [40]
let-7 family	Insulin-PI3K-mTOR signaling pathway	Glucose tolerance and insulin sensitivity [44]
let-7a-5p let-7b-5p	TRIM71, IL6-induced signal activation of transcription,	Stem cells proliferation, fetal development [43], activation of metalloproteinases [45]

[1] The most expressed miRNA of human milk.

Literature data provide information on the content of 16,304 different mRNAs in porcine milk exosomes [2] and up to 19,230 mRNAs in bovine milk exosomes [32]. As with thousands of proteins, it is difficult to imagine how these thousands of mRNAs can fit in a 40–100 nm vesicle [6]. Interestingly, 18S and 28S ribosomal RNAs are practically absent in the milk-derived exosomes of human [12] and porcine milk [29], which corresponds to the current knowledge biogenesis of these vesicles.

The mechanism by which these nucleic acids can co-isolate with vesicles is not as apparent as for proteins. The potential interaction of nucleic acids in milk exosomes (especially the anti-inflammatory effect and weakening of the

immune response [39]) should also be taken into account when planning experiments on the delivery of therapeutic nucleic acids to cells.

3. Lipids of Milk Exosomes

Exosomes are vesicles rounded by the lipid bilayer containing proteins directed to the extracellular space. In this regard, the delivery of pharmacologically significant compounds is possible both inside and outside exosomes, both for hydrophilic (bound to surface proteins) and hydrophobic (as part of the lipid bilayer) molecules. The exosomal membrane is known to contain cholesterol, phosphatidylcholine, phosphatidylinositol, sphingomyelin, and ceramides; most phosphatidylcholine and sphingolipids has been shown to be located in the outer layer of the exosomal membrane [46]. The thickness of the lipid bilayer is at least 5 nm, so the exosome's minimum diameter cannot be less than 40 nm. Depending on the source and the methods used, the composition of exosome lipids in milk differs from one source to another. A detailed overview of exosome lipids is given in [46][47].

The problem of exosome preparation's contamination with lipid droplets, lipoproteins, and other particles [47] during their isolation by centrifugation and ultracentrifugation limits the study of intrinsic exosomal lipids. Exosomes isolated from cell cultures contain arachidonic acid, prostaglandins, enzymes of fatty acid metabolism [18], and leukotrienes [48]. This data indicates the need for further study of lipids in highly purified exosome preparations.

Liposomes have been widely used as targeted delivery vehicles for over 40 years. Liposome membranes can be modified to improve the targeting properties with monoclonal antibodies, aptamers, proteins, and small molecules [49]. A promising way of changing the membrane of natural exosomes is its fusion to liposomes with artificially functionalized lipids [50]. Polymer nanoparticles might have higher stability compared to liposomes, but their biocompatibility and safety in the case of long-term administration remain an unresolved problem [51]. Natural lipid components of milk exosomes are more compatible and less toxic than artificial liposomes for use as drug delivery vehicles [52].

To increase the efficiency of drug delivery by milk exosomes, a more intensive study of the lipid composition of exosomes, proteins that are located in the lipid membrane, is required. Modification of exosomal surface for targeted delivery and prolonged half-life in the body is an essential task for biopharmacology and biomedicine [53].

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