Article

Deposition of Polyphenols in Mare's Milk Exosomes and Assessment of Their Bioavailability

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Abstract: Polyphenols are a group of chemicals synthesized in plants (fruits, stems, roots, seeds). Polyphenols have geroprotective properties, but the use of polyphenols is limited due to their poor solubility in water. Milk-derived exosomes can be used as a transport system. The aim of the study was to evaluate the bioavailability of polyphenols in mare's milk exosomes. Materials and methods: using isoelectric precipitation and ultracentrifugation, exosomes were made from mare's milk. Quercetin was used as polyphenols. Encapsulation of quercetin in exosomes was carried out according to the method of encapsulation of medicinal substances in exosomes from cow's milk. The capacity and efficiency of loading exosomes with antioxidants were evaluated. The release of quercetin from exosomes in vitro was carried out by equilibrium dialysis, then the percentage of binding in each time interval was calculated. Results: when studying the stability of the preservation of antioxidants in exosomes, we obtained a relative ratio (mV) for the quercitin standard - 69.4; for quercetin in exosomes immediately after inclusion - 90.1; for quercetin on the 12th day after storage at -80 ° C - 90.4. The efficiency of loading exosomes with quercetin was 55%. The proportion of quercetin released in total for 72 hours was 67.3%. Conclusion: The stability of quercetin in exosomes was confirmed for 12 days when stored at -80 °C. The results obtained during the dissociation of quercitin from exosomes in vitro using the equilibrium dialysis method indicate a gradual course of the release process.

Keywords: polyphenols, quercetin, exosomes, bioavailability of polyphenols.

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1. Introduction

Polyphenols are a group of chemicals that are mainly synthesized in plants as secondary metabolites, which are structurally characterized by the presence of one or more hydroxyl groups (-OH) linked to phenols. They are widely distributed in nature and are extremely diverse in their molecular structure. They are found in fruits, leaves, stems, roots and seeds, and more than 8000 polyphenols have been identified to date [1].

The main food sources are fruits and beverages such as juices, wine, tea, coffee, hot chocolate and beer, and in smaller amounts vegetables, legumes and cereals. The total intake of polyphenols in a standard diet is approximately 1 g/day. Of these, phenolic acids account for one-third of the total, flavonoids account for two-thirds, and other variants are ingested in negligible amounts [2].

Polyphenols have been the subject of much research as geroprotective agents, and in recent years attempts have been made to utilize their putative senolytic activity to inhibit some cellular aging processes [3,4]. However, bioavailability studies in humans are still scarce. Plasma concentrations rarely exceed 1 μM after ingestion of 10 to 100 mg of a single phenolic compound. However, the total plasma phenol concentration may increase depending on the presence of metabolites formed in body tissues or colonic microflora. The chemical structure of polyphenols determines the rate of its absorption, the extent and nature of metabolites circulating in the plasma.

It is generally recognized that the use of polyphenols in many cases is limited due to their poor water solubility, chemical instability and low bioavailability [5]. But it is also known that chemical instability due to interaction with substances and metabolic products of the intestinal microbiome as well as pharmacokinetic defects can be overcome by using transport delivery systems in the form of liposomes, nanoparticles, micelles or conjugates [6,7,8]. Milk-derived exosomes have come to be considered as a modern variant of the transport system in recent years [9,10]. Extracellular vesicles (30-160 nm) called exosomes are known to be present in the milk of farm animals [11]. Milk exosomes have been characterized as relatively stable vesicles [12].

There are publications proving the possibility of increasing the bioavailability of polyphenols when deposited in exosomes from cow milk [13,14]. This fact allows us to suggest the prospect of

using exosomes from milk and other animal species for the creation of transport systems. In particular, mare milk is of interest as a source of exosomes production. Among the types of milk of farm animals, horse milk occupies a special place [15,16]. Mare's milk is similar to human breast milk and therefore may have some valuable therapeutic properties [17].

Objective: to evaluate the bioavailability of polyphenols in mare milk exosomes.

2. Materials and Methods

Fresh milk obtained from a certified farm within a day was used as raw material for the production of exosomes from mare's milk (Figure 1).

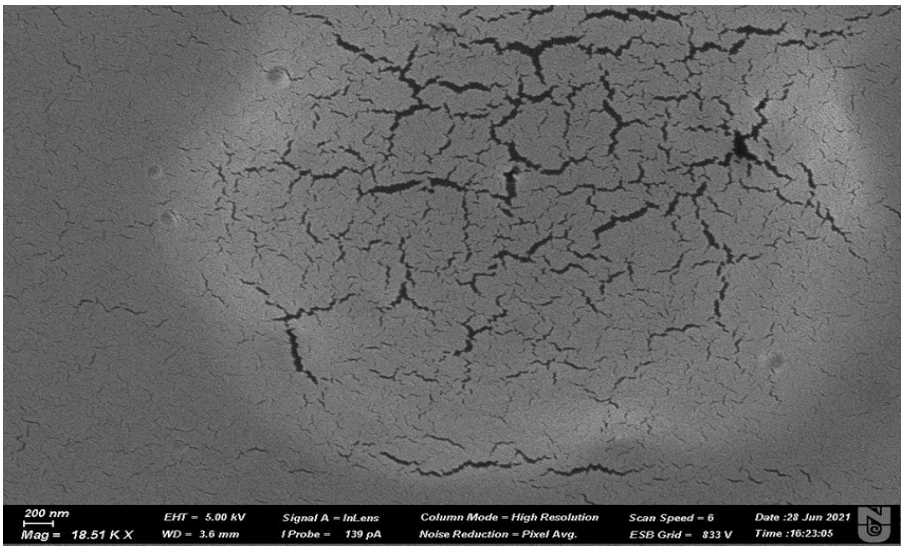

Figure 1: Electron micrographs of mare's milk exosome (scale 200 nm)

Two methods were used to obtain exosomes from horse milk: the isoelectric precipitation method as described by M. Yamauchi et al., for cow's milk [18] with some variants specific for mare's milk and the ultracentrifugation method (Figure 2).

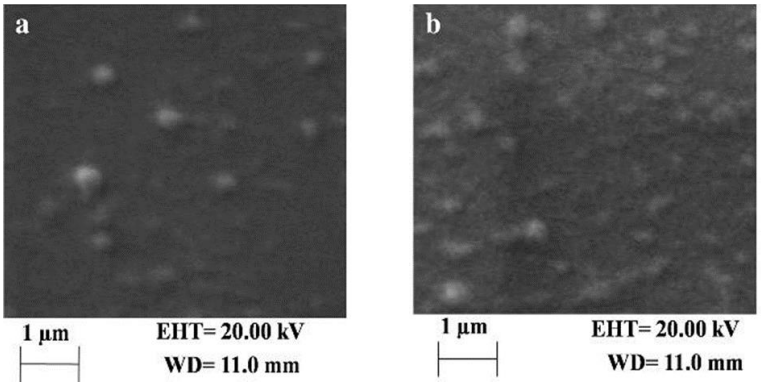

Figure 2. Mare's milk exosomes isolated by ultracentrifugation: (a). Mare's milk exosomes isolated using precipitation (b). Images obtained using scanning electron microscopy, magnification ×35000

In the isoelectric precipitation method, contaminating caseins were removed at the isoelectric point for caseins.

For encapsulation into exosomes, we selected quercetin as a sample of polyphenols. When encapsulating quercetin in exosomes isolated from horse milk, we followed the method described by R. Munagala, F. Aqq. Munagala, F.Aqil, J.Jeyabalan and R.C Gupta for encapsulating drug substances into exosomes from cow milk [19]. Quercetin loading is achieved by mixing the test agent (dissolved in ethanol) with exosome suspension in a 1:9 ratio at room temperature (22°C). 1 mg of quercetin was weighed and dissolved in 200 µL of DMSO containing 2% Tween-80, and then 1.5 mg of exosomes were added and incubated under continuous shaking at 4°C for 14 hours. The unbound substance was removed by low-speed centrifugation (10000×g) for 10 minutes, and the drug-loaded exosomes were collected by centrifugation at 135000×g for 2 hours.

The resulting complex of antioxidant and exosomes was washed with PBS to remove the unencapsulated substance. The concentration of quercetin was then determined in the supernatants after low-speed and high-speed centrifugation, in the supernatant after washing, and in the precipitate by high-performance liquid chromatography (HPLC).

The capacity and antioxidant loading efficiency of exosomes were calculated using the following equations: % loading of exosomes with the tested substance = (amount of antioxidant initially added to exosomes - amount of antioxidant in the supernatant after low- and high-speed centrifugation - amount of washed substance) × 100. Loading efficiency, % = amount of antioxidant loaded to exosomes × 100 / amount of antioxidant initially added to exosomes.

The in vitro release of quercetin from exosomes was investigated using the equilibrium dialysis method [19]. Exosomes containing the tested antioxidants were dialyzed against phosphate-salt buffer solution (PBS) for 1, 3, 8, 24 and 72 hours. Dialysis membranes (20/32-32/32, Chicago, USA) were used in the dialysis cell. After equilibrium was established, free quercetin concentrations were measured and the fraction of substance bound to exosomes was calculated. Residual drug levels were measured by HPLC.

The percentage of binding at each time interval was calculated using the following equation: concentration of antioxidant in dialysis bag - concentration in buffer / concentration in dialysis bag. Percentage of free antioxidant at each time interval: concentration in buffer / concentration of antioxidant in dialysis bag × 100. Percentage of antioxidant released cumulatively over 72 hours, % = amount of unbound antioxidant in buffer / amount of bound antioxidant contributed to the dialysis bag in exosomes × 100.

3. Results

When examining the stability of antioxidant retention in exosomes, we obtained a relative ratio (mV) for quercitin standard, 69.4; for quercetin in exosomes immediately after incorporation, 90.1; and for quercetin on day 12 after storage at -80°C, 90.4 (Figure 1).

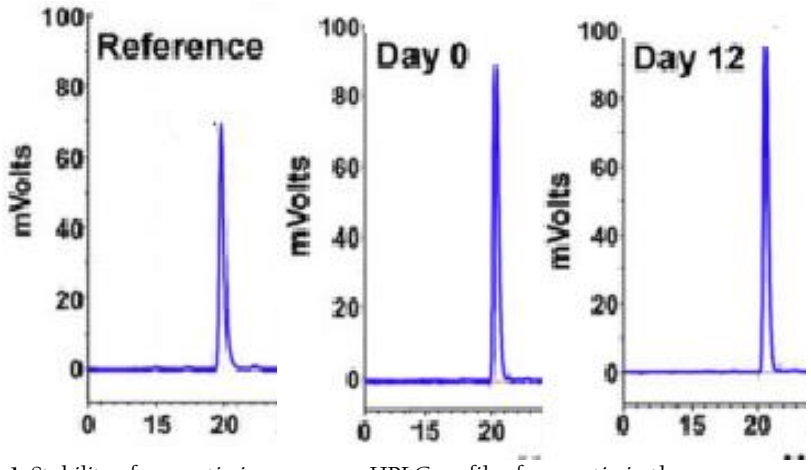

Figure 1: Stability of quercetin in exosomes. HPLC profile of quercetin in the exosomes up to day 12

We established arithmetic mean concentration values for quercitin:

1) Concentration of quercetin initially added to exosomes - 0.114 mg/mL;
2) Concentration of quercetin in the supernatant after centrifugation - 0.041 mg/mL;
3) Quercetin concentration in supernatant after washing - 0.017 mg/mL;
4) Quercetin concentration in exosome sediment - 0.063 mg/mL;

Based on the obtained data on quercetin concentration in test media, the percentage of quercetin loading of exosomes was calculated. Amount of quercetin initially added to exosomes (1.14 mg) - amount of quercetin in the supernatant after centrifugation (0.41 mg) - amount of washed quercetin (0.17 mg) × 100 = 56%. Quercetin loading efficiency of exosomes: amount of quercetin loaded into exosomes (0.63 mg) ×100 / amount of quercetin initially added to exosomes (1.14 mg) = 55%. The results obtained in the study of quercetin release from exosomes in vitro are presented in Table 1.

Table 1. Quercetin concentration level (mg/mL) in dialysis system (arithmetic mean of three repetitions)

Concentration in the dialysis bag	Dialysis duration, hrs	Quercitin concentration in buffer
0,055	0	-

0,052	1	0,007
0,033	3	0,020
0,019	24	0,038
0,016	72	0,037

Proportion of quercetin released cumulatively in 72 hours (% dissociation) = 100 × (amount of unbound antioxidant in buffer/number of bound antioxidant in exosomes introduced into the dialysis bag) = 67.3%.

4. Conclusions

The stability of quercetin in exosomes was confirmed during 12 days storage at -80°C.

The percentage of loading of exosomes with quercetin was 56%, and the efficiency of loading of exosomes with quercetin was determined at the level of 55%.The results obtained in the study of dissociation of antioxidants from exosomes in vitro, obtained using the method of equilibrium dialysis indicate a rather gradual course of the release process. The dissociation rate of quercetin total for 72 hours (% dissociation) was 67.3%.

Application of artificial intelligence:

The article is written without the use of artificial intelligence technologies.

Conflicts of Interest: The authors declare no conflict of interest.

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