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ISOLATION AND CHARACTERISATION OF BACTRIAN CAMEL MILK-DERIVED EXOSOMES

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ABSTRACT

The morphological features were identified by TEM and total exosome protein concentration was determined by the BCA Protein assay. Under the electron microscope, it was observed that Bactrian camel milk exosomes were a typical vesicle enrichment with a large bilayer lipid membrane structure and the size of 30-200 nm round or oval membranous vesicles in the shape of a cup holder. The centre was evenly dyed black, the film was lightly dyed white and the edges were clear. The total protein concentration of the exosome extract measured by the BCA Protein assay was 18.6404 ± 1.7297 μg/μL. The study provided new ideas for exploring the biological function and principle of action of biological nano-vehicles in Bactrian camel milk.

Key words: Bactrian camel, exome, milk, TEM

Exosomes are extracellular vesicles (EVs) secreted by cells with a diameter of 30-150nm (Beuzelin and Kaeffer, 2018). It is a carrier for transmitting biological information such as nucleic acids, lipids and proteins and can transport various biologically active molecules. Recipient cells are widely involved in the exchange of information between cells and play an important role in various physiological and pathological processes, especially in immune responses (Zeng *et al,* 2021a) and tumours (Zeng *et al,* 2021b). Exosomes are derived from the intracellular membrane and are released into the extracellular environment when multivesicular bodies (MVB) fuse with the plasma membrane. Many cells can release exosomes, including reticulocytes (Johnstone *et al,* 1987), dendritic cells (Jung *et al,* 2020), B cells (Calvo and Izquierdo, 2020), T cells (Lopez *et al,* 2020, Wen *et al,* 2021), mast cells (Lecce *et al,* 2020), mesenchymal stem cells (Kim *et al,* 2021), epithelial cells (Du *et al,* 2021) and tumours cells (Srivastava *et al,* 2021, Zeng *et al,* 2021b). After the release of exosomes, these are widely present in blood, lymph (Saunderson *et al,* 2014), urine, saliva (Witwer *et al,* 2013), milk (Mirza *et al,* 2019), amniotic fluid, ascites (Jayaseelan, 2020) and other physiological or pathological fluids. Indeed, there are certain differences in the composition and function of exosomes from different sources.

Research has confirmed the physiological functions of milk-derived exosomes play an active role in infant intestinal development, innate immunity and prevention of inflammation (Martin *et al,* 2018). Camel milk and its exosomes successfully improved CTX-induced immunosuppression and oxidative stress in albino rats (Ibrahim *et al,* 2019). Exosomes isolated from milk can prevent experimentally induced necrotic intestinal damage by increasing goblet cell production and endoplasmic reticulum function. Milk exosomes provide a possible preventive strategy for human infants at risk of necrotising enterocolitis (Li *et al,* 2019). Through RNA sequencing and proteomics analysis of porcine milk exosomes, many mRNAs and proteins have been predicted to be involved in immunity, proliferation

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and cell signal transduction (Chen *et al,* 2017). The mRNA profiles of milk-derived exosomes were analysed from buffalo, dairy cow, swine, human and panda, in which several candidate genes that regulate disease resistance, immune response and metabolism were selected (Chen *et al,* 2020). In addition, the mRNAs of porcine milk exosomes have also been found to have the potential to protect intestinal epithelial cells from deoxynivalenol (DON) damage by regulating cell proliferation and tight junction proteins (TJs) (Xie *et al,* 2020). There has been no report on the research of Bactrian camel milk-derived exosomes. In this study, Bactrian camel milk was used as the research object to study the extraction and identification of exosomes and determination of total protein content by the BCA protein assay.

Materials and Methods

Milk sampling

The samples were taken from the mixed milk of 6 adult Bactrian camels transport on dry ice and stored at -80°C ultra-low temperature.

Preparation of exosomes from milk

The ultracentrifugation was used for separation, some changes were made (Badawy *et al,* 2018). Camel milk was centrifuged at 8,000 g at 4 °C for 30 minutes to remove fat, casein, cell debris and other crumbs. Skimmed milk supernatant was taken and centrifuged at 13,000 g at 4 °C for 30 minutes to remove the remaining fat and cell debris. The fat-free supernatant was ultracentrifuged at 100,000g at 4 °C for 120 minutes and then the supernatant was removed to obtain exosome pellets. The particles were suspended in PBS to obtain a uniform suspension. Bacteria were filtered with a 0.22 μm filter and either used immediately or stored in a freezer -80 °C.

Transmission electron microscope (TEM)

The isolated exosomes were identified by TEM (JEM2100, JEOL Ltd.) at a voltage of 80-120 kV. The exosome suspension were put in an ice bath, pipetted 5 L, fixed with a special fixative for exosomes, dropped on a copper mesh and allowed to dry, rinse with PBS 3 times and excess liquid was absorbed with filter paper. Observed by TEM after staining with uranyl acetate for 3 minutes, the 30-200 nm cup-holder-like vesicle structure in the field of view was typical exosome morphology.

BCA Protein assay

The standard protein was completely dissolved and diluted to 0.5 mg/mL. Depending on the number of standard wells, BCA reagent A and reagent B (V50: V1) were mixed and 0, 1, 2, 4, 8, 12, 16, 20 μL standards were added to the wells in order and PBS were topped up to 20 μL. The concentration was set to 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 μg/μL, respectively. The fully mixed exosome solution (2 μL) was added to the sample well of the plate and up to 20 μL was repeated 6 times using PBS. 200 μL BCA working solution was added to each well and was put in a 37 °C thermostat for 30 min. The absorbance was measured at the wavelength of A562 with a microplate reader and calculated the protein concentration of exosomes after making a calibration curve.

Results and Discussion

Under the TEM, many vesicle-like concentrates with a two-layer membrane structure was seen. A circular or oval membranous vesicle sized 30-200 nm in the shape of a saucer and cup holder was typical (Fig 1B). The slightly larger translucent vesicles over 200 nm in diameter were EVs (Fig 1). Since the periphery of the exosome was coated with a bilayer lipid membrane, the centre was uniformly stained black, the membrane was stained lightly white, the periphery was stained darkly and the edges were clear. This was consistent with the size and shape of exosomes secreted by other cells reported so far (Fig 1). According to the BCA Protein assay, the fitting equation is $y = 20.693 x - 2.3014$, $R^2 = 0.9948$. The measured total protein concentration was 18.6404 ± 1.7297μg/μL, based on the results shown in Fig 2.

Morphological observations to identify exosomes rely primarily on TEM, which allowed direct observation of morphology and measurement of exosome size and this method was simple and practical. In this experiment, we directly observed elliptical vesicle-like nanoparticles with a horizontal diameter of 76 nm and a vertical diameter of 89 nm, with band-like fragments on the top. The background was darker, the spheres were uniformly stained white, the membrane boundaries were visible and stained black uniformly. Dense black bands that were darker than the background were visible around the exosomes. Due to the adhesion phenomenon of some exosomes, it was inferred that the attachments on the surface of the membrane body may be part of the membrane fragments that broke the exosomes, as shown in Fig 1.

The two exosomes were round membranous capsules with an average diameter of about 90 nm. The exosome membrane in the lower right foot of the visual field clearly showed that a darker, wider, uniformly stained ring banded surrounded the vesicle membrane (Fig 1).

Interestingly, in this study, a typical bilayer cup holder-like exosome structure was directly observed under the TEM (Fig 1). After software measurement and analysis, the observed exosomes were 156 nm in lateral diameter and 178 nm in vertical diameter, with oval and disc-shaped, complete membrane structure, clear outlines and hollow vesicle-like shapes. The background of the visual field was clear, the film was divided into two layers, an inner layer and an outer layer, there was a gap between the homogeneous white dyed film, a cavity was formed between the inner and outer sheet and the gap was uniform in texture and black and the colour was lighter than the background. In the centre of the membrane, a uniform black circular cavity can be seen. The capsule containing the material carried by the exosomes was highly consistent with the size and shape of the exosomes reported in previous literature (Wang *et al,* 2020).

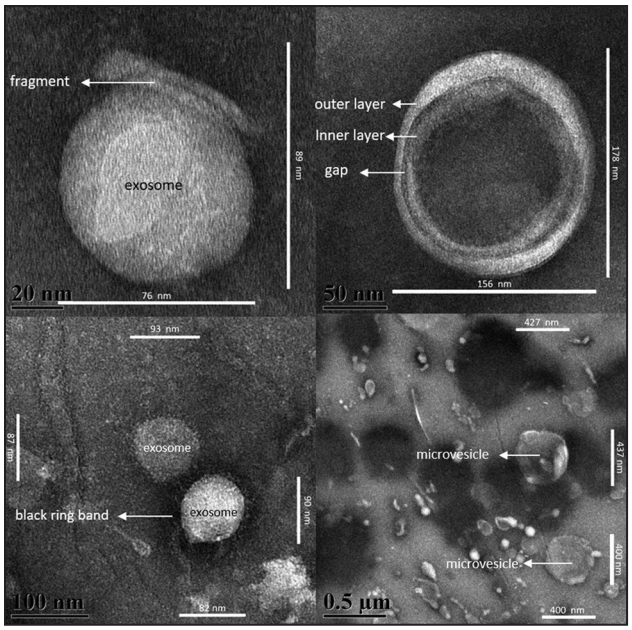
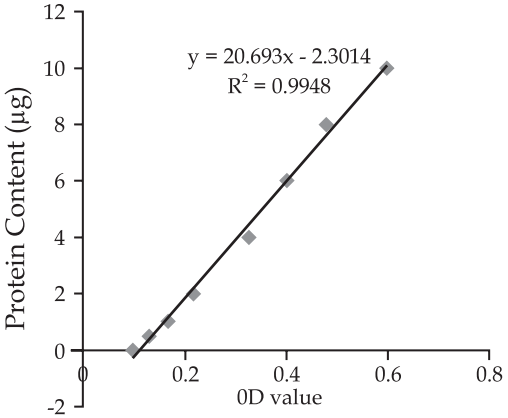


Fig 1. Observation of the exosomes by TEM. (A): Exosomes adhere to each other after disintegration and death (B): Typical double-layer cup holder-like exosomes (C): Dense staining (black ring band) around the exosome membrane (D): Extracellular vesicles larger than 200 nm.

EVs can be divided into 3 categories, depending on their size and source: exosome (30-200nm), microvesicle (100nm-1000nm) and apoptotic body (50-5000nm) (Galley and Besner, 2020). In this study, a transparent vesicle structure with a size of about 400 nm was successfully observed. The surface of the layers was rough and the overall shape was irregular (Fig 1). It is preliminarily inferred that this structure is typical microvesicle. The size and shape were consistent with previous studies (Ibrahim *et al,* 2019).

Exosomes from the same source were similar in shape and size and the diameter of exosomes from different sources may be different, but the diameter was between 30-100 nm. Serum-derived exosomes have the same diameter as other cell-derived exosomes, both of which were lipid bilayer-coated secretions. The exosomes observed in this study showed a typical model vesicle structure with



Number	0D value	Protein Content (µg)	Protein concentration µg/mL
1	0.098	0	0
2	0.13	0.5	25
3	0.166	1	50
4	0.216	2	100
5	0.326	4	200
6	0.4	6	300
7	0.478	8	400
8	0.598	10	500

Standard Curve Data Sheet

Number	1	2	3	4	5	6
0D value	1.975	1.597	2.106	1.805	2.019	1.975
Protein concentration (µg/µL)	19.2836	15.3727	20.6390	17.5247	19.7389	19.2836
Mean ± Sem (µg/µL)	18.6404 ± 1.7297					

Fig 2. Determination of the total protein concentration by BCA Protein assay.

round or oval morphology, darkly stained periphery and low-density regions within the cavity, with a diameter between 30-150 nm, with an average of about 50 nm in diameter, consistent with previous reports (Badawy *et al,* 2018).

Currently, people do not have a uniform standard for exosome extraction and various extraction methods have their strengths and weaknesses. The use of a series of ultracentrifugation methods to extract exosomes is the choice of most researchers. The physiological state of exosomes can be maintained as much as possible without the use of chemical reagents. Ultracentrifugation is the current "gold standard" for extracting exosomes, but it also has its flaw. After pretreatment, some large pollutants can be removed, but there are still some other small molecule proteins, which will affect the quantification and analysis eventually. The electron microscope can directly observe the structure, morphology and particle size of exosomes, which is a piece of direct evidence for the macroscopic identification of the presence of exosomes. Exosomes can also be comprehensively identified by nanoparticle tracking analysis, western blotting, flow cytometry, etc.

There is no doubt that camel milk has a significant positive effect in treating chronic diseases and improving body immunity. Studies show that camel milk can be used as a natural nutritional supplement to improve the body's immune system and help treat diabetes and its metabolic complications. Camel milk is a natural medicine for lowering blood glucose and suppressing lipids, which can significantly reduce the levels of total cholesterol, triglycerides and high-density lipoprotein in the blood of diabetic rats (Mansour *et al,* 2017). Camel milk exosomes have the properties of insulin-like protein and are not easily destroyed by gastric acid, which creates the possibility of oral insulin preparation (Shori, 2015). However, it remains to be seen whether camel milk neuropeptides and exosomes act in cells through specific signaling pathways or regulate physiological functions by binding to specific cell surface receptors. This also offers more possibilities for our next study. By increasing the molecular level of exosomes and studying their effects and signaling pathways, we can also better understand the internal mechanisms of the anti-diabetic properties of camel milk. Therefore, additional *in vivo* and *in vitro* experimental studies will provide new therapeutic ideas and theoretical support further for clinical T2D (Mansour *et al,* 2017), which can be employed.

As exosomes are new stars in the study of intercellular signal transduction, researchers have continuously discovered their important roles in the development of various diseases, physiological conditions and body immunity. The electron microscope observation of the shape and size of exosomes allows researchers to fully understand its own unique basic structural characteristics, thereby laying a certain foundation for better application in clinical disease treatment. certainly, the extraction and purification of exosomes from different species and origins have greatly enriched the breadth and innovation of exosomes research.

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