Oral administration of quercetin-loaded mare's milk derived exosomes reversed age induced organ damage in rats

Shynggys Sergazy[1], Sanzhar Zhetkenev[2], Zarina Shulgau[2], Laura Chulenbayeva[2], Yevgeniy Kamyshanskiy[3], Madiyar Nurgaziyev[2], Ayaulym Nurgozhina[2], Zhanel Mukhanbetzhanova[2], Kulzhan Berikkhanova[2], Alexander Gulyayev[2], and Mohamad Aljofan[1]

[1]Nazarbayev University School of Medicine
[2]Nazarbayev University National Laboratory Astana
[3]Karaganda State Medical University

August 26, 2024

Abstract

Exosomes are cell derived and membrane-surrounded particles that deliver bioactive molecules to various cells. Their small size, low immunogenicity, extended blood circulation, and involvement in cellular communication make them a potentially effective drug carrier. Exosomes found in different biological fluids including mare's milk, a traditional drink in central Asia. Therefore, the aim of this study is to compare exosomes isolation methodology and determine the stability of mare's milk-derived exosomes as potential therapeutic carrier. Three extraction methods namely, immunoprecipitation, size exclusion chromatography, and total exosome isolation were compared in terms of exosome characteristics, purity, and content. The isolated exosomes then loaded with quercetin and their ability of increasing its bioavailability were tested in vitro and in vivo. Out of the three tested methods, total exosome isolation appeared to be the most efficient method that produced good quality exosomes, which were then loaded with quercetin and compared to free quercetin and exosomes only. Interestingly, exosomes loaded with 80μM quercetin significantly restored β-galactosidase activity and cellular viability in doxorubicin treated cells more than negative control and exosomes only, with a potency similar to that of 160μM free quercetin. Interestingly, aged model animals treated with exosomes loaded with quercetin showed significantly less frequent patterns of acute and subacute damage in the myocardium, kidneys, and liver compared to the untreated control group of aged models. The current study is a proof-of-concept that shows mare's milk-derived exosomes are able to be absorbed by cells and animal tissues, which support their potential use as drug carrier.

Oral administration of quercetin-loaded mare's milk derived exosomes reversed age induced organ damage in rats.

Shynggys Sergazy[1,*], Sanzhar Zhetkenev[1], Zarina Shulgau[1], Laura Chulenbayeva[1], Yevgeniy Kamyshanskiy[3], Madiyar Nurgaziyev[1], Ayaulym Nurgozhina[1], Zhanel Mukhanbetzhanova[1], Kulzhan Berikkhanova[1], Alexander Gulyayev[1], Mohamad Aljofan [1,2]

[1]*Private Institution, National Laboratory Astana, Nazarbayev University, Astana, Kazakhstan*

[2]*Department of Biomedical Science, Nazarbayev University School of Medicine, Astana, Kazakhstan*

[3]*Karaganda State Medical University, Karaganda, Kazakhstan*

**Correspondence to:*

Dr. Shynggys Sergazy, PhD

Address: National Laboratory Astana, Nazarbayev University

Kabanbay Batyr 53, Astana 010000, Republic of Kazakhstan

Email: shynggys.sergazy@gmail.com

Phone: +7 702 1999758

Abstract

Background and Purpose.

Exosomes are cell-derived, membrane-surrounded particles that deliver bioactive molecules to various cells. Due to their small size, low immunogenicity, extended blood circulation, and involvement in cellular communication, they hold potential as effective drug carriers. Exosomes are present in various biological fluids, including mare's milk, a traditional drink in Central Asia. This study aims to compare exosome isolation methodologies and determine the stability of mare's milk-derived exosomes as potential therapeutic carriers.

Experimental Approach.

Three extraction methods—immunoprecipitation, size exclusion chromatography, and total exosome isolation—were compared in terms of exosome characteristics, purity, and content. The isolated exosomes were then loaded with quercetin, and their ability to increase its bioavailability was tested in vitro and in vivo.

Key Results

Total exosome isolation was identified as the most efficient method, producing high-quality exosomes. These exosomes were loaded with quercetin and compared to free quercetin and exosomes alone. Exosomes loaded with 80 μM quercetin significantly restored β-galactosidase activity and cellular viability in doxorubicin-treated cells, exhibiting similar potency to 160 μM free quercetin. In aged model animals, treatment with quercetin-loaded exosomes resulted in significantly less acute and subacute damage to the myocardium, kidneys, and liver compared to untreated control animals.

Conclusions & Implications

This study provides proof-of-concept that mare's milk-derived exosomes can be effectively absorbed by cells and animal tissues, supporting their potential use as drug carriers.

Keywords

Exosomes; extracellular vesicles; quercetin; ageing; ROS

Introduction

Extracellular vesicles (EVs) including exosomes, microvesicles, and apoptotic vesicles, are membrane-bound small structures released from cells into the surrounding environment (1). They play essential roles in intercellular communication, particularly exosomes, which contain several constituents from the cells that secrete them, and appear to be involved in the pathogenesis of various disorders, including cancer, neurodegeneration, and inflammatory diseases (2). Interestingly, the biocompatibility of exosome, their circulating stability, and bioavailability in vivo, allowed them to gain increasing attention as an emerging drug delivery methodology over the last decade (3). They represent an important pathway to transfer information between cells, and thus, might be developed to package and deliver therapeutic molecules. Although exosomes are not nanoparticles derived from the nanotechnology due to its non-mankind nature, they may act as a Nano-carrier owing to their particle diameter (4), which is estimate to be between 30-100nm, and thus, can be used to load a variety of small bioactive molecules to improve bioavailability. Therefore, exosome's particle size allows them penetrate deep into the tissues and overcome barriers such as the blood-brain barrier and the deformable cytoskeleton. Another important character is that they have a slightly negative zeta potential, which guarantees their long circulation (5). In addition, some exosomes are capable of escaping from the immune system and have shown to have a low immunogenicity, and high stability in the blood, which prolongs drug circulation within the body (6).

Generally, exosomes are excreted from different types of cells and found abundantly in animal milk, which confirmed to contain relatively a stable structure and were shown to contain different miRNAs, including miRNA-148a-3p (7). Since bovine milk exosomes are protected from degradation by stomach acids (2, 8), milk exosomes are, therefore, considered to be useful for drug-delivery systems (DDSs), hence, milk exosome-encapsulated formulations can be used for therapeutic purposes (9). Therefore, milk-derived exosomes are seeing as promising new drug carriers for reaching distant tissues (10, 11).

Mare's milk is the national drink of the indigenous population in Central Asia, including Kazakhstan. Recent scientific data on the characteristics of the composition of horse milk and their potential properties that contribute to improving health have increased interest in this dietary source (12). Mare's milk shares some similarities to human breast milk and therefore, may have some valuable therapeutic properties (13). Mare's milk has a very good hygienic and sanitary status, differs from the milk of other farm animals in that it has the lowest somatic cell content and a very low total number of microorganisms (14). There are no studies examine the use of mare's milk-derived exosomes as a form of therapeutic carriers. Therefore, the current study aims to extract mare's milk-derived EVs, and isolate high content exosomes to test their suitability as a reliable form of therapeutic drug carrier. However, milk-derived EVs significantly different in size ranging from 30nm-10μm, and possibly contain various constituents, thus we do not exactly know which EVs extraction methods would allow us to have the optimum exosomal size. Thus, there is a need to compare and characterize different methods that allows isolation of the highest concentration of exosomes with the most homogenous shapes and sizes.

Materials and Methods

Mare's Milk purchase

Fresh mare's milk purchased from Saumal factory located at Karaganda region, Kazakhstan, and aliquoted in 50 ml tubes then stored at -80 ºC. To remove milk fat globules and cell debris, thawed samples were centrifuged at 10000 × g for 30 minutes at 4 ºC. To obtain large number of good quality exosomes, three exosome isolation methods were used.

Exosomes isolation methods

First method is Total Exosome Isolation (TEI), where milk samples centrifuged at 2000 × g for 30 min, then supernatant was collected and 2.5 mL of TTEI reagent was added (1:2 ratio), mixed well until homogeneity and incubated overnight at 4 ºC. The solution was then centrifuged at 10,000 × g for 1 hour at 4 °C. Supernatant was discarded; pellet and sediment exosomes were re-suspended in equal volume of 1 × PBS. The second method, isoelectrical precipitation (IP), which was performed according to Yamauchi M. et all (2018). Briefly, skimmed milk was diluted with distilled water, and pH was adjusted to 4.6. Samples were then centrifuged at 5100 × g for 20 min at 25 ºC, and supernatant was collected and filtered using 0.45 μm filters. The third isolation method was size exclusion chromatography (SEC) and performed according to manufactures instructions. This involves isolating exosomes from the supernatant of cell cultures and complex biological fluids. Since the separation is based on size, the vesicles pass through the column, are retained and eluted in the void. Proteins and other contaminants that are smaller than the pores are retained by the column and eluted later. Isolation using original size exclusion columns removes >99% of background protein contaminants and up to 95% of high-density lipoprotein contaminants from samples in a single isolation. Column qEV original 70 (IZON Science, New Zealand) that has an optimal recovery range from 70 nm to 1000 nm was used in the experiment. Columns were washed by filtered 1 × PBS, followed by a second wash containing 19 mL of filtered 1 × PBS and 0.5 mL of supernatant. A total of 5-10 concentrated fractions were collected for the study.

Characterization of exosomes

Transmission electron microscopy

Approximately, five μL of isolated exosomes using SEC, IP and TEI added onto a Carbon Film Supported Copper Grid (Sigma-Aldrich) for Transmission Electron Microscopy (TEM) analysis. The concentrated

samples, maintained in a sterile environment, and kept still in order to allow the liquid to evaporate. Samples then were fixated and characterized using JEM-1400 Plus electron microscope.

Nanoparticle determination

Exosome particle size determination was performed using Malvern Zetasizer Nano-ZS ZEN 3600 (Malvern Panalytical, UK). Approximately, 350µL of obtained exosomes were used in the experiment and measured at 25°C ambient temperature.

Protein determination

The Thermo Scientific Pierce BCA Protein Assay Kit was used to determined the protein concentrations in each of the exosome samples obtained using the different isolation methods, IP, TEI, and SEC. Bovine Serum Albumin (BSA) in 1% SDS was used as a standard with final concentrations of 2µM, 1µM, 0.5µM, 0.25 µM and 0.125µM. The BCA reagent (50 ml of solution A and 1 ml of solution B) was added into triplicates of exosome containing media from different isolation method. Series of standards and 10 µl of each sample added into a 96-well plate, followed by 190µl of 1% SDS and 200µl of BCA reagent. The plate was incubated for 30 minutes, and then read at wavelength of 562nm. BCA Total Protein Standard Curve is obtained and the protein concentrations were calculated. The Kruskall-Wallis test is performed to assess whether there is a statistically significant differences in medians of protein content in each of the media obtained by different methods.

Western Blot analysis

All of the following antibodies were purchased from ThremoFisher Scientific, USA; MFGE8 (Lactadherin) Monoclonal Antibody (EDM45), MFGE8 (Lactadherin) Polyclonal Antibody, and CD63 goat polyclonal antibody and used for Western Blot analyses. Samples containing EV pellets were suspend in PBS and diluted with lysis buffer, then were further mixed with 6x SDS sample buffer and placed at 95°C for 5 min. Gel (12% Mini-PROTEAN TGX Stain-Free, Bio-Rad, USA) run 20-25 minute at 90 V for stacking, then 1-1.5 hour at 110 V for separating. The proteins were transferred using Transfer-Blot Turbo Transfer System with Transfer pack (Bio-Rad). The membranes were blocked with 5% non-fat dried milk dissolved in TBST. The proteins were detected by incubation with secondary antibody conjugated with HRP (anti-rabbit IgG goat antibody, anti-rabbit IgG donkey antibody, and anti-goat IgG donkey antibody all by Invitrogen, USA). The membranes were washed thrice prior to imaging. Detected the peroxidase activity using a Clarity ^{TM}Western ECL substrate (Bio-Rad) and visualized using an ChemiDoc^{TM} Imaging system (Bio-Rad).

Exosome loading with quercetin

Approximately, 99ml of exosomes extracted from the mare's milk mixed with 14ml of quercetin dissolved in 1% DMSO solution to a achieve a final concentration of 160µM. The solution was left to mix with magnet stirrer for 24 hours. In order to get rid of unincorporated quercetin and purify the solution, IZON ASF and qEV original 70 (IZON Science, New Zealand) columns were used.

In vitro Assays

Cell cultures

Neonatal human dermal fibroblasts (Gibco) cells were cultured in DMEM medium high glucose with L-glutamine and with sodium pyruvate (Biowest, France), supplemented with 10% fetal bovine serum exosome-depleted (Gibco; Thermo Fisher Scientific, Inc.) and 1% Penicillin-Streptomycin solution (Sigma) in a 37°C, 5% CO^2 incubator (Binder CB-150, Binder, Germany). To induce aging, cells with 3µM of doxorubicin (DOX) for 3 hours, and then washed with HBSS. To determine the effect of exosomes, cells were incubated with exosomes alone from different isolation method (IP, SEC, or TEI) and loaded with quercetin and compared to quercetin only using different concentrations 20, 40, 80, and 160µM. For vehicle control cell were treated with 1% DMSO, and for negative control, cells were treated with 1 × PBS.

Cell proliferation assay

To determine cellular proliferation, MTT Cell Proliferation assay kit (10009365, Cayman, USA) was used. Briefly, cells were seeded in 96-well plate (10^5 cells/well) in 200µL DMEM medium for 24 h. Untreated cells were used as a negative control. After incubation 10µL of MTT reagent was added to each well, gently mixed for 1 minute on orbital shaker, and then incubated 3 hours at 37°C in a 5% CO^2. Further 100µL of crystal dissolving solution was added to each well to dissolve the formazan crystals, producing a purple solution, and incubated for 18 hours in a 37°C at CO2 incubator. The absorbance of each sample were measured at 570nm on BioTek Citation 5 Cell Imaging Multimode reader.

β-γαλαςτοσιδασε ασσαψ

β-galactosidase assay was performed using Mammalian β-Galactosidase Assay Kit (Thermo Fisher Scientific, Inc.). Cells were plated in 96-well plate for 24 hours, after that washed with 100µL PBS (pH 7.2). A 100µL of β-Galactosidase Assay Reagent was added to each well and incubated for 30 minutes at 37°C. The reaction was stopped by adding 100µL of β-Galactosidase Assay Stop Solution. Absorbance of cells were measured at 405nm on BioTek Citation 5 Cell Imaging Multimode reader.

Animal experimentations

Efficacy of quercetin-loaded exosomes from mare's milk compared to free quercetin in an aging male rat model

The efficiency of quercetin-loaded exosomes derived from mare's milk versus free quercetin were evaluated using an aging model of male Wistar outbred rats. Experimental groups consisted of males older than 12 months, with young male Wistar rats aged 8 weeks serving as the negative control. On average, the rats weighted 200 ± 20 g and housed in the animal facility of the National Center for Biotechnology, Astana, Kazakhstan. After one-week adaptation period, the rats were randomly divided into their groups (3 rats/cage) and housed in a room with controlled temperature and a 12-h light–dark cycle with unlimited access to standard food and drinking water ad libitum.

Experimental design

The experiment was conducted on total of fifteen male rats (12 months old as ageing sample, N=12), plus young rats as a control (N=3), divided into five groups of three rats per group as follows: Group 1 (exosomes only group) contains old rats that were administered intragastrically with empty exosome from mare's milk once daily for a week. Group 2 (quercetin-loaded exosomes) has old rats administered intragastrically with quercetin-loaded exosomes from mare's milk (quercetin concentration 2 mg/ml, volume administered was 0.5ml per rat, equating to 1mg of quercetin per rat) once daily for a week. Group 3 (quercetin only) has old rats administered intragastrically with free quercetin (quercetin concentration 2 mg/ml, volume administered was 0.5 ml per rat, equating to 1 mg of quercetin per rat) once daily for a week. Group 4 (control- untreated old rats) contains old rats that were administered intragastrically with drinking water (manipulation control) at a volume of 1 ml per rat. The fifth group (control- untreated young rats) has young rats that were administered intragastrically with drinking water once daily for a week. On the conclusion of the experiment day 8, animals were killed and three major organs were harvested for analysis including the heart, kidneys, and liver. Organs were preserved by immersion in 10% neutral buffered formalin.

Histopathological Examination

Collected tissues were fixed and sectioned using a standard protocol developed by the Veterinary Diagnostic Laboratory at Washington University (15), ensuring each animal was examined for the same organ slice. After sectioning, the samples underwent processing with isopropyl alcohol, xylene, and paraffin using a tissue processor. Once placed in the histo-cassettes, the material was embedded in paraffin forming blocks for histological sectioning. Histological sections of approximately, 3µm were stained with hematoxylin and eosin to determine the general morphological pattern and cellular infiltration in the heart, kidneys, and liver. Also, Masson's trichrome staining to identify collagen fibers, staining blue, and connective tissue in the heart and liver. To detect mucopolysaccharide accumulation in the mesangium and basal membranes of the glomeruli and tubules of the kidneys and for assessing glycogen accumulation in hepatocytes, tissue were stained with periodic acid-Schiff (PAS). Sections were examined using light microscopy using a Zeiss AxioLab

4.0 microscope at magnifications of ×40, ×100, ×200, and ×400. AxioVision 7.2 software for Windows was used for image capturing.

Morphometric Study

The morphometric study was conducted by two independent histopathologists experienced in animal models. They were not aware of which animal group the images come from and analyzed each criterion separately. Lesions identified in each organ were described according to the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) (16, 17).

A semiquantitative scoring analyses of electron microscope images was used to evaluate the degree of ultrastructural damage, as previously described by Sergazy et al., 2020, which utilizes a four-point scale (Grade 1–4) for the morphological assessment of subcellular structures (18, 19). In brief, slides assessed for different morphologic changes, and lesions were classified as present/absent on a scale from 0 to 1, or assessed for severity on a scale from 0 to 4. Histopathological lesions of the heart evaluated as present – "1" or absent – "0", included cardiomyocyte hypertrophy, focal hyper-eosinophilia, enlargement of cardiomyocyte nuclei/multinucleated cardiomyocytes, cellular infiltrate, and atherosclerosis. Fibrosis (perivascular and interstitial) was assessed on a scale: "0" – none, "1" – minimal changes, "2" – mild changes, "3" – moderate changes, "4" – severe lesions. Kidney lesions evaluated as present – "1" or absent – "0", included tubular epitheliocyte vacuolization and hyperplasia, formation of protein cylinders in tubules, interstitial cellular infiltrate, thickening and fibrosis of glomerular membranes, hyaline glomerulopathy, and glomerulosclerosis. Liver lesions evaluated as present – "1" or absent – "0", included focal hepatocyte vacuolization, presence of hyperchromatic/double-nucleated hepatocytes, lymphocytic infiltration, hyperplasia (cysts) of bile ducts, and periportal fibrosis. Glycogen accumulation was differentiated on a scale from 0 to 3: "0" – less than 10% of hepatocytes, "1" – 11-30% of hepatocytes, "2" – 31-60%, "3" – more than 61% of hepatocytes.

Statistical analysis

All data are expressed as mean +- standard error of the mean (S.E.M.) unless stated otherwise. Data were analyzed using one-way analysis of variance (ANOVA) and Student t -tests. Results with p -values less than 0.05 ($p < 0.05$) were considered statistically significant.

Ethics approval

The animal study was reviewed and approved by Local Ethics Commission of "National Center for Biotechnology" (IRB00013497 National Center of Biotechnology IRB #1) on 7 August 2020 (Protocol No. 3). The reporting in this study is in accordance with guidelines published by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (ARRIVE guidelines).

Results

Comparison of exosome isolation methods

A comparison of the three different methods of exosome isolation performed to evaluate the morphology, properties of the obtained exosomes and their applicability as drug carriers. The morphology assessment of the isolated exosomes performed using TEM, shows that exosomes isolated using the different methods have morphologically dense vesicular structure indicated with arrows (Figure 1). It can be clearly observed that some debris can be observed in EVs obtained by IP and SEC, but not the in the EVs isolated using TEI method, which might be due to the PBS dilution during the isolation process. Nanoparticle tracking illustrating the size distribution of the particles shows the distribution of particles close to 100 nm (Figure 2). Particles isolated by SEC showed less variation in particle distribution than IP and TEI. No significant difference in the mean size of exosomes across the different isolation methods SEC, IP and TEIK (110nm, 118nm and 131.5nm, respectively).

Western blot using the membrane markers, MEFG-8 monoclonal, MEFG-8 polyclonal and CD63 antibodies performed to measure confirm the identity of exosomes present in each of the isolates (Figure 3). The protein content in IP isolate is shown to be the highest as measured by MEFE-8 and CD63 antibodies.

The TEI showed a significant band against MEGD-8 monoclonal antibody, but no significant differences in other antibodies tested in comparison with SEC. However, for protein content, isolates from TEI showed the highest, followed by IP and then SEC (Figure 4). Protein contents from TEI method (mean protein concentration of 0.1914) are significantly higher (p= 0.012) than the negative control PBS (mean protein concentration is 0.0085 mg/ml).

Therapeutic cargo properties of isolated exosomes

The cargo properties of the isolated exosomes from different methods were investigated using quercetin-loaded exosome mixtures on pre-cultured and doxorubicin-induced senescent neonatal human dermal fibroblasts. The results show no significant differences in relative β-galactosidase activity between 160μM of free quercetin compared to 80μM quercetin loaded exosomes isolated by SEC (P>0.05) and TEI (P>0.05) (Figure 5), but significantly higher than the quercetin loaded with IP and exosomes alone. This indicates the potential increase (almost doubling) of the intracellular concentration of quercetin loaded in exosomes isolated by SEC and TEI in par with that of 160μM free quercetin. Compared to negative control, all isolated EVs loaded with quercetin significantly increased cell viability (Figure 6). However, quercetin loaded into TEI isolated exosomes showed a significantly higher cell viability percentage than the other two isolation methods. The viability is similar to that of the 160μM free quercetin and that of 150μM resveratrol, a potent antioxidant used as a positive control in this experiment. This finding confirms the concept of exosome's ability in increasing bioavailability as well as the reliability of TEI as the most suitable exosome extraction method. Therefore, TEI extraction method was selected as the most suitable method and, thus, used for the subsequent in vivo testing.

Organ histological and histochemical analysis

Heart:

The analysis of the histopathological characteristics of the control untreated group of young male rats showed that the myocardium structure of both the left and right ventricles corresponded to the histological norm. With hematoxylin and eosin staining, the histoarchitecture of the cardiac muscle in all histological sections of all animals was preserved (Table 1). Rows of cardiomyocytes without hypertrophy, significant eosinophilia, or cross striation were identified, with a centrally located basophilic oval vesicular nucleus (Figure 7). With Masson's trichrome staining, a small amount of collagen fibers was observed in the perivascular space, predominantly located in the vessel wall (Table 1). Collagen fibers were mostly absent in the interstitial space. In individual fields of view of some sections, threadlike thin and short blue collagen fibers were noted (Figure 7).

In the control group of old untreated rats, all animals exhibited bundles of hypertrophied cardiomyocytes, the presence of pyknotic nuclei, focal hyper-eosinophilia of cardiomyocytes, as well as increased size of cardiomyocyte nuclei and the appearance of multinucleated cardiomyocytes (Figure 7). In some fields of view, individual nuclei were enlarged, from oval to round, hyperchromatic, and had a dense central longitudinal septum (Figure 7), and atherosclerotic changes were identified in the walls of large arteries (Figure 7). One rat, isolated mononuclear cells were identified in the myocardium (Figure 7).

With Masson's trichrome staining, collagen fibers in both the left and right ventricular wall were noted in the perivascular zone and interstitial space, arranged chaotically and disorderly (Table 1). Thick and twisted dark-blue collagen fibers in the myocardial interstitial space formed complex anastomoses. A minimal degree of perivascular fibrosis was found in one animal, and a mild degree was found in two of the animals (Figure 7). Mild interstitial fibrosis was observed in all the animals in this group (Figure 7, Table 1).

In the exosome only group, all the rats exhibited cardiomyocyte hypertrophy, bundles of large longitudinally arranged hypertrophied heart muscle cells with large basophilic reticular nuclei

(Figure 7). In addition, two rats showed focal hyper-eosinophilia and increased size of cardiomyocyte nuclei with the appearance of multinucleated cardiomyocytes were observed, with one animal exhibiting perivascular lymphoplasmacytic infiltration (Figure 7). Mild atherosclerotic changes in large vessels were observed in all cases. A minimal perivascular fibrosis observed in one of the animals and the other two rats showed mild cases of perivascular fibrosis (Figure 7). Interestingly, mild form of interstitial fibrosis (Figures 7) was observed in all three animals in this group (Table 1).

All of the three rats in the experimental group that received quercetin loaded exosomes showed increase in the size of cardiomyocyte nuclei with the appearance of multinucleated cardiomyocytes, two exhibited cardiomyocyte hypertrophy and focal hyper-eosinophilia, and one animal displayed perivascular lymphocytic infiltration (Table 1). Focal myocytolysis was also observed: some cardiomyocytes appeared "empty," i.e., without nuclei and cross striation, and their cytoplasm stained more intensely eosinophilic (Figure 7). Mild myocardial artery atherosclerosis and interstitial fibrosis were observed in all animals, and a minimal degrees of perivascular fibrosis was found in one and a mild form in another (Table 1). However, all of the animals in the quercetin only group exhibited cardiomyocyte hypertrophy; increased size of cardiomyocyte nuclei with the appearance of multinucleated cardiomyocytes, mild myocardial artery atherosclerosis, and mild interstitial fibrosis (Table 1). One of the animals in this group had focal hyper-eosinophilia, and using Masson's trichrome staining, one animal showed minimal perivascular fibrosis, and the other two showed mild perivascular fibrosis (Table 1).

Kidneys

The comparative histomorphometric characteristics of kidney of the untreated control group of young male rats, showed normal histological structures of the renal cortex and medulla (Table 2). Hematoxylin and eosin staining revealed a normal kidney structure with Malpighian bodies consisting of a glomerulus surrounded by Bowman's capsule. Proximal convoluted tubules with a narrow lumen were lined by pyramidal epithelial cells with acidophilic cytoplasm and basal round vesicular nuclei. Distal convoluted tubules were lined by a relatively larger number of cuboidal epithelial cells with light acidophilic cytoplasm and central round vesicular nuclei (Table 2).

Periodic Acid-Schiff (PAS) histochemical staining identified light PAS staining (purple color) of the glomerular mesangial matrix and basal membranes, minimal PAS staining of tubular basal membranes and tubular casts, with no glomerulosclerosis (Figure 8).

In the control group of old untreated male rats, all animals exhibited progressive age-related nephropathy, including regenerative hyperplasia of tubular epithelial cells and formation of protein casts (Figure 8). Additionally, aging was associated with increased lymphoplasmacytic infiltration in the interstitium of the kidneys. No histopathological signs of tubular epithelial vacuolization were detected in any of the animals in this group.

Sections stained with Periodic Acid-Schiff (PAS) revealed a PAS-positive reaction (staining purple-red) in the basal membranes and areas of thickened Bowman's capsule in all animals, an increase in the number of PAS-stained glomeruli with thickened mesangial matrix and basal membranes, glomerulosclerosis, tubular casts, and PAS-positive thickened tubular basal membranes (Figure 8).

All of the exosomes only group, showed hyperplasia of tubular epitheliocytes, formation of protein casts, presence of lymphoid infiltrates in the renal interstitium, but no tubular vacuolization (Figure 8). Sections stained with Periodic Acid-Schiff (PAS) revealed thickening and fibrosis of the glomerular basal membranes and glomerulosclerosis in all animals (Table 2). Notably, pronounced eosinophilic infiltration in the kidney capsule was observed in all animals (Table 2), but focal hyalinosis was observed in two animals only (Table 2).

Interestingly, animals in the quercetin loaded exosomes, showed no tubular epithelial vacuolization, but two animals, showed hyperplasia of tubular epitheliocytes, focal cellular infiltration in the renal interstitium, predominantly consisting of lymphatic and plasma cells, thickening and fibrosis of glomerular membranes and focal hyalinosis (Table 2). Formation of protein casts, glomerulosclerosis, and eosinophilic infiltration were observed in the kidney capsules of all 3 animals (Table 2).

Animals in the free quercetin treated group, showed no tubular, but two animals exhibited hyperplasia of tubular epitheliocytes and formation of protein casts, and one other animal in the group showed cellular infiltrates predominantly consisting of lymphatic and plasma cells (Table 2). Periodic Acid-Schiff (PAS) histochemical staining identified glomerulosclerosis in all animals, focal hyalinosis in one, and in two animals, edema, thickening, and fibrosis of glomerular basal membranes, sclerosis, and hyalinosis were detected (Figures 8).

Liver

The comparative histopathological characteristics of age-related changes in the liver in the studied groups of rats are presented in Table 3. In liver sections of the control group of young male rats, all animals had liver architecture corresponding to the histological norm. With hematoxylin and eosin staining, the liver consisted of numerous hepatic lobules formed by cords of hepatocytes radiating outward from the central vein to the lobule periphery, separated by sinusoids (Table 3). Hepatocytes had a polygonal shape with eosinophilic granular cytoplasm and vesicular-basophilic nuclei. Masson's trichrome staining showed collagen fibers in the walls of the portal tract vessels but absent in the extracellular matrix (Table 3). Histochemical staining (PAS) revealed glycogen in the form of violet-purple fragments in the cytoplasm of most hepatocytes in all animals (Figure 9).

Animals in the old untreated group, showed evidence of age-related changes with hematoxylin and eosin staining including; hepatocyte vacuolization, inflammatory/immune changes with lymphocyte and plasma cell infiltration (Figure 9 or Table 3). Hepatic sections of all animals in the group were characterized by microvesicular vacuolization of hepatocyte cytoplasm (Table 3). Two out of three animals in the group, displayed some hepatocytes contained nuclei of various shapes and sizes, and binucleated hepatocytes in some fields (Figure 9). No signs of central venous congestion were observed in any animal in the group. Portal tracts in all three animals of the group were characterized by dilated and congested portal veins and hyperplasia (cysts) of bile ducts, and an increase in the number of lymphocytes and plasmacytic cells infiltrating between hepatocytes in the portal areas was observed (Table 3). Masson's trichrome staining showed proliferation of collagen fibers in the portal tracts with individual thick and long collagen fibers in the extracellular matrix of all animals (Table 3). Histochemical staining (PAS) showed that most hepatocytes were PAS-negative or contained a few scattered glycogen granules of violet-purple color in less than 10% of hepatocytes in all rats (Figure 9).

However, focal microvesicular vacuolization of hepatocytes and focal lymphocytic infiltration were observed in all animals in the exosome only group. Cellular pleomorphism, part of hepatocytes contained nuclei of variable size and shape, as well as binucleated hepatocytes and hyperplasia (cysts) of bile ducts observed in two of the animals in the group (Table 3). Masson's trichrome staining in all animals showed perioportal proliferation of collagen fibers (Table 3). Histochemical staining (PAS) showed that most hepatocytes were PAS-negative or contained a small amount of glycogen of violet-purple color, and in one animal glycogen was present in less than 61% of hepatocytes (Table 3) and (Figure 9).

Interestingly, all animals in the group that was treated with quercetin loaded exosomes exhibited focal microvesicular vacuolization of hepatocytes, focal lymphocytic infiltration, and hyperplasia or cysts of bile ducts (Table 3). Two of the animals showed abnormalities in nuclear size and chromatin distribution, and binucleated hepatocytes. Masson's trichrome staining in all animals showed perioportal proliferation of collagen fibers (Table 3). Histochemical staining (PAS) in histological liver sections of two animals revealed glycogen in less than 10% of hepatocytes, and in one case – in 11-30% of hepatocytes (Table 3). While

two of the animals that received free quercetin, exhibited focal microvesicular vacuolization of hepatocytes, binucleated hepatocytes, lymphoplasmacytic infiltration, and hyperplasia (cysts) of bile ducts, all of animals exhibited perioportal fibrosis (Table 3). Glycogen in all animals was detected in less than 10% of hepatocytes (Table 3).

Discussion

The current study investigated the feasibility of isolating milk-derived exosomes from mare's milk derived EVs and tested their suitability as therapeutic carriers. There are other milk and dietary derived EVs available, but this study focuses on testing the concept of mare's milk, a widely consumed milk in central Asia. Different exosome isolation methods were used and compared based on the presence of exosomal markers confirming particle isolation using immunoblotting, particle size as confirmed by proteomic profile (Zetasizer), exosome morphology using TEM, and protein concentration (bicinchoninic acid assay, BCA). There are a number of methods available to extract milk-derived exosomes including centrifugation, and ultracentrifugation- density gradient (20), but this study aims to find a cheap, easy to use and reliable extraction method. Thus, out of the three tested methods IP, SEC, TEI, the latter proved to be the most suitable methodology capable of isolating high quantity and good quality exosomes.

The suitability of mare's milk-derived exosomes as therapeutic carriers was tested in terms of an increase in the therapeutic effects compared to free drug (quercetin only). For example, quercetin loaded exosomes showed significantly higher antioxidant activity compared to quercetin alone in doxorubicin treated cells, demonstrating the potential of exosomes in improving drug bioavailability in vitro. This is due to their ability to easily pass through biological barriers, such as blood-brain barrier, intestinal and placental barriers (21), which allows them to play an important role in cellular communication and enable them to deliver their mRNA, miRNAs, proteins, and small molecules into other cells (22).

This theory was further supported in the animal work, where exosomes isolated from mare's milk were loaded with quercetin and administered to ageing rats. The analysis showed that exosomes loaded with quercetin has more bioavailability than quercetin alone and that they were efficiently absorbed in different organs. This finding is in an agreement with previous reports that showed the use of milk-derived exosomes as Nano-carriers increased drug bioavailability. For example, Munagala et al. 2016, reported that milk-derived exosomes loaded with withaferin A significantly enhanced the tumor reduction of the drug (23), as well as Aqil et al., 2016, which showed the use of exosomes increased the therapeutic response of Celastrol against lung cancer (24).

Interestingly, a range of histopathological changes identified in the heart, liver, and kidney sections of old rats across all groups compared to the group of young rats. These changes included cellular damage (tinctorial and morphological), tissue structure alterations, and cellular infiltrate associated with organismal aging. For example, in the heart, the most common age-related lesion observed was fibrosis with loss and hypertrophy of cardiomyocytes. The kidneys exhibited focal chronic progressive glomerulonephropathies, including mild sclerosis of the capsule and mesangium of glomeruli. The liver cells displayed widespread variations in the size of hepatocyte nuclei, chromatin distribution within the nucleus, vesicular nuclei, basophilic, fine-grained or vacuolated cytoplasm. The observed damages in the old animals were of acute, subacute and chronic damages. While the latter is consistent with age-related damage (25), the acute and subacute are the results of doxirubucin treatment, confirming the success of the establishing an ageing model using this drug.

However, animals treated with exosomes loaded with quercetin showed significantly less frequent patterns of acute and subacute damage in the myocardium, kidneys, and liver compared to the control group of old males without treatment ($p<0.05$). These findings observed in the exosomes loaded with quercetin treatment group, but not with quercetin only or exosomes only groups. While, this might be used as a supporting evidence for the suitability of mare's milk-derived exosomes as potential drug carriers, it must be interpreted with caution, as exosomes alone were reportedly involved in regulating oxidative stress (OS) (26). A number of studies indicated that exosomes might not only transport proteins, RNA, and other molecules, but also participate in OS-related conditions, such as ischemia–reperfusion, atherosclerosis, and cardiac remodeling

by reducing reactive oxygen species (ROS) through inhibiting protein synthesis and mRNA degradation (27, 28). For instance, Wang et al., 2019, reported that mir-126 derived from exosomes reduced apoptosis and lessened OS in ischemia and reperfusion injuries (29).

However, the amounts of proteins, mRNA, and RNA found in the exosomes as well as their therapeutic potential depend on the source of the exosomes (e.g. milk) and the isolation methodology used. There were approximately 200 different proteins found in dendritic cell derived exosomes extracted using crude preparation (30), and more than 2000 different proteins, including major protein markers, identified from bovine milk-derived exosomes isolated by centrifugation (31). While, the protein components of the isolated exosomes were not analyzed as this is beyond the scope of the current study, we can assume that no proteins or any other component was involved in OS. Thus, it is safe to assume that due to their ability in crossing biological barriers, mare's milk-derived exosomes increased the bioavailability of quercetin, and hence, they might be used as a reliable form of a therapeutic carrier to improve drug delivery.

Nevertheless, the findings confirm the absorption of exosomes by different organs and show an increase of quercetin bioavailability at the target site. However, was the observed effect due to the increased bioavailability of quercetin? Alternatively, did the drug and the exosome achieve it synergistically? Further research is needed to determine whether synergistic effects between exosome and quercetin exist. It would also be interesting to investigate the roles of exosomes isolated from mare's milk alone, or their components in different pathologies with or without drugs. This will likely shed more lights on their biological roles.

In conclusion, the current study supports the concept of using exosomes as a potentially reliable form of a drug carrier. The results demonstrated that mare's milk-derived exosomes, can easily be absorbed by different tissues, and that their use as a drug carrier, presumably, increased the bioavailability of quercetin. However, follow up studies are needed to identify the components of mare's milk-derived exosomes such as proteins, mRNA and miRNA, as well as to determine their biological roles.

Data Availability Statement. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests' Statement. None

Funding. This research was funded by the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan (Grant No. AP13067844) and Nazarbayev University Collaborative Research Program 2021–2023 (Award no.OPCRP2021006).

Conflict of Interest. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

References

1. Izumi H, Tsuda M, Sato Y, Kosaka N, Ochiya T, Iwamoto H, et al. Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. J Dairy Sci. 2015;

2. Izumi H, Kosaka N, Shimizu T, Sekine K, Ochiya T, Takase M. Bovine milk contains microRNA and messenger RNA that are stable under degradative conditions. J Dairy Sci. 2012;

3. Zempleni J, Sukreet S, Zhou F, Wu D, Mutai E. Milk-Derived Exosomes and Metabolic Regulation. Annu Rev Anim Biosci. 2019;

4. Golan-Gerstl R, Elbaum Shiff Y, Moshayoff V, Schecter D, Leshkowitz D, Reif S. Characterization and biological function of milk-derived miRNAs. Mol Nutr Food Res. 2017;

5. Reif S, Elbaum Shiff Y, Golan-Gerstl R. Milk-derived exosomes (MDEs) have a different biological effect on normal fetal colon epithelial cells compared to colon tumor cells in a miRNA-dependent manner. J Transl Med. 2019;

6. Trams EG, Lauter CJ, Norman Salem J, Heine U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. BBA - Biomembranes. 1981;

7. Vader P, Mol EA, Pasterkamp G, Schiffelers RM. Extracellular vesicles for drug delivery. Advanced Drug Delivery Reviews. 2016.

8. Malhotra H, Sheokand N, Kumar S, Chauhan AS, Kumar M, Jakhar P, et al. Exosomes: Tunable nano vehicles for macromolecular delivery of transferrin and lactoferrin to specific intracellular compartment. J Biomed Nanotechnol. 2016;

9. Hood JL. Post isolation modification of exosomes for nanomedicine applications. Nanomedicine. 2016.

10. Pieters BCH, J.Arntz O, Bennink MB, Broeren MGA, Van Caam APM, Koenders MI, et al. Commercial cow milk contains physically stable extracellular vesicles expressing immunoregulatory TGF-β. PLoS One. 2015;

11. Groenen MAM, van der Poel JJ. Regulation of expression of milk protein genes: a review. Livest Prod Sci. 1994;

12. Munagala R, Aqil F, Jeyabalan J, Gupta RC. Bovine milk-derived exosomes for drug delivery. Cancer Lett. 2016;

13. Betker JL, Angle BM, Graner MW, Anchordoquy TJ. The Potential of Exosomes From Cow Milk for Oral Delivery. J Pharm Sci. 2019;

14. Vaswani K, Koh YQ, Almughlliq FB, Peiris HN, Mitchell MD. A method for the isolation and enrichment of purified bovine milk exosomes. Reprod Biol. 2017;

15. Snyder JM, Ward JM, Treuting PM. Cause-of-Death Analysis in Rodent Aging Studies. Vet Pathol. 2016 Mar;53(2):233-43. doi: 10.1177/0300985815610391. Epub 2015 Oct 27. PMID: 26508696.

16. Keenan CM, Baker J, Bradley A, Goodman DG, Harada T, Herbert R, Kaufmann W, Kellner R, Mahler B, Meseck E, Nolte T, Rittinghausen S, Vahle J, Yoshizawa K. International Harmonization of Nomenclature and Diagnostic Criteria (INHAND): Progress to Date and Future Plans. Toxicol Pathol. 2015 Jul;43(5):730-2. doi: 10.1177/0192623314560031. Epub 2014 Dec 21. PMID: 25530274.

17. Snyder JM, Snider TA, Ciol MA, Wilkinson JE, Imai DM, Casey KM, Vilches-Moure JG, Pettan-Brewer C, Pillai SPS, Carrasco SE, Salimi S, Ladiges W. Validation of a geropathology grading system for aging mouse studies. Geroscience. 2019 Aug;41(4):455-465. doi: 10.1007/s11357-019-00088-w. Epub 2019 Aug 29. PMID: 31468322; PMCID: PMC6815299.

18. Sergazy S, Shulgau Z, Fedotovskikh G, Chulenbayeva L, Nurgozhina A, Nurgaziyev M, Krivyh E, Kamyshanskiy Y, Kushugulova A, Gulyayev A, Aljofan M. Cardioprotective effect of grape polyphenol extract against doxorubicin induced cardiotoxicity. Sci Rep. 2020 Sep 7;10(1):14720. doi: 10.1038/s41598-020-71827-9. PMID: 32895481; PMCID: PMC7477547.

19. Sergazy S, Shulgau Z, Kamyshanskiy Y, Zhumadilov Z, Krivyh E, Gulyayev A, Aljofan M. Blueberry and cranberry extracts mitigate CCL4-induced liver damage, suppressing liver fibrosis, inflammation and oxidative stress. Heliyon. 2023 Apr 17;9(4):e15370. doi: 10.1016/j.heliyon.2023.e15370. PMID: 37128328; PMCID: PMC10147973.

20. Sedykh S, Kuleshova A, Nevinsky G. Milk Exosomes: Perspective Agents for Anticancer Drug Delivery. Int J Mol Sci. 2020 Sep 11;21(18):6646. doi: 10.3390/ijms21186646. PMID: 32932782; PMCID: PMC7555228.

21. Kim MS, Haney MJ, Zhao Y, Mahajan V, Deygen I, Klyachko NL, Inskoe E, Piroyan A, Sokolsky M, Okolie O, Hingtgen SD, Kabanov AV, Batrakova EV. Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. Nanomedicine. 2016 Apr;12(3):655-664. doi: 10.1016/j.nano.2015.10.012. Epub 2015 Nov 14. PMID: 26586551; PMCID: PMC4809755.

22. Sun Z, Wang L, Zhou Y, Dong L, Ma W, Lv L, Zhang J, Wang X. Glioblastoma Stem Cell-Derived Exosomes Enhance Stemness and Tumorigenicity of Glioma Cells by Transferring Notch1 Protein. Cell Mol Neurobiol. 2020 Jul;40(5):767-784. doi: 10.1007/s10571-019-00771-8. Epub 2019 Dec 18. PMID: 31853695.

23. Munagala R, Aqil F, Jeyabalan J, Gupta RC. Bovine milk-derived exosomes for drug delivery. Cancer Lett. 2016 Feb 1;371(1):48-61. doi: 10.1016/j.canlet.2015.10.020. Epub 2015 Nov 18. PMID: 26604130; PMCID: PMC4706492.

24. Aqil F, Kausar H, Agrawal AK, Jeyabalan J, Kyakulaga AH, Munagala R, Gupta R. Exosomal formulation enhances therapeutic response of celastrol against lung cancer. Exp Mol Pathol. 2016 Aug;101(1):12-21. doi: 10.1016/j.yexmp.2016.05.013. Epub 2016 May 24. PMID: 27235383.

25. Shubin AV, Demidyuk IV, Komissarov AA, Rafieva LM, Kostrov SV. Cytoplasmic vacuolization in cell death and survival. Oncotarget. 2016 Aug 23;7(34):55863-55889. doi: 10.18632/oncotarget.10150. PMID: 27331412; PMCID: PMC5342458.

26. Zhang C, Wang J, Ma X, Wang W, Zhao B, Chen Y, Chen C, Bihl JC. ACE2-EPC-EXs protect ageing ECs against hypoxia/reoxygenation-induced injury through the miR-18a/Nox2/ROS pathway. J Cell Mol Med. 2018 Mar;22(3):1873-1882. doi: 10.1111/jcmm.13471. Epub 2018 Jan 24. PMID: 29363860; PMCID: PMC5824419.

27. Poe AJ, Knowlton AA. Exosomes as agents of change in the cardiovascular system. J Mol Cell Cardiol. 2017 Oct;111:40-50. doi: 10.1016/j.yjmcc.2017.08.002. Epub 2017 Aug 3. PMID: 28782514; PMCID: PMC5600701.

28. Jansen F, Nickenig G, Werner N. Extracellular Vesicles in Cardiovascular Disease: Potential Applications in Diagnosis, Prognosis, and Epidemiology. Circ Res. 2017 May 12;120(10):1649-1657. doi: 10.1161/CIRCRESAHA.117.310752. PMID: 28495995.

29. Wang W, Zheng Y, Wang M, Yan M, Jiang J, Li Z. Exosomes derived miR-126 attenuates oxidative stress and apoptosis from ischemia and reperfusion injury by targeting ERRFI1. Gene. 2019 Mar 30;690:75-80. doi: 10.1016/j.gene.2018.12.044. Epub 2018 Dec 28. PMID: 30597234.

30. Morelli AE, Larregina AT, Shufesky WJ, Sullivan ML, Stolz DB, Papworth GD, Zahorchak AF, Logar AJ, Wang Z, Watkins SC, Falo LD Jr, Thomson AW. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. Blood. 2004 Nov 15;104(10):3257-66. doi: 10.1182/blood-2004-03-0824. Epub 2004 Jul 29. PMID: 15284116.

31. Reinhardt TA, Lippolis JD, Nonnecke BJ, Sacco RE. Bovine milk exosome proteome. J Proteomics. 2012 Feb 16;75(5):1486-92. doi: 10.1016/j.jprot.2011.11.017. Epub 2011 Nov 23. PMID: 22129587.

Hosted file

`Figures.docx` available at https://authorea.com/users/819356/articles/1218502-oral-administration-of-quercetin-loaded-mare-s-milk-derived-exosomes-reversed-age-induced-organ-damage-in-rats

Hosted file

`Tables.docx` available at https://authorea.com/users/819356/articles/1218502-oral-administration-of-quercetin-loaded-mare-s-milk-derived-exosomes-reversed-age-induced-organ-damage-in-rats