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Bactrian Camel (*Camelus bactrianus*) Milk Exosomes Promote Glucose Consumption in L6 Cells

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ABSTRACT

Background: Camel milk can treat diabetes, but the exact anti-diabetes components of camel milk are still unclear. It is not clear whether camel milk exosomes can regulate glucose metabolism. Therefore, the aim of this study was to determine whether camel milk exosomes can promote glucose consumption in L6 cells.

Methods: Camel milk exosomes were isolated by ultracentrifugation and identified by transmission electron microscopy (TEM). Cell Counting Kit-8(CCK-8) assay was used to detect the effects of different concentrations of camel milk exosomes on the viability of L6 cells. Glucose oxidase activity assay was used to detect the effect of camel milk exosomes on glucose consumption in L6 cells. RNA sequencing was used to detect differentially expressed genes and enriched pathways in L6 cells treated with camel milk exosomes.

Result: The size of isolated camel milk exosomes was 30-100 nm by TEM. 3-12 ng/uL camel milk exosomes had no significant effect on L6 cell viability (P>0.05). 6-24 ng/uL camel milk exosomes can significantly increase glucose consumption in L6 cells (P<0.05). A total of 401 differentially expressed genes (DEGs) were identified. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of downregulated genes suggested inhibition of mitochondrial respiratory chain complex I. Camel milk exosomes may promote glucose consumption by inhibiting mitochondrial respiratory chain complex I.

Key words: Camel milk, Exosomes, Glucose consumption, Mitochondrial respiratory chain complex I.

INTRODUCTION

In recent decades, type 2 diabetes mellitus (T2DM) has become a global pandemic, which has brought a heavy burden to the global health and economy (Reed *et al.* 2021). The etiology of T2DM is mainly due to insufficient insulin secretion of pancreatic β cells and insulin resistance (IR) of peripheral organs(Galicia-Garcia *et al.* 2020). IR in peripheral organs, including skeletal muscle and adipose tissue, results in decreased glucose uptake and utilization(Laakso 2001). Therefore, alleviating IR in peripheral organs plays a very important role in the treatment of T2DM (Hao *et al.* 2011). At present, there are contraindications and adverse reactions of various drugs used in clinical treatment of diabetes and long-term application can cause secondary failure (Wang *et al.* 2019). Recent studies have found that milk exosomes have various biological functions and can be used as drug carriers for targeted therapy of diseases (Galley and Besner 2020).

Bactrian camels feature two humps on their backs and are adapted to live in desert areas (Bai *et al.* 2020). Studies have proved that camel milk has antioxidant, anti-infection, anti-diabetes, anti-cancer and anti-hypertensive effects (Sharma *et al.* 2022; Singh *et al.* 2019). Studies have found that camel milk has the effects of lowering blood sugar and IR and can be used as an adjuvant in the treatment of diabetes. However, the exact anti-diabetic components contained in camel milk and the molecular mechanisms are still not fully understood (Ayoub *et al.* 2018).

Exosomes are nanoscale vesicles released from various cells into the extracellular space. The nucleic acids, proteins and lipids contained in exosomes can be taken up by adjacent or distant cells and regulate the function of recipient cells (Zhang *et al.* 2020; Zhao and Zhao 2015). Mammalian milk exosomes have been isolated and identified (Admyre *et al.* 2007; Yun *et al.* 2021). Milk-derived exosomes contain non-coding RNA, including miRNA, lncRNA and circular RNA, which are involved in regulation of immune response and development of metabolic diseases such as obesity and diabetes (Jiang *et al.* 2021). In addition, it has been reported that camel milk exosomes have anti-tumor and anti-oxidative stress effects (Badawy *et al.* 2018; Ibrahim *et al.* 2019). Now studies have shown that miRNAs in milk exosomes play an important role in anti-inflammatory, immune regulation and

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metabolic regulation(Chen *et al.* 2020; Melnik *et al.* 2021). However, the function of miRNAs in exosomes of camel milk remains to be studied and developed.

Based on the above, we hypothesized that camel milk exosomes could regulate glucose metabolism. We detected the changes of glucose consumption and DEGs in L6 cells treated with camel milk exosomes. The results suggested that camel milk exosomes promoted glucose consumption by inhibiting mitochondrial respiratory chain complex I.

MATERIALS AND METHODS

Experiment

The study was conducted at college of veterinary medicine of Inner Mongolia Agricultural University and Novogene company from 2021 to 2022. Camel milk was collected from Siziwang Banner, Inner Mongolia.

Exosomes isolation and characterization

Fresh camel milk was collected from female Bactrian camels at mid lactation period. The camel milk exosomes were isolated by differential ultracentrifugation. Camel milk was centrifuged at 8000 g at 4°C for 30 min to remove fat, casein and cell debris. The supernatant was taken and centrifuged at 13,000 g at 4°C for 1 h to remove the remaining fat and cell debris. The supernatant of skim milk was ultracentrifuged at 120,000 g at 4°C for 120 min to remove the supernatant and obtain exosome precipitation. The exosome particles were suspended in phosphate buffered saline (PBS) to obtain homogenous suspension. The milk exosomes were filtered by a 0.22 μm filter and then stored in a refrigerator at -80°C. The total protein concentration of milk exosomes was determined by Bicinchoninic Acid (BCA) method. The exosomes were fixed in 2.5% glutaraldehyde in cacodylate buffer at 20°C for 1 h and stained with 2% uranyl acetate. The exosomes were identified by transmission electron microscopy (TEM)(JEM2100, Joel Inc., Japan).

Materials

The rat skeletal muscle cell line L6 myoblasts were purchased from Chinese Type Culture Collection(CTCC). Dulbecco's modifified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (USA). BCA protein quantitation assay kit was purchased from Keygen Biotech (China). CCK-8 assay kit was purchased from Meilunbio (China). Glucose oxidase activity assay kit was purchased from Applygen Technologies (China).

Cell culture and CCK-8 assay

L6 myoblasts were cultured in DMEM supplemented with 10% FBS and 100U/mL penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

L6 myoblasts were seeded in 96-well plates at the density of 3×10^3 per well. The cells were treated with medium containing different doses of camel milk exosomes. The treatment doses were 0, 3, 6, 12 and 24 ng/μL, respectively and the treatment time was 24 h.10 μL of CCK-8 solution was added to each well and continue to culture for 0.5 h. The OD values were read at 450 nm.

Detection of glucose consumption

L6 myoblasts were seeded in 96-well plates at a density of 5×10^3 per well. The cells were washed three times with PBS. The cells were treated with medium containing different doses of camel milk exosomes. The treatment doses were 0, 3, 6, 12 and 24 ng/μL, respectively, metformin (2 mM) was used as the positive control and the treatment time was 24 h. Glucose concentration of cell culture supernatant was detected by Glucose oxidase activity assay kit according to the manufacturer's instructions and calculated the glucose consumption.

RNA sequence

L6 myoblasts were seeded in 25 cm^2 cell culture flask. L6 cells were cultured with medium containing 12 ng/μL camel milk exosomes for 24 h and the control group was not added with exosomes. There were three replicates in each treatment group and control group. Trypsinized cells were frozen at -80°C for RNA sequencing.

The samples were sequenced on the Illumina Hiseq 2500 platform (Novogene, Beijing, China). Data quality was checked using the fastq software. The reads were compared with rat genome and treated by Hisat2. Differential gene expression analysis was performed by the DESeq2 R package. Genes with $p<0.05$ and $|\log_2 \text{Fold change}| > 1$ are defined as DEGs. The statistical enrichment of DEGs in GO terms and KEGG pathways was tested by clusterProfiler R package. GO terms with corrected $p<0.05$ were considered significantly enriched by DEGs. KEGG pathway with corrected $p<0.05$ were significantly enriched by DEGs.

Statistical analysis

Statistical analysis was performed by ANOVA (GraphPad Prism 9). The data are presented as means±SD. Significant differences between or among groups are indicated by *p< 0.05 and **p<0.01.

RESULTS AND DISCUSSION

TEM showed that the diameter of camel milk exosomes ranged from 30 nm to 100 nm (Fig 1). The size of camel milk exosomes observed by TEM in this study is consistent with the results previously reported (Ibrahim *et al.* 2019).

In this study, CCK-8 assay was used to detect the effect of different concentrations of camel milk exosomes on the viability of L6 cells. The results of CCK-8 assay showed that there was no significant change in the cell viability after 3, 6 and 12 ng/μL camel milk exosomes treated L6 cells for 24 h ($P>0.05$). The viability of cells treated with 24 ng/μL was significantly decreased for 24 h ($P<0.05$) (Fig 2).

In this study, the effect of camel milk exosomes on glucose consumption in L6 cells was detected. The results showed that 6, 12 and 24 ng/μL camel milk exosomes significantly increased the glucose consumption of L6 cells for 24 h ($P<0.05$) (Fig 3). Camel milk can be used to treat

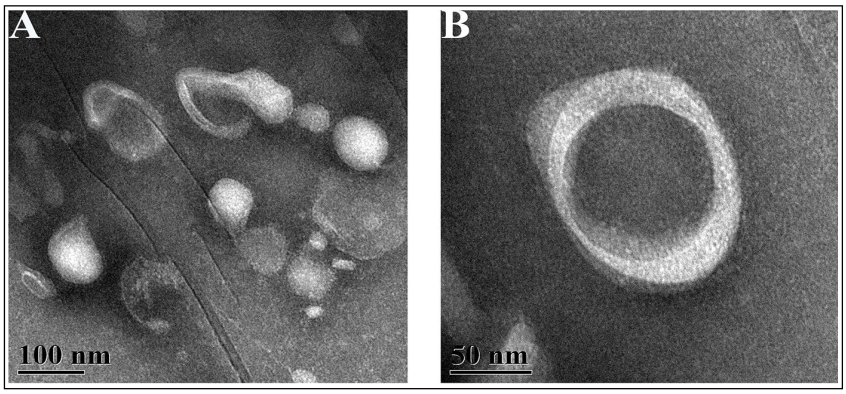

Fig 1: TEM images of exosomes extracted from camel milk.

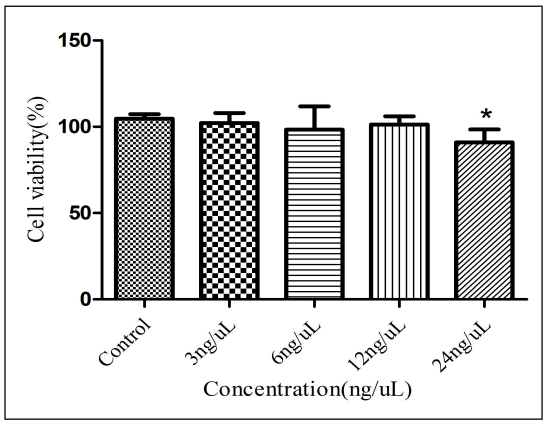

Fig 2: The effect of different concentrations of camel milk exosomes on viability of L6 cells.*p<0.05.

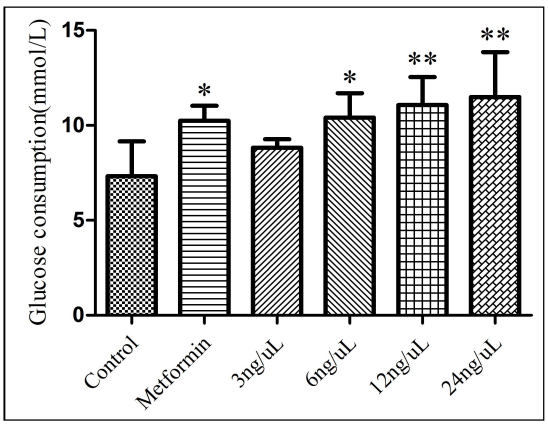

Fig 3: The effect of camel milk exosomes on glucose consumption in L6 cells. *p<0.05*,*p<0.01.

diseases such as diabetes, cancer and gastrointestinal disease (Sabha *et al.* 2020). Furthermore, camel milk exosomes have been shown to have anti-cancer effects by inhibiting inflammation and oxidative stress(Badawy *et al.* 2018). These results suggest that camel milk exosomes have a potential role in treating T2DM.

We sequenced 6 cDNA libraries from three adipose depots from the control group and treatment group. We obtained 42.39-46.92 million raw reads by high-throughput sequencing. The raw reads were filtered to obtain clean reads, which were then aligned to the rat reference genome using Hisat2. 95.14%~95.64% of the total sequenced fragments could be mapped to the reference genome (Table 1).

A total of 401 DEGs were identical between control group and treatment group, of which 135 DEGs were upregulated and 266 DEGs were downregulated in treatment vs. control. 135 upregulated genes were not significantly enriched in GO terms. 266 downregulated genes were significantly enriched in respiratory chain, mitochondrial respiratory chain complex I and NADH dehydrogenase complex for cellular component (CC) category; NADH dehydrogenase activity for molecular function (MF) category (Table 2). Pathway annotation of DEGs was performed using the KEGG database. 135 upregulated genes were not significantly enriched in KEGG terms. 266 downregulated genes were significantly enriched in oxidative phosphorylation and so on (Table 3).

From the functional analysis of DEGs, we found that downregulated differential genes were significantly enriched in respiratory chain complex I (GO analysis) and oxidative phosphorylation (KEGG analysis). From these results, we speculated that the promotion of glucose consumption by camel milk exosomes may be due to the inhibition of mitochondrial respiratory chain complex I. It has been reported that metformin, the first-line anti-diabetic drug, exerts anti-diabetic effects by inhibiting mitochondrial respiratory chain complex I (Owen *et al.* 2000). In addition, the research has proposed that mitochondrial respiratory chain complex I can be used as a target for the treatment of diabetes (Hou *et al.* 2018).

Milk exosomes contain miRNAs (Melnik *et al.* 2021), they act a key player for intracellular communication by carrying their contents (*e.g.*, miRNA) to target cells (Yun *et al.* 2021). we hypothesized that miRNAs released from the exosomes of camel milk after their entry into cells inhibited the mitochondrial respiratory chain complex I and thus promoted the glucose consumption of cells.

Table 1: Statistics for filtering and mapping reads.

Sample	Raw reads	Clean reads	Total map	Clean bases	Error rate	Q20	Q30	GC
C1	45756112	43751728	41842685 (95.64%)	6.56G	0.03	97.21	92.29	48.21
C2	46325248	44498030	42339978 (95.15%)	6.67G	0.03	97.04	92	49.84
C3	46926238	45555928	43472405 (95.43%)	6.83G	0.03	97.19	92.29	50.33
T1	45415620	44194144	42176120 (95.43%)	6.63G	0.03	97.24	92.46	51.31
T2	42393228	41432252	39417689 (95.14%)	6.21G	0.03	97.36	92.74	51.54
T3	45765016	44745196	42742464 (95.52%)	6.71G	0.03	97.08	91.99	50.16

Table 2: The significantly enriched GO terms from downregulated DEGs.

Category	GO ID	Description	Corrected p	Count	Gene name
CC	GO:0070469	respiratory chain	0.002919191	8	ND2/ND4/ND5/ND4L/COX2/LOC679739/ND6/ND3
CC	GO:0005747	mitochondrial respiratory chain complex I	0.002919191	6	ND2/ND4/ND5/ND4L/LOC679739/ND3
CC	GO:0030964	NADH dehydrogenase complex	0.002919191	6	ND2/ND4/ND5/ND4L/LOC679739/ND3
CC	GO:0045271	respiratory chain complex I	0.002919191	6	ND2/ND4/ND5/ND4L/LOC679739/ND3
CC	GO:0098803	respiratory chain complex	0.003261593	7	ND2/ND4/ND5/ND4L/COX2/LOC679739/ND3
CC	GO:0000793	condensed chromosome	0.00630917	8	Smc4/Smc6/Rif1/Cenpe/Stag2/Atrx
CC	GO:0098800	inner mitochondrial membrane protein complex	0.00676564	8	ATP6/ND2/ND4/ND5/ND4L/ATP8/LOC679739/ND3/Mis18bp1/Tex12
CC	GO:0044455	mitochondrial membrane part	0.007562291	10	Bnip3/ATP6/ND2/ND4/ND5/ND4L/Coq4/ATP8/LOC679739/ND3
CC	GO:0005746	mitochondrial respiratory chain	0.016446715	6	ND2/ND4/ND5/ND4L/LOC679739/ND3
CC	GO:1990204	oxidoreductase complex	0.037839494	6	ND2/ND4/ND5/ND4L/LOC679739/ND3
MF	GO:0008137	NADH dehydrogenase (ubiquinone) activity	0.000106114	6	ND2/ND4/ND5/ND4L/ND6/ND3
MF	GO:0050136	NADH dehydrogenase (quinone) activity	0.000106114	6	ND2/ND4/ND5/ND4L/ND6/ND3
MF	GO:0003954	NADH dehydrogenase activity	0.000145563	6	ND2/ND4/ND5/ND4L/ND6/ND3
MF	GO:0016655	oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor	0.00016829	6	ND2/ND4/ND5/ND4L/ND6/ND3
MF	GO:0016651	oxidoreductase activity, acting on NAD(P)H	0.012719905	6	ND2/ND4/ND5/ND4L/ND6/ND3

Table 3: The significantly enriched KEGG pathways from downregulated DEGs.

KEGG ID	Description	Corrected p	Count	Gene name
rno05168	Herpes simplex virus 1 infection	5.67E-13	28	Zfp758/-/Zfp560/-/AABR07001910.1/Zfp317/LOC100365363/LOC103691005/LOC102551340/LOC102552527/RGD1566386/Zfp51/LOC680200/AABR07001905.2/Zfp458/LOC102549842/Zfp386/Zfp455/AABR07001923.1/Giot1/AABR07002774.2/AABR07002784.1/Zfp40/Zfp52/LOC100910577/Cfp/Zfp182/-
rno00190	Oxidative phosphorylation	0.000259626	11	ATP6/ND2/ND4/ND5/ND4L/-/ATP8/COX2/LOC679739/ND6/ND3
rno04723	Retrograde endocannabinoid signaling	0.002459032	9	ND2/ND4/ND5/Faahl/ND4L/-/LOC679739/ND6/ND3
rno05012	Parkinson disease	0.008220925	12	ATP6/ND2/ND4/ND5/ND4L/-/ATP8/COX2/LOC679739/ND6/ND3/Slc18a2
rno04714 ND3	Thermogenesis	0.010573256	11	ATP6/ND2/ND4/ND5/ND4L/-/ATP8/COX2/LOC679739/ND6/
rno05016	Huntington disease	0.023590343	12	ATP6/ND2/Rb1cc1/ND4/ND5/ND4L/-/ATP8/COX2/LOC679739/ND6/ND3

CONCLUSION

This study demonstrated for the first time that camel milk exosomes can promote glucose consumption in L6 cells. The reason why camel milk exosomes promote cellular glucose consumption may be due to the inhibition of mitochondrial respiratory chain complex I. Therefore, camel milk exosomes may be used as adjuvant in the treatment of T2DM. However, further studies are needed to clarify the mechanism behind it.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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