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Camel milk exosomes had a selective anticancer effect on PANC1 cells and a proliferative effect on H6c7 cells

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Abstract

Background/aim: In the Middle East, there is a common belief that the daily intake of camel milk prevents cancer and diabetes. However, the actual anti-cancer and anti-diabetic mechanisms of camel milk remain elusive. This study aimed to evaluate the effect of camel milk exosomes on the viability of human pancreatic cancer cells PANC1 and normal pancreatic duct epithelial cells H6c7.

Methods: Exosomes were isolated from freshly collected camel milk by gradient ultracentrifugation and were characterized by transmission electron microscope (TEM) and further confirmed by western blot. Cell viability was assessed by MTT assay and relative expression of the pro-apoptotic genes (*Bax* and caspase3) and the anti-apoptotic gene (*Bcl2*) were determined by real-time PCR.

Results: Interestingly, camel milk exosomes had a selective effect on the cell viability depending on the type of the target cell. These exosomes induced a potent cytotoxic effect on the cancer cells (PANC1), while they improved the viability of the normal pancreatic cells (H6c7). These incredible results were confirmed on a molecular basis. Camel milk exosomes induced a significant increase in *Bax* and caspase3 and a significant decrease in *Bcl2* mRNA levels in PANC1 cells. However, the effect on these markers was reversed when exosomes were added to H6c7 cells.

Conclusion: To the best of our knowledge, this may be the first study to report that exosomes derived from camel milk had a selective anticancer effect on pancreatic cancer cells and a proliferative effect on normal pancreatic cells.

Keywords: Camel milk exosomes; PANC1; H6c7; MTT assay; Apoptosis

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by increased blood glucose levels. There are two main types of DM: DM type 1 which is mainly caused due to decreased insulin secretion and DM type 2 which is mainly caused due to increased insulin resistance where cells are unable to utilize insulin properly due to disrupted insulin receptors and/or transduction signaling pathway. If DM is left without treatment, many macro-and microvascular complications, including cardiopathy, nephropathy, and retinopathy could occur (Abdellatif et al., 2019). Because DM is a chronic metabolic disorder, diabetic patients should take anti-diabetic drugs forever. This long-lasting treatment induces resistance to these drugs, causes severe side effects, and increases the predisposing risk factors for pancreatic cancer (Gupta et al., 2016). DM is probably the third regulating risk factor for pancreatic cancer after cigarette smoking and obesity (Li, 2012). About 50-80 % of patients with pancreatic cancer were diabetic (Pannala et al., 2008). The more suspected mechanisms underlying the relation between DM and pancreas cancer are insulin resistance, compensatory hyperinsulinemia, and high levels of circulating insulin-like growth factors (Li, 2012). Increased understanding of diabetes-pancreatic cancer relations would help develop new preventive and therapeutic policies on that deadly disease. Therefore, many attempts by researchers were done to find effective alternative therapies for anti-diabetic drugs, especially synthetic insulin.

Camel milk contains unknown (to date) biologically active components that have an anti-cancer effect (Malik et al., 2012; Mirmiran et al., 2017). Diabetic patients who drink camel milk regularly have significantly lower blood glucose levels than those did not drink camel milk (Agrawal et al., 2007). Moreover, diabetic rats administrated camel milk for 1-2 months had reducing blood glucose levels and insulin resistance (Agrawal et al., 2011; Khan et al., 2013; Korish, 2014; Mohamad et al., 2009; Sboui et al., 2010). Some other studies showed a potent cytotoxic effect of camel milk on a large variety of cancer cells including human liver cancer HepG2 cells, breast cancer MDA-MB-231 and MCF7 cells, and murine hepatoma Hepa 1c1c7 cells (Badawy et al., 2018; Homayouni-Tabrizi et al., 2017; Korashy et al., 2012a; Korashy et al., 2012b; Shariatikia et al., 2017).

Exosomes are extracellular vesicles (diameters 40 to 100 nm) surrounded by phospholipid membrane and originated from endosomes of almost all cell types. They play a crucial role in cell-to-cell

communication through their nucleic acid cargo (mRNA, microRNA, LncRNA, DNA), proteins, and lipids (Alzahrani et al., 2018). Cow milk exosomes can be up-taken by macrophages, splenocytes, and intestinal cells *in vitro* (Arntz et al., 2015). In our two recent publications, we extracted camel milk exosomes and investigated their role in immune response and against cancers. We found that the administration of camel milk exosomes resulted in the induction of immunity in cyclophosphamide-immunocompromised rats and a reduction in the proliferation of breast cancer MCF7 cells both *in vitro* and *in vivo* (Badawy et al., 2018; Ibrahim et al., 2019).

Although several previous studies investigated the anti-diabetic effect of camel milk, to date, no available data in literature addressed the anti-diabetic effect of camel milk-derived exosomes. Also, it remains unknown whether these exosomes have an anticancer effect on pancreatic cancer cells and whether they are safe on normal pancreatic cells. Therefore, this study was conducted to investigate whether camel milk exosomes could affect the viability of human pancreatic cancer cells, PANC1, and normal pancreatic duct epithelial cells, H6c7.

2. Materials and methods

2.1. Animals and milk samples

This study was reviewed and approved by the Animal Care and Welfare Committee of Kafrelsheikh University. Milk samples (200 ml/animal) were collected from 5 healthy camels (Camelus dromedaries) at the early lactation stage. Animals source was a national local farm in Marsa Matruh, Egypt.

2.2. Camel milk exosomes isolation

The isolation of exosomes was done using differential ultracentrifugation as previously detailed (Badawy et al., 2018; Ibrahim et al., 2019). In brief, milk samples were centrifuged twice the first at 3000 *g* at 4 °C for 30 min (to eliminate fat and debris), and the second at 10000 *g* at 4 ºC for 30 min (to get clear milk serum). Then, the exosomal pellets were obtained from milk serum samples by twice centrifugation each at 100,000 *g* at 4 °C for 60 min using Optima L-90K ultracentrifuge (Beckman Coulter, Brea, California). The exosome pellets from the four animals were used directly for total RNA extraction, while the fifth pellet was used for transmission electron microscopy (TEM) examination and western blot analysis.

2.3. Camel milk exosomes characterization by TEM

TEM was used to characterize the exosomes using a previously detailed protocol (Badawy et al., 2018). Briefly, following fixation in 2.5% glutaraldehyde for 60 min at room temperature, the exosome pellet was treated with 1% osmium tetroxide for 90 min before being fixed in epoxy resin. Fixed samples were cut into ultrathin sections and fixed on appropriate grids. These sections were stained with 2% uranyl acetate and examined by TEM (JEM2100, Joel Inc., Japan) at 80 kV.

2.4. Camel milk exosomes characterization by western blot

Western blot was conducted to confirm the isolation of exosomes through the identification of their specific surface markers CD63 and CD81. We followed the procedure of our previously published protocol (Abou-Easa et al., 2014) with some modifications to adapt to exosomal proteins. Exosomal pellets were lysed in a RIPA buffer containing Tris (50 mM, pH 7.4), EDTA (20 mM), NP-40 (0.5%), NaCl (150 mM), phenylmethylsulfonyl fluoride (0.3 mM), NaF (1 mM), $NaVO_4$ (1 mM), dithiothreitol (1 mM), aprotinin (1l g/ml), leupeptin (1l g/ml), and pepstatin (1l g/ml). The cell lysates were centrifuged for 10 min at 10,000 g at 4ºC. Bio-Rad protein assay kit (Bio-Rad, Hercules, and CA) was used to measure the total exosomal protein concentrations of the obtained supernatants. These proteins were led into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a concentration of 17 µg per lane and then were transferred to nitrocellulose membranes. Following incubation with 5% skimmed milk in Tris-buffered saline, the immunoblots were incubated with two specific surface exosomal markers; the anti-CD63 (1:200) and anti-CD81 (1: 200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washes, membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1: 2000 in 1% PBS Cell Signaling Technology, Beverly, MA, USA). The detection was achieved with enhanced chemiluminescence reagents (Santa Cruz).

2.5. MTT assay

MTT assay was used to investigate the effect of camel exosomes on the viability of both human pancreatic cancer cell PANC1 (purchased from VACERA, Egypt) and human normal pancreatic duct epithelial cells H6c7 (purchased from Kerafast, and provided by the laboratory of Ming-Sound Tsao, MD, FRCPC, University Health Network, Canada). This assay proceeded as detailed by previous protocols (Abdelwahab et al., 2019; Azaam et al., 2018; Khodair et al., 2019). Briefly, cells were cultured in a 96-well plate [1× 10^4 cells/well, 100 µl/well]. The culture medium contained DMEM, 10% fetal bovine serum, and 1% penicillin/streptomycin (GIBCO, USA). Incubation (37 °C for 24 h, 5% CO_2, 95% air) was done till getting 70-80% confluence. Exosomal total RNA concentrations were measured by a nanodrop (Quawell nanodrop Q5000, USA). Cells were incubated with exosomes at different RNA concentrations (0, 3.125, 6.25, 12.5, 25, 50, and 100 µg/ml) for 24 h before addition of 5 mg/ml of MTT (Sigma). Following incubation for 4 h, the medium was changed by 100 µl DMSO (Sigma) and absorbance was set at 570 nm. Sigmoidal curve using GraphPad (Prism) statistic software was used to determine the concentration of exosomes inhibiting 50% of cells (IC_{50}).

2.6. Real-time PCR

Total RNA was extracted from PANC1 and H6c7 cells after treatments with exosomes. using RNeasy Mini kit following the manufacturer's guidelines (Qiagen, #74104) and as previously described (Shaban et al., 2018). The cDNA was synthesized from RNA by reverse transcription using a Quantiscript reverse transcriptase (Qiagen, #205310) and as previously detailed (Abd-Allah et al., 2015). The qPCR reaction mixture included 2 µl cDNA, 1 µL from each primer, 12.5 µL QuantiTect SYBR Green qPCR Master Mix, and 8.5 µL RNase-free water. Primer sequence was as follows: *Bax* (F 5'-CCTGTGCACCAAGGTGCCGGAACT -3' and R 5'-CCACCCTGGTCTTGGATCCAGCCC-3'), Bcl2 (F 5'-AGGAAGTG AACATTTCGGTGAC -3' and R 5'- GCTCAGTTCCAGGACCAGG C -3'), caspase 3 (F 5'- TGTTGTGCCACTAATTGGAAGT -3' and R 5'- GCCCTGATCTAACTGCCACC -3') and *β-actin* (F 5'-CACCAACTGGGACGACAT -3' and R 5'- ACAGCCTGGATAGC AACG -3'). The thermal conditions of qPCR, melting curves, and calculation of gene expression fold change using the $2^{-\Delta\Delta Ct}$ method was done as previously detailed (El-Adawy et al., 2018; Saleh and El-Magd, 2018). The quantities critical threshold (Ct) of target genes were normalized with quantities (Ct) of the internal control (*β-actin*).

2.7. Statistical analysis

For statistical analysis, GraphPad Prism 7 (GraphPad Software, Inc, La

Jolla, CA) computer program was used. The statistical analysis was performed by unpaired student t-test. Data were presented as mean ± standard error of the mean (SEM) and significance was set at $P < 0.05$.

3. Results

3.1. Characterization of camel milk-derived exosomes

In this study and similar to our previous publications (Badawy et al., 2018; Ibrahim et al., 2019), we successfully extracted exosomes from camel milk samples using the ultracentrifugation method. As seen by TEM, the extracted camel milk exosomes appeared as small nanovesicles with an average size of 30-100 nm (Fig. 1). This extraction was further confirmed by western blot and the results confirmed the expression of two surface marker exosomal proteins CD63 and CD81 (Fig.2). The total yield of the exosome pellet was 380 mg from 1liter milk. The concentrations of total RNA extracted from the first four exosome pellets were 153, 180, 142, and 129 ng/ml, respectively, as revealed by Nanodrop measures (Fig. 3).

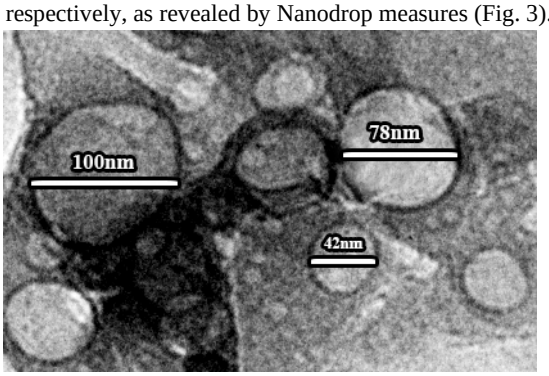

Fig.1. Characterization of camel milk-derived exosomes by TEM. Diameters of some selected exosomes were displayed with scale bars of 100, 78, and 42 nm.

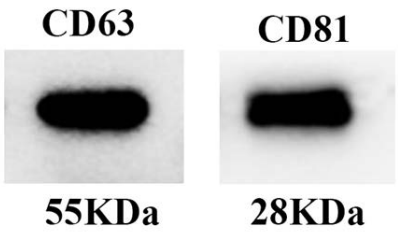

Fig.2. Characterization of camel milk-derived exosomes by western blot. Western blot data shows the presence of exosomal specific surface marker proteins, CD63 and CD81 with the size of 55KDa and 28KDa, respectively.

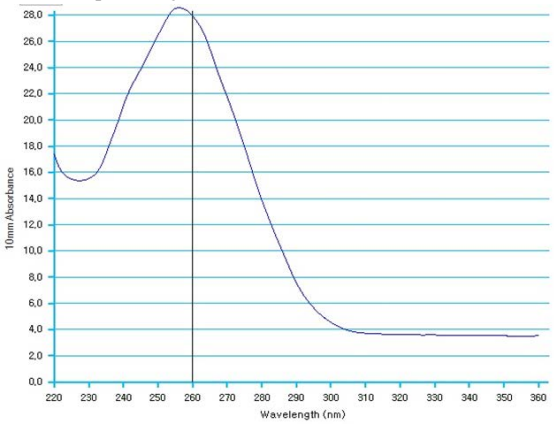

Fig. 3. A representative Nanodrop curve shows the concentration and purity of extracted RNA from the first exosome pellet which is 153 ng/ml. In this curve, the upper top presents at 260 and the bottom at 230 which indicates the presence of pure RNA.

3.2. Effect of camel milk exosomes on cell viability of PANC1 and H6c7 cells

The MTT assay was achieved to assess the effect of camel milk exosomes on human pancreatic cancer PANC1 and normal pancreatic H6c7 cells. The obtained results revealed a significant dose-dependent cytotoxic effect for exosomes on PANC1 with IC_{50} values of 37.8±1.14 µg/ml and a significant proliferative effect on H6c7 relative to vehicle (DMSO)-treated cells (Fig.4). Exosomes IC_{50} on pancreatic cancer PANC1 cells was higher than that of the standard anticancer drug cisplatin (IC_{50} = 16.15±0.93 µg/ml). However, unlike exosomes, cisplatin showed a higher cytotoxic effect on normal pancreatic H6c7 cells with an IC_{50} of 60.89±2.55 µg/ml.

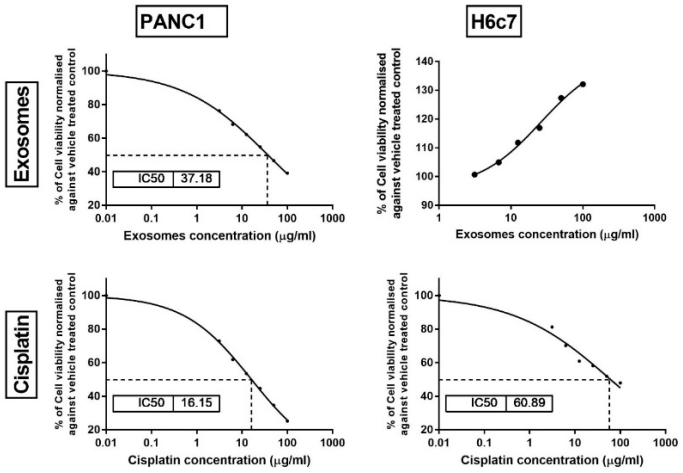

Fig.4. Sigmoidal curves for the MTT assay show IC_{50} values and the inhibition/viability percentage (%) of camel milk exosomes and cisplatin on PANC1 and H6c7 cells.

3.3. Effect of camel milk exosomes on the expression of apoptotic genes

Real-time PCR was applied to determine the changes in the relative expression of apoptotic genes (*Bax* and caspase 3) and the anti-apoptotic gene *Bcl2* in PANC1 and H6c7 cells following treatment with camel milk exosomes. PNC1 cells treated with camel milk exosomes at concentrations equal to their IC_{50} (37.18 µg/ml) showed a significantly upregulated expression of *Bax* and caspase 3 and a significantly downregulated expression of *Bcl2* as compared to vehicle(DMSO)-treated control group (Fig. 5). However, H6c7 cells treated with the same concentrations of exosomes exhibited a significant decrease in *Bax* and caspase 3 and a significant increase in *Bcl2* expression.

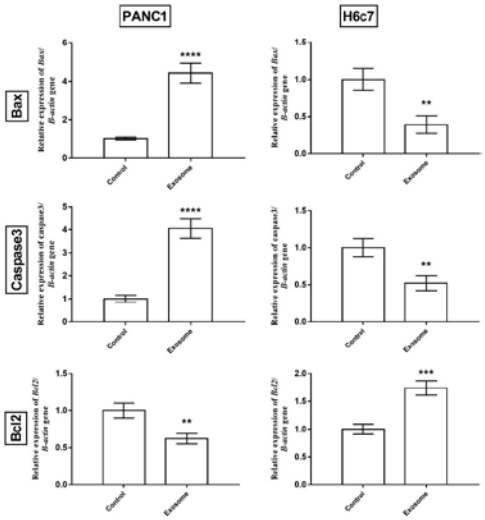

Fig.5. Effect of exosomes on expression of apoptotic (*Bax* and caspase3) and anti-apoptotic (*Bcl2*) genes as detected by real-time PCR in PANC1 and H6c7 cells. Fold change in gene expression are expressed as mean ± SEM, n = 7. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 vs control.

4. Discussion

Previous studies reported anti-cancer (Badawy et al., 2018; Homayouni-Tabrizi et al., 2017; Korashy et al., 2012a; Korashy et al., 2012b; Shariatikia et al., 2017) and anti-diabetic (Agrawal et al., 2011; Khan et al., 2013; Korish, 2014; Mohamad et al., 2009; Sboui et al., 2010) effects for camel milk. Other than our previous study which reported a potent anti-cancer effect for camel milk exosomes on breast cancer MCF7 cells both *in vitro* and *in vivo* (Badawy et al., 2018), no available data in the literature investigated the influence of camel milk exosomes on cancer and normal pancreatic cells. This prompts us to conduct this experiment to evaluate this effect.

We first isolated exosomes from camel milk through gradient ultracentrifugation and got nanovesicles with a size of 30-100 nm. Similar camel milk exosomal size was also obtained using the same method of extraction by other researchers (Badawy et al., 2018; Ibrahim et al., 2019; Yassin et al., 2016), indicating the efficiency of ultracentrifugation in exosomes isolation from milk. Further, we performed an MTT assay to detect the exosomes effect on PANC1 and H6c7 cells. Interestingly, camel milk exosomes had a selective effect on the cell viability depending on the type of the target cell. These exosomes induced a potent cytotoxic effect on the cancer cells (PANC1), while they improved the viability of the normal pancreatic cells (H6c7). In contrast, the standard anticancer drug cisplatin showed a higher cytotoxic effect on PANC1 and H6c7. This indicates that exosomes are not only safer than cisplatin but also could have a regenerative effect on the normal pancreatic cells. This selective action provides camel milk exosomes as an alternative therapy for pancreatic cancer, with not only a non-cytotoxic effect but also a proliferative effect against normal pancreatic cells. However, because the IC50 of exosomes was higher than that of cisplatin, which indicates less anti-cancer activity for exosomes, exosomes might be not used as a sole therapeutic agent against cancer instead, they could be used as a vehicle/carrier for other conventional anticancer drugs. In support, exosomes derived from bovine milk were successfully used as neoadjuvant/carriers to improve the oral bioavailability, efficiency, and biosafety of some conventional anticancer drugs (Munagala et al., 2016).

Lastly, we investigated the underlying molecular mechanism for this selective anti-cancer effect of camel milk exosomes. The obtained real-time PCR data showed that camel milk exosomes induced a significant increase in *Bax* and caspase3 and a significant decrease in *Bcl2* mRNA levels in PANC1 cells. These results were consistent with the result of the MTT assay and confirmed the selective anti-cancer effect of camel milk exosomes on PANC1. These data also explained that the cytotoxic effect of exosomes on PANC1 could be, at least in part, mediated by the induction of apoptosis in these cells. Although several previous studies investigated the anti-cancer effect of camel milk against a large variety of cancer cell lines (Badawy et al., 2018; Homayouni-Tabrizi et al., 2017; Korashy et al., 2012a; Korashy et al., 2012b; Shariatikia et al., 2017), none of these studies evaluated the effect of either camel milk or its exosomes on PANC1 cells. Thus, our study is the first to report this camel milk exosomes' effect on these cells.

Unlike PANC1, H6c7 treated with camel milk exosomes showed lower expression of *Bax* and caspase 3 genes and higher expression for the *Bcl2* gene. This is compatible with the results obtained by the MTT assay and both indicate a proliferative effect for exosomes on H6c7. This proliferative effect could be due to the inhibition of apoptosis and the induction of anti-apoptotic pathways in these cells. Similarly, it was recently reported that bovine milk exosomes could initiate the proliferation of rat normal intestinal epithelial cells and protect them from damage by free radicals oxidative effect (Wang et al., 2020). Although the actual anticancer effect of camel milk remains elusive, the proliferative effect of camel milk exosomes on normal pancreatic cells could theoretically explain this anti-diabetic effect. However, this assumption should be experimentally proven on lab animals (preclinical approach) and further on diabetic patients (clinical trial). These *in vivo* experiments are also very important to assess whether these exosomes have any adverse effect on various body systems. Also, it is not well-known whether RNA cargos in milk exosomes can bypass digestion by gastric and intestinal enzymes, circulate in the blood, and are functionally expressed in the body of milk consumers (animal or human). Therefore, further investigations are encouraged to use a suitable exosomal *in vivo* labeling system to track the pathway of these exosomes within the body. Although, it remains unknown how DM type 2 increases the prevalence of pancreatic cancer (Li, 2012), understanding the mechanisms of diabetes and pancreatic cancer interaction would help in developing new preventive and therapeutic approaches for pancreatic cancer. In that regard, we provided camel milk exosomes as new biological agents that could increase our knowledge of DM-pancreatic cancer interaction.

5. Conclusion

To the best of our knowledge, this may be the first study to report that exosomes derived from camel milk had a selective apoptosis-dependent anticancer effect on pancreatic cancer cells and an anti-apoptosis-based proliferative effect on normal pancreatic cells. This indicates a possible role in cancer treatment and prevention for camel milk exosomes. However, further investigations are required to confirm this role *in vivo*.

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