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DEMONSTRATION OF HEPATOPROTECTIVE ACTION OF CAMEL MILK THROUGH IMPROVING ANTIOXIDANT ACTIVITY AND REGULATING GENE EXPRESSION IN MICE

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ABSTRACT

The composition of camel milk includes antioxidants that are beneficial for liver function and regulate gene expression. Here we evaluated the protective effects of camel milk in mice. Our results revealed that camel milk protected the liver by decreasing levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and malondialdehyde (MDA), and increasing the activity of superoxide dismutase (SOD) and glutathione (GSH) in mice. Histopathological examination demonstrated that the livers of mice receiving camel milk were not different to those of control mice. The mRNA expression of SAA1, TGF-α, TNF-α, and LCN2 was down-regulated in the livers of mice receiving camel milk. These results indicate that camel milk significantly increases the antioxidant capability of the liver and regulates gene expression in mice. Thus it is opined that regular consumption of camel milk has hepato-protective effect.

Key words: Camel milk, Hepatoprotective, Antioxidant activity, Regulating gene expression

The liver plays a key role in the metabolism of foreign compounds entering the body; contact with polluted environments, consumption of contaminated food and exposure to toxic chemicals can lead to a variety of different types of liver disease in humans (Rajesh and Latha, 2017). In recent years, there has been much research into the therapeutic effects of camel milk for the amelioration of liver disease symptoms and the side effects of treatments. For example, El-Bahr (2014) reported that camel milk protected the liver against carbon tetrachloride (CCl_4)-induced hepatic toxicity by modulating the extent of lipid peroxidation and by boosting the antioxidant defense system both at the activity and the gene expression level. Darwish *et al* (2012) found that treatment with camel milk alleviated alcohol-associated liver disfunction and protected hepatic tissues from alcohol-induced toxicity. Regular consumption of camel milk could also provide a natural way to protect against non-alcoholic fatty liver disease induced by a high-fat diet (Korish *et al,* 2013), and halt the progression of hepatocellular carcinoma (Miniawy *et al,* 2014). Overall, camel milk contains factors that have the potential to be protective and therapeutic in the liver.

Camel milk has a wide range of antioxidative, antimicrobial and immuno-modulatory properties (Mihic *et al,* 2016). In view of this, the present study was done to demonstrate the hepato-protective effects of camel milk on the regulation of gene expression and activity of hepatic antioxidant enzymes in mice.

Materials and Methods

Collection and processing of camel milk. Milk was collected from 35 camels (Alxa League, Inner Mongolia, China), stored in sterilised containers on ice, and immediately transported to the laboratory. Once in the laboratory the milk was frozen at -80°C prior to pasteurisation at 65°C for 30 minutes and then freeze-dried into milk powder. The milk powder was pressed into pellets for long-term storage at -80°C prior to use in experiments. During experiments the milk powder was reconstituted with sterile water (dry matter was 15%) in a sterile environment.

Animal treatments. Twenty male C57BL/6J mice (12 weeks old) were housed in a room maintained

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under standard laboratory conditions (12 h light/dark cycle; temperature of 21-23°C; relative humidity of 45-65%). Mice had free access to sterilised standard chow and distilled water throughout the experiment. The animals received humane care and all protocols were approved by Animal Care and Use Committee at Inner Mongolia Agricultural University.

After acclimatisation to standard laboratory conditions for 7 days, mice were randomly assigned to two groups (10 per group) as follows:

Control group (C) received sterile distilled water (10 ml/kg body weight/day) intra-gastrically.

Camel milk group (M) received camel milk (10 ml/kg body weight/day) intra-gastrically.

After 4 weeks, mice were euthanised using isoflurane gas. Blood samples were collected into heparin-containing blood sampling tubes, centrifuged at 2500 g for 15 min at 4°C and the supernatant transferred to a clean tube and frozen at -80°C until further analysis. The liver of each mouse was dissected out quickly. A portion of the left lobe of each liver was excised and fixed in a 4% paraformaldehyde solution for histopathological analysis.

Measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum. In order to evaluate the liver-protection capacity of camel milk, the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in mice receiving camel milk were compared with control mice. The levels of ALT and AST in mouse serum were determined using commercial assay kits (Roche Diagnostics, Switzerland) according to the manufacturer's protocols.

Determination of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) levels in the liver. After homogenisation and centrifugation, the supernatants of liver tissues were evaluated for the activity of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) by ELISA (Sangon Biotech Co., Ltd., Shanghai, China). Results were calculated by following the manufacturer's instructions. This was because MDA level is widely used as a marker for free radical mediated lipid peroxidation injury and SOD and GSH are used as indices for the antioxidant status of tissues.

Quantitative RT-PCR Analysis. Liver tissue (50-100 mg/ mouse) was homogenised in 1 mL of TriZol reagent (Invitrogen) and total RNA was extracted. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed as follows: cDNA was synthesised with a ReverTra Ace-a Kit (Toyobo) from total RNA after DNase I treatment, and real-time PCR was performed using a QuantiFast SYBR Green PCR Kit (Qiagen). Every plate included the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control. Particular interest in the expression of genes for cancer-associated factors and inflammatory factors was kept in mind. The primer sequences used for each gene are listed in Table 1. The $2^{-(\Delta\Delta Ct)}$ method was used to calculate the results.

Histopathological examination. The fixed liver tissue samples (4% paraformaldehyde, 4°C) were embedded in paraffin, cut into 5 µm sections and stained for histopathological examination.

Statistical analysis. Unless otherwise indicated, all values are expressed as the mean ± SEM. Statistical analyses were performed using Student's t test. Statistically significant differences between groups were defined as $p < 0.05$.

Results

Effects of camel milk on AST and ALT levels in serum

Levels of ALT (P<0.01) and AST (P<0.01) were significantly lower in the mice receiving camel milk compared to the control mice (Fig 1).

Effects of camel milk on MDA, SOD and GSH levels in liver tissue

The levels of MDA were lower in the livers of mice receiving camel milk compared with control mice, although this was not statistically significant (P>0.05) (Table 2). The levels of SOD (P<0.05) and GSH (P<0.05) increased significantly in the livers of mice receiving camel milk compared with control mice (Table 2).

Quantitative RT-PCR Analysis

The mRNA expression of serum amyloid A 1 (SAA1) was significantly down-regulated in the livers of mice receiving camel milk compared with control mice (Fig 2A, P<0.01). This was also the case for transforming growth factor-α (TGF-α) (Fig 2B, P<0.01), tumor necrosis factor-α (TNF-α) (Fig 2C, P<0.01) and lipocalin 2 (LCN2) (Fig 2D, P<0.01).

Histopathological examination

Histopathological changes in the liver are shown in Fig 3. In normal control animals, gross macroscopic evaluation of the liver showed red,

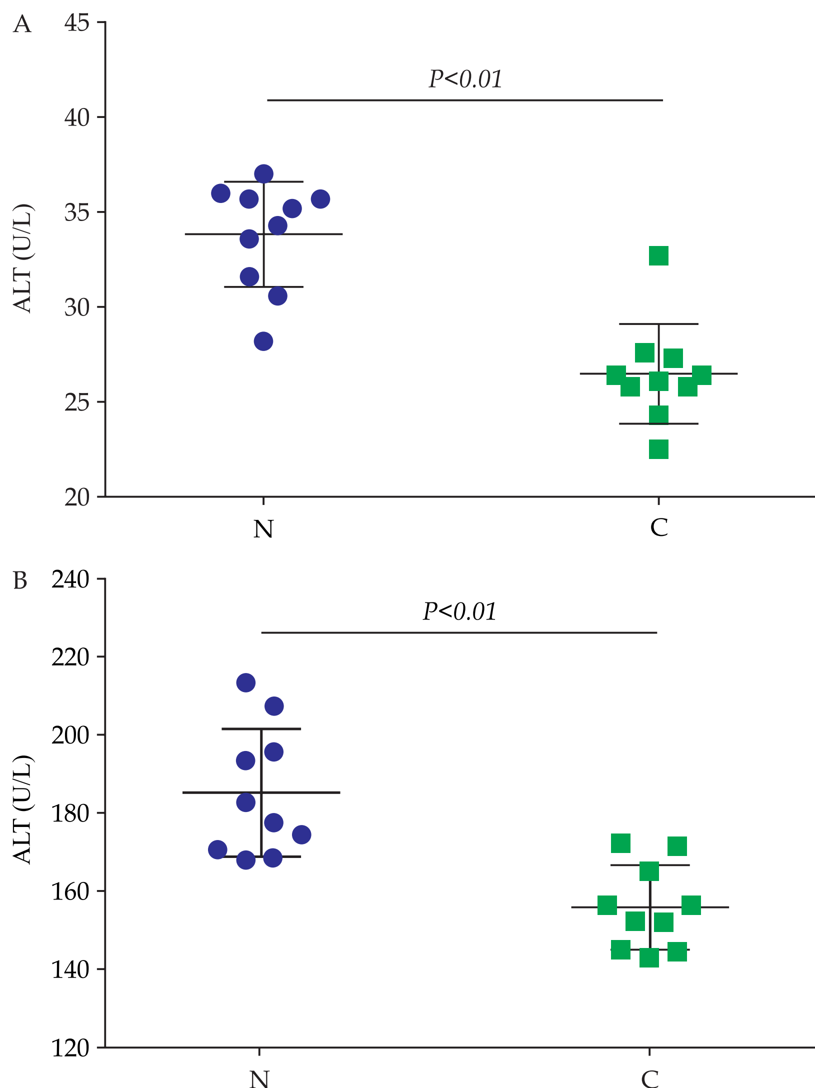

Fig 1. Effects of camel milk on serum AST and ALT activities.

smooth and shiny liver tissues (Fig 3A), and liver sections showed normal hepatic cells with well preserved cytoplasm, prominent nucleus and nucleolus and central vein (Fig 3C). Liver gross macroscopic and sections of the mice receiving camel milk group were similar to the control group mice (Fig 3B and 3D).

Discussion

Camel milk is widely used in various populations for the treatment and prevention of diseases (Yagil, 2013). Our results revealed that camel milk could protect liver by decreased levels of MDA, and increase activities of SOD and GSH in mice. An increase in the levels of MDA in the liver enhances peroxidation and can lead to tissue damage and failure of the antioxidant defence mechanisms that prevent formation of excessive free radicals (Sun *et al,* 2013). Consumption of camel milk is known to have beneficial antioxidative properties in the treatment of many diseases, and also that it inhibits lipid peroxidation (MDA) in mice (Mihic *et al,* 2016). Our findings are in accordance with these previous studies.

GSH, is the most abundant thiol in mammals, was discovered a century ago, and has a central function in the detoxification and protection of oxidants (Li *et al,* 2012). GSH is an extremely efficient intracellular buffer for oxidative stress (Hsu *et al,* 2008). Furthermore, SOD can also reduce oxidative stress, and is an effective defense enzyme that converts the dismutation of superoxide anions into hydrogen peroxide (Li *et al,* 2012). Several studies have found that antioxidant enzymes such as SOD and GSH provide protection against oxidative tissue-damage (Hsu *et al,* 2008). We found that the activity of GSH and SOD in liver tissue of mice receiving camel milk increased significantly compared with control mice and suggest that these two enzymes contribute to the hepato-protective effects of camel milk in mice.

The liver is an important organ of the human body, and internal mechanisms for protection of the liver are the main way that damage to liver cells is prevented. Serum amyloid A (SAA) is a pro-inflammatory molecule that induces leukocyte infiltration and promotes neutrophil adhesion to endothelial cells under inflammatory conditions (Young *et al,* 2015). In a case study of a patient with alcoholic liver cirrhosis, an existing liver nodule was diagnosed as SAA-positive by immunohistochemistry (Kim *et al,* 2014). SAA1 is an isoform of SAA that has been reported in mice (Young *et al,* 2015). SAA1 increases expression of AST and ALT as well regulating secretion of pro-inflammatory cytokines during hepatitis (Young *et al,* 2015). In our study, the expression of SAA1 was down-regulated in the livers of mice receiving camel milk, thus contributing to protection of the liver from inflammation.

TGF-α is a cellular factor that plays a role in regulation of healthy and tumour cell proliferation

Table 1. Primer sequences used for quantitative RT-PCR.

Gene	Sense	Anti-sense
SAA1	CAGCTACCAATCAGGCATGTC	ATGTCTGCTCGAAGCATTAAC
TGF-α	CTGGCTGTCCTCATTATCACCT	AAATTCCTCCTCTGGGATCTTC
TNF-α	AAGCCTGTAGCCCACGTCGT	CGTAGTCGGGGCAGCCTTGTC
LCN2	AAAGACCCGCAAAAGATGTATG	AACCTGGAACAAAAGTCCTGAT
GAPDH	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC

Table 2. Effects of camel milk on MDA, SOD and GSH levels in mouse liver.

Group	MDA(nmol/g)	SOD(U/g)	GSH(ng/g)
N	120.193±10.123	1752.153±84.678	52.661±3.162
C	115.119±8.265	1816.094±78.356*	55.574±2.406*

Each value represents the mean±SD. * $p<0.05$, compared with N group.

and differentiation (Koshibu and Levitt, 2005). In human cancers, studies have shown that TGF-α could serve as a tumour marker and as a marker for the malignant potential of a tumour (Grigioni, 2002). To date, the types of carcinomas with which abnormal TGF-α expression has been associated include liver, breast and ovarian cancers (Zhang *et al,* 2004). Our result showed that the expression of TGF-α was down-regulated in mice receiving camel milk and this could explain why consumption of camel milk can halt the progression of hepatocellular carcinoma.

Liver injury is reportedly associated with a chronic inflammatory response involving TNF-α (Farinati *et al,* 2006). Hepatocyte apoptosis induced by TNF-α is a common pathological phenomenon and the mechanism driving many liver pathologies in the early stage. TNF-α is associated with NAFLD and induced inflammatory cytokines formation (Tilg *et al,* 2011). More importantly, TNF-α-mediated hepatocyte injury not only leads directly to hepatocyte necrosis, but also mediates apoptosis (Guicciardi *et al,* 2001). Our result showed that the expression of TNF-α was down-

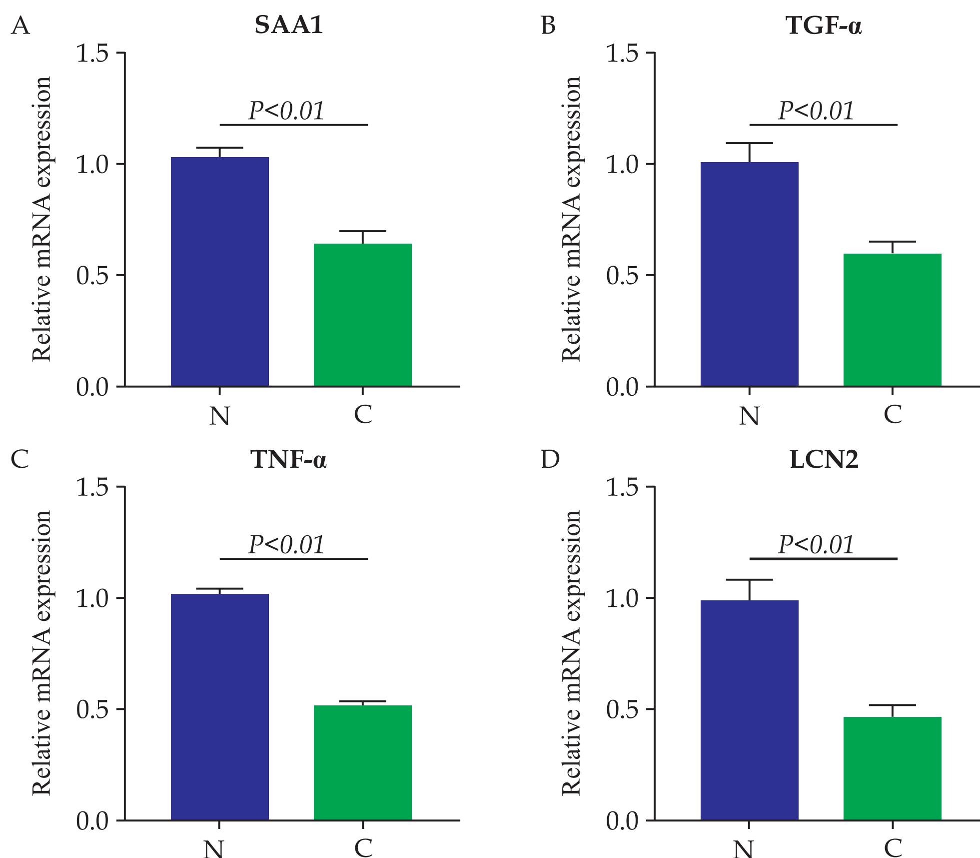

Fig 2. Relative mRNA expressions of SAA1, TGF-α, TNF-α and LCN2 in mouse liver.

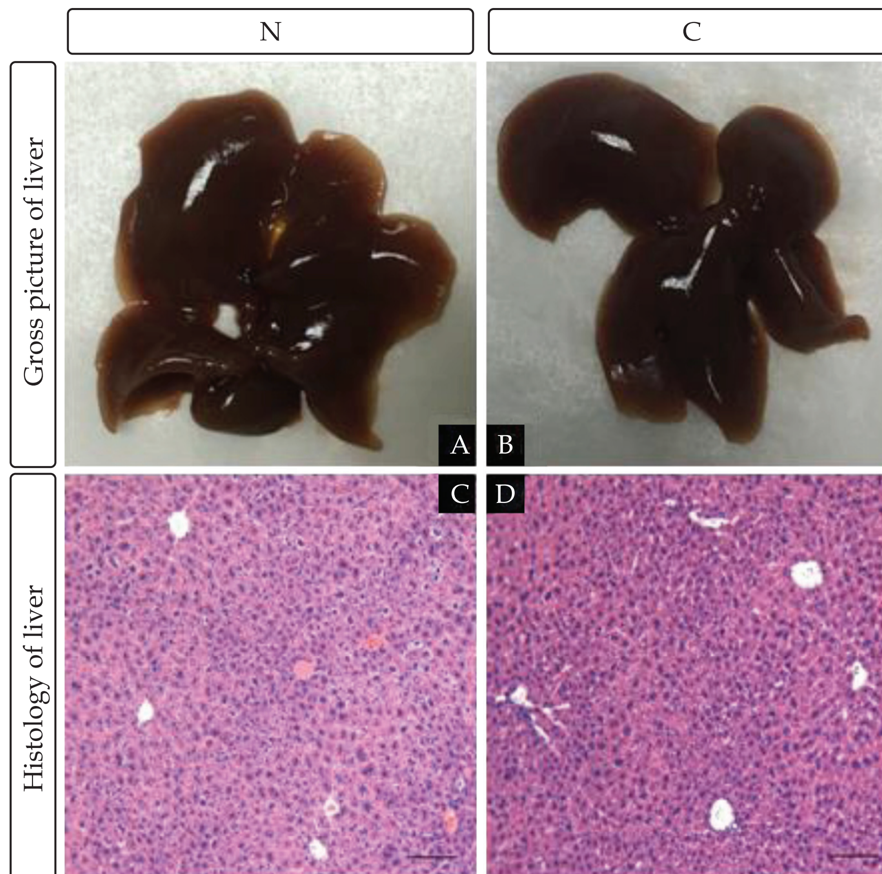

Fig 3. Gross and histology changes in the liver from normal group and camel milk group mice. HE staining (20X).

regulated in the livers of mice receiving camels milk, again explains hepato-protective action of camels milk.

The protein LCN2 is a secretory glycoprotein that is secreted by liver cells and may play a protective role when stimulated by inflammatory factors; it is up-regulated during many cellular stresses (Zhang *et al,* 2014). There is increasing evidence that LCN2 plays a protective role in liver injury. Wan *et al* (2017) found that inhibition of inflammation by curcumin was related to LCN2 down-regulation. We found down-regulation in the expression of LCN2 in the livers of mice receiving camel milk, showing that camel milk can protect liver cells.

Our results in mice have revealed that camel milk treatment could protect the liver by decreasing the levels of ALT, AST and MDA, increasing SOD and GSH activity and down-regulating mRNA expression of SAA1, TGF-α, TNF-α and LCN2. Thus, regular consumption of camel milk could increase the antioxidant capacity of the liver and regulate gene expression; in this way camel milk can prevent the liver from being damaged. Camel milk is a potential liver-protective food without any side effects.

In conclusion, this study has indicated the protective effect of camel milk in mice. The mechanism for liver protection was restoration/ enhancement of the activities of antioxidant enzymes and the inhibition of lipid peroxidation. By enhancing the antioxidant ability of hepatocytes camel milk decreased various toxic substance-induced oxidative stresses in the liver. Further studies with individual active compounds that exist in camel milk are underway which will enable us to understand the exact mechanisms responsible for liver protection.

Acknowledgements

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Ethical Approval

All animal procedures were approved by Animal Care and Use Committee at Inner Mongolia Agricultural University.

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