Milk Exosomes

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Milk contains various extracellular vesicles and non-vesicular structures: exosomes (with diameter 40-100 nm), vesicles of other size, fat globules (with diameter 4-6 mkm, containing milk fat globule membranes), and their aggregates. Due to the biocompatibility of milk exosomes, these vesicles have a wide potential as vehicles for oral delivery of therapeutically relevant molecules (drugs and therapeutic nucleic acids).

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1. Introduction

Exosomes are natural extracellular nanovesicles that participate in intercellular communication by carrying biologically active substances like proteins, microRNA, mRNA, DNA, and other molecules [1][2]. The carried compounds can be transported both inside and on the surface of exosomes [3]. Due to their small size (diameter 40–100 nm), exosomes are promising delivery tools in personalized therapy [4]. Because artificial exosome synthesis methods are not developed yet, the urgent task is to effectively and safely obtain exosomes in mass quantities from natural sources. Tumor cell cultures can hardly be considered as a promising source of vesicles for pharmacology applications. The exosome content is shown in various biological fluids: blood plasma, urine, saliva, milk, amniotic fluid, ascites, cerebrospinal fluid, and others [2]. Among them, milk is the only biological liquid containing exosomes that is available on an industrial scale.

Human milk exosomes were first described in 2007. To date, exosomes have been isolated (chronologically) from human [5], bovine [6], porcine [7], wallaby [8], camel [9], rat [10], horse [11], panda [12], yak [13], sheep [14], and goat milk [15]. Databases provide more than 100 articles on exosomes and other milk vesicles (more than half of them are on human and cow milk vesicles). Large volumes of milk can be obtained from a cow and relatively large from horse, sheep, goat, and camel. Due to possible prion content, bovine milk should not be considered a source of protein-containing structures [16].

Milk exosomes are promising candidates in developing new therapeutic approaches to the treatment of various diseases, including cancer. However, currently, there is only incomplete data in the literature on the use of milk vesicles, particularly exosomes, to deliver biologically active molecules to cells [17]. In the application of milk exosomes for cancer treatment, two directions can be highlighted: the delivery of antitumor drugs [18] and therapeutic nucleic acids [19][20]. Various side effects occurring during the use of cytotoxic and cytostatic agents are unresolved problems of cancer chemotherapy [21]. Cells with a high mitotic index are most susceptible to the action of cytostatic agents; thus, the use of cytostatic drugs leads to the death of not only the tumor cells but also cells of

the bone marrow, skin, hair, epithelium of the gastrointestinal tract, etc. [22]. Targeted chemotherapeutic agent delivery can be a potential solution to the occurrence of toxic effects in chemotherapy [4][23].

Because exosomes contain mRNA, microRNA, and proteins that may harm cells, some experts disagree that exosomes (including milk exosomes) can be used as delivery vehicles. Analysis of literature data shows that many papers devoted to the protein and nucleic components of exosomes may have overestimated the number of proteins and/or nucleic acid molecules in exosomes due to contamination of the preparations with co-isolating molecules [24][25]. Therefore, it is essential to analyze highly purified exosome preparations to identify biopolymers that are intrinsic components of exosomes and the proteins and nucleic acids that co-isolate with exosomes. Exosomes can be used as drug delivery systems, carrying the drugs of therapeutic nucleic acids on the surface or after “loading” of these components using electroporation, sonication, or ligands on the surface of exosomes [26]. Another method is the fusion of exosomes with liposomes, loaded with necessary contents to form “chimeric” exosomes [27].

2. Isolation of Milk Exosomes

Various physical, physicochemical, and immunological approaches are used to isolate exosomes from milk. General issues concerning the isolation and characterization of exosomes are described in the guidelines of the International Society for Extracellular Vesicles [28][29]. Because the volume of milk obtained from single humans or animals may vary from milliliters to liters, the general protocol [1] of exosome isolation from milk samples usually starts with a series of centrifugations [6]: first, several sequential centrifugations at low speed of 600–1000× *g* to defat the milk and precipitate the cells, then 10,000–16,000× *g* to precipitate milk proteins, followed by one or several ultracentrifugations at 100,000–200,000× *g* [30] to isolate the exosomes. Some protocols include the stage of ultracentrifugation in the sucrose density gradient [31].

Patented and commercially available sets of reagents using volume-excluding polymers are also used: for example, ExoQuick (System Biosciences, Palo Alto, CA, USA)—contains PEG 8000 kDa; Total Exosome Isolation (Thermo Fisher Scientific, Waltham, MA, USA)—includes dextrans, polyethylene glycol, polyvinyl with a molecular weight above 1000 kDa, and their combinations with other methods [32]. Different exosome isolation methods include filtration through a semipermeable membrane and isolation on immunomagnetic particles [4].

One- or two-time centrifugation of milk at low speeds before subsequent optional storage of milk plasma preliminary to exosome isolation is considered fundamental, as described in many protocols, for example, [30]. Because the composition of nucleic acids, proteins [33], and lipids [34] of milk exosomes and milk fat globule membrane (MFGM) in various articles often coincides, it cannot be excluded that storage of nondefatted milk samples leads to contamination of exosome preparations with MFGM. Refrigeration of nondefatted milk samples may lead to exosomes sticking to MFGM, resulting in further MFGM contamination of the exosome samples obtained.

Exosome preparations isolated by centrifugation and ultracentrifugation from blood plasma, urine, milk, lacrimal, and cell culture fluid usually contain “nonvesicles” with 20–100 nm diameter. These “nonvesicles” are morphologically attributed to intermediate- and low-density lipoproteins (20–40 nm) and very low-density (40–100 nm) lipoproteins [35]. These have a different structure according to transmission electron microscopy and should not be confused with exosomes.

The possibility of exosome isolation using gel filtration after or instead of ultracentrifugation [36] has been shown for human [37], bovine [38], and horse milk. Because the gel filtration allows us to effectively separate sample volumes that do not exceed 1–5% of the column volume, this method is not suitable for isolating exosomes directly from large amounts of milk. Gel filtration allows purifying the sample from co-isolating proteins in preparations obtained after ultrafiltration and/or ultracentrifugation. Our data suggest that sequential centrifugations and ultracentrifugations followed by the ultrafiltration throw 0.22 μm filter are not enough to get purified exosomes [39]. Moreover, gel filtration of sediments obtained by ultracentrifugation results in exosome preparations without admixtures of co-isolating proteins [11].

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