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CAMEL MILK ALLEVIATES ALCOHOL INDUCED LIVER INJURY IN MICE

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ABSTRACT

The present study aimed to investigate the effectiveness of camel milk (CM) in alleviating alcohol-induced hepatotoxicity as a model of clinical liver illness. Male mice were divided into 4 groups: pair-fed control (PE), ethanol (ET), camel milk treated group (CM) and cow milk treated group (NM). Mice from the PE ingested a Lieber-DeCarli controls liquid diet, while mice from the ET, CM and NM groups ingested a Lieber-DeCarli alcohol liquid diet of 8 weeks. Camel milk and cow milk were orally administered from the beginning of the 4th week until the end of the experiment. Mice from camel milk group was observed to significantly reduce the levels of serum ALT, AST and LPS compared with other groups. The hepatic steatosis was improved after camel milk administration and attenuated alcohol-induced oxidative stress by declining the level of hepatic oxidants. In addition, the production of apoptosis was inhibited by preventing the formation of TNF-α in the CM group. These results showed that camel milk may alleviate alcohol-associated hazards and protect hepatic tissue from alcohol-induced toxicity.

Key words: Alcohol liver injury, camel milk, hepatoprotective, lipopolysaccharide

Alcoholic liver disease (ALD) is one of the most prevalent liver diseases in Europe and the United States. The disease can be caused by the chronic consumption of alcohol exceeding a certain daily amount (>40g/day) (Seitz *et al*, 2018), which varies from different individuals (Akinyemiju *et al*, 2017). In the early stages of the disease, alcoholic fatty liver (an accumulation of triglycerides in hepatocytes) usually develop and progress to alcoholic hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma. According to the statistics, chronic heavy alcohol consumption (>40g of alcohol per day) of a sustained period (months or years) will result in 90-100% individuals developing alcoholic fatty liver. Among them, only 10-35% individuals who continue with chronic heavy alcohol consumption will develop alcoholic steatohepatitis, which is characterised by inflammation of the liver with concurrent fat accumulation in liver. While, only 8-20% chronic heavy drinkers develop alcoholic liver cirrhosis, of these patients, 2% develop hepatocellular cancer per year.

Comprehensive research shows that camel milk has inestimable value to human disease prevention, health and longevity (Hammam, 2019). In recent years, a great deal of research has been conducted on the therapeutic effects of camel milk on the improvement in liver disease symptoms and treatment side effects. The treatment of ethanol-induced hepatotoxicity in rats with camel milk had a tendency to alleviate liver damage by its antioxidant activity or to its chelate effects on toxicants (Elhag *et al*, 2017). A research on rats revealed that camel milk may improve hypercholesterolemia (Meena *et al*, 2018). Overall, camel milk contains factors that may be protective and therapeutic to the liver disease. Camel milk has a wide range of an antioxidant, antibacterial and immunomodulatory properties. However, scarce research has been done on the protective effect of camel milk on alcoholic liver injury. In this study, we investigated the therapeutic and repairing effects imparted by camel milk in a mouse model of ALD.

Materials and Methods

Chemicals

Ethanol (≥99.9%, absolute ethanol) was purchased from FUCHEN Chemical Reagents Company (Tianjin, China), while camel milk and cow milk were purchased from camel farm in Bayan Nur City, Inner Mongolia, China.

Alanine transaminase (ALT), aspartate transaminase (AST), malondialdehyde (MDA),

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superoxide dismutase (SOD), glutathione (GSH) and triglyceride (TG) kits were provided from Nanjing Jiancheng Bioengineering Institute, China.

Caspase-3, lipopolysaccharide (LPS), interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-a(TNF-α) were determined using enzyme-linked immunosorbent assay (ELISA) kits that were provided by Shanghai Enzyme-linked Biotechnology Co, Ltd, China.

Animal Treatment

Male C57BL/6N mice weighing 18-20 g were obtained from Charles River (Beijing Vital River Laboratory Animal Technology Co. Ltd, China). The animals were housed in standard plastic cages in an environmentally controlled room which was housed in a temperature (25±2°C) and relative humidity (65%-75%) with a 12 : 12 light dark cycle (Yu *et al*, 2016). The animals received humane care and all protocols were approved by Animal Care and Use Committee at Inner Mongolia Agricultural University. After one week of acclimatisation with chow diet, the animals were randomly divided into 4 groups of 6 animals in each group. Pair-fed with isocaloric Lieber-DeCarli liquid diets (TROPHIC Animal Feed High-Tech Co. Ltd, China) for 8 weeks as the following:

(1) Pair-fed control group (PF, n=6), receiving regular Lieber–DeCarli liquids diets;

(2) Ethanol group (ET, n=6), receiving ethanol containing Lieber–DeCarli liquid diets (28% of total calories as ethanol);

(3) Camel milk treated group (CM, n=6), receiving ethanol containing Lieber–DeCarli liquid diets (28% of total calories as ethanol) and administrated camel milk by the gavage (3g/kg body weight);

(4) Cow milk treated group (NM, n=6), receiving ethanol containing Lieber–DeCarli liquid diets (28% of total calories as ethanol) and administrated milk by the gavage (3g/kg body weight); The ethanol content of the diet gradually increased over a 7-day period (no ethanol for day 2, one fourth of the given amount for days 3-4, half of the given amount for days 5-6, two-thirds of the given amount for days 7-8 and full amount for the rest). At the ninth week, ethanol-fed and pair-fed mice were gavaged in the early morning with a single dose of ethanol (5 g kg- 1 body weight) or isocaloric maltose dextrin and euthanised 9 h later. Blood samples were collected by the retrobulbar vessels and allowed to clot for 45 min at room temperature. After standing for 3 h, the serum was separated by centrifugation (1500 rpm, 10 min and 4°C) and stored at - 80°C for biochemical analysis. In addition, body weight of the mice was weighed once a week, before and after the experiment. After the experiment, the mice in different groups were sacrificed promptly by cervical vertebra dislocation. Livers were dissected quickly, washed twice with phosphate buffer saline, blotted dry on a filter paper and wet weight were measured then stored at - 80°C (Li *et al*, 2015).

Biochemical 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 the ET group. The levels of ALT and AST in mouse serum were determined using commercial assay kits according to the manufacturer's protocols.

Measurement of LPS level in serum

The LPS level in serum was measured by using enzyme-linked immunosorbent assay (ELISA) kit according to the procedure supplied by the manufacturer.

Determination of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) levels in the liver

After homogenisation and centrifugation, the supernatants of liver tissues were evaluated for the content of malondialdehyde (MDA), the activity glutathione (GSH) and superoxide dismutase (SOD) were measured according to the commercial assay kits.

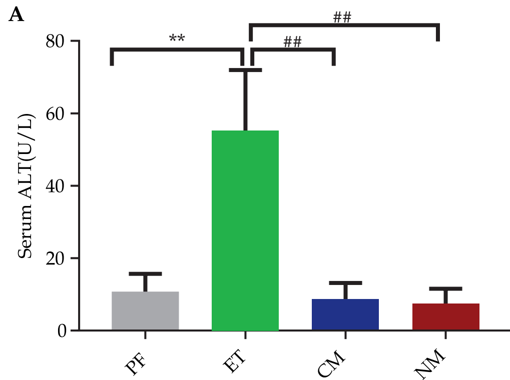
Determination of triglyceride (TG), Caspase-3 activity and TNF-α, IL-1β and IL-6 levels in liver tissue

The level of TG in liver was measured according to the commercial assay kits.

Caspase-3 activity, TNF-α, IL-1β and IL-6 levels were measured by using enzyme-linked immunosorbent assay (ELISA) kit according to the procedure supplied by the manufacturer.

Statistical Analysis

The data were expressed as mean ± SD values. One-way analysis of variance (ANOVA) was used for



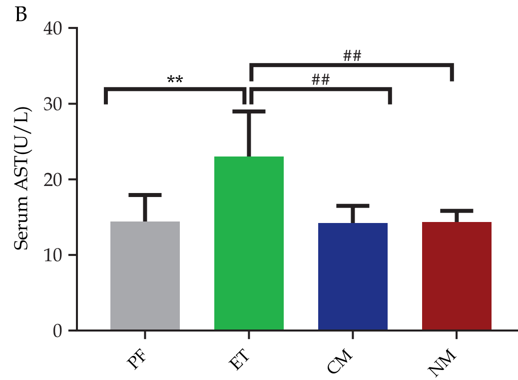


Fig 1. Effects of camel milk on serum ALT(A) and AST(B) activities. All data are expressed as mean ± SEM. ** $p < 0.01$ *versus* PF group, ## $p < 0.01$ *versus* ET group. PF= Pair-fed control group, ET= Ethanol group, CM= Camel milk treated group, NM=Cow milk treated group.

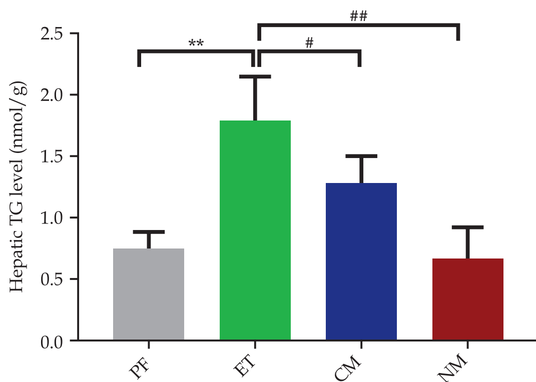


Fig 2. Effects of camel milk on TG level in liver tissue. All data are expressed as mean ± SEM. **$p < 0.01$ *versus* PF group, # $p < 0.05$, ##$p < 0.01$ *versus* ET group. PF= Pair-fed control group, ET= Ethanol group, CM= Camel milk treated group, NM= Cow milk treated group.

the comparison of more than 2 mean values. Results were considered to be statistically significant when $p < 0.05$ and $p < 0.01$.

Results and Discussion

The body weight of the mice from ET group did not change in comparison with PF group but in CM group it showed a decreasing trend ($p>0.05$) (Table 1).

Organ index of the liver was evaluated in mice. Liver index was increased in mice from ET group compared with the PF group. While the liver index from CM group had the decreasing trend ($p>0.05$) (Table 1).

The above results showed that consumption of the ethanol diet had a significant effect on mice weights, resulting in decreased body weight compared with PF group. There are no significant changes in final body weight, liver weight, or liver weight as a percentage of body weight of mice in CM group.

Table 1. Effects of camel milk on body weight and organ index in mice.

Group	Initial Wts (g)	Final Wts (g)	Liver Index (×100, $mg \cdot g^{-1}$)
PF	23.03±1.53	34.79±4.85	4.33±0.81
ET	24.91±0.63	29.78±2.33	4.16±0.23
CM	24.12±1.02	29.18±2.40	4.09±0.23
NM	22.7±2.04	26.32±3.11	4.49±0.50

All data are expressed as mean ± SEM. * $p < 0.05$ PF group, # $p < 0.05$ ET group. PF= Pair-fed control group, ET= Ethanol group, CM= Camel milk treated group, NM=Cow milk treated group.

The increase of AST and ALT in the blood indirectly reflects liver failure caused by ethanol induced hepatotoxicity. The levels of ALT and AST in serum were significantly elevated at 9 h following gavages of alcohol to mice ($p< 0.01$) (Fig 1A). This indicated liver cell damage in the mice and the model of alcoholic liver injury had been established successfully. The levels of ALT and AST were significantly decreased in the CM group and NM group of mice compared to the ET group ($p< 0.01$) (Fig 1B), indicating that the gavage of camel milk and cow milk can protect the liver of mice from the damage to ethanol and reduced the content of ALT and AST in serum.

Camel milk is widely used in various populations for the treatment and prevention of diseases (Dubey *et al*, 2016). 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 (Lbrahim *et al*, 2017). Our results revealed that compared with the PF group, SOD and GSH levels in the liver of the ET group of mice showed a downward trend and the MDA level significantly increased

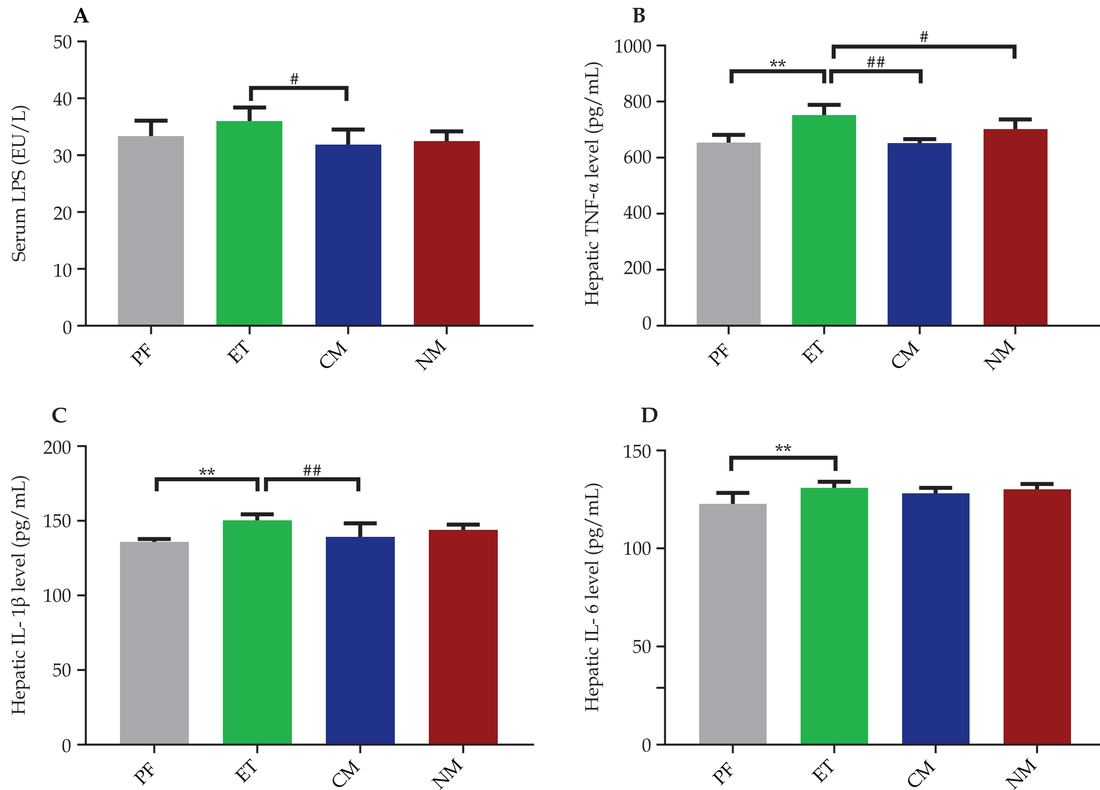


Fig 3. Effect of camel milk on alcohol-induced inflammation and on serum LPS level. All data are expressed as mean ± SEM. ** p < 0.01 *versus* PF group, # p < 0.05, ## p < 0.01 *versus* ET group. PF= Pair-fed control group, ET= Ethanol group, CM= Camel milk treated group, NM= Cow milk treated group.

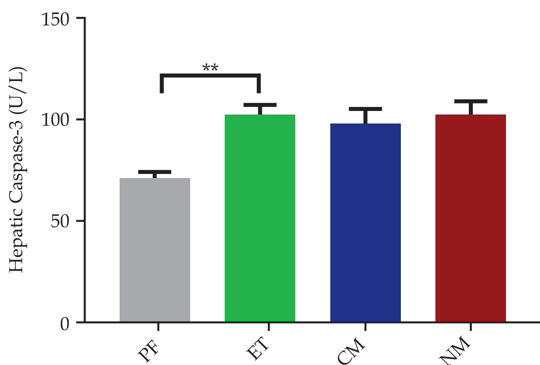


Fig 4. Effects of camel milk on Caspase-3 levels in liver tissue. All data are expressed as mean ± SEM. **p < 0.01 *versus* PF group. PF= Pair-fed control group, ET= Ethanol group, CM= Camel milk treated group, NM= Cow milk treated group.

(p<0.05) (Table 2). The results of this experiment indicated that a more severe lipid peroxidation occurred in the mice of the ET group. Compared with the ET group, the levels of SOD and GSH in the liver of the CM and NM groups of mice showed an upward trend. There was no difference in MDA levels in liver of NM group mice, while MDA levels in liver of CM group decreased significantly (p<0.05). We found that our results are consistent with previous studies (Mihic *et al*, 2016). Camel milk could protect the liver by decreasing the levels of MDA and increasing the activities of SOD and GSH in mice. An increase in the level of MDA in the liver enhances peroxidation and can lead to tissue damage and failure of the antioxidant defense mechanisms.

Chronic exposure to ethanol induces glutathione depletion, which makes hepatocytes more sensitive to oxidative stress (Wheeler *et al*, 2001) as reducing (not oxidised) glutathione protects cells against ROS (Louvet and Mathurin, 2015). 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 protect against oxidative tissue-damage (Diaz Castro *et al*, 2013) and suggest that these two enzymes contribute to the hepatoprotective effects of the mice CM group.

When alcohol is ingested excessively, fat metabolism in the body is impaired. The decomposition of fatty acids was inhibited and synthesis was strengthened (Ni *et al*, 2013), which would lead to fat accumulation in liver cells and

clinically manifested as liver triglyceride, high cholesterol levels (Wang *et al*, 2016). The level of TG was significantly increased in the liver of the mice from ET group compared with the PF group (p<0.01) (Fig 2). The level of TG was significantly decreased in the livers of the mice from CM group (p<0.05) and NM group (p<0.01) compared with the ET group. The above results effectively illustrated the inhibitory effect of camel milk and cow milk on liver lipid accumulation caused by alcohol.

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

Group	MDA (nmol/mg prot)	SOD (U/mg prot)	GSH (μmol/g prot)
PF	1.47±0.22	1137.61±74.43	14.16±6.35
ET	2.10±0.44*	981.82±143.65	9.69±2.23
CM	1.56±0.20#	1018.62±44.87	27.30±3.67##
NM	1.63±0.27	956.33±49.19	21.23±6.86##

All data are expressed as mean ± SEM. * p < 0.05 versus PF group, # p < 0.05, ## p < 0.01 *versus* ET group. PF= Pair-fed control group, ET= Ethanol group, CM= Camel milk treated group, NM= Cow milk treated group.

Increased serum level of LPS was commonly found in patients with ALD. Alcohol consumption not only caused enteric dysbiosis and bacterial overgrowth, but also increased gut permeability and the translocation of bacteria-derived LPS from the gut to the liver (Yan *et al*, 2011). LPS can stimulate Kupffer cells to produce reactive oxygen species and cytokines (including TNF-α) that causes hepatocellular damage and activate TLR4 signaling in liver sinusoidal endothelial cells, resulting in the regulation of angiogenesis and subsequent promotion of fibrogenesis (Tamai *et al*, 2002).

Collectively, the role of bacterial translocation in the pathogenesis of ALD had been clearly established (Crabb, 1999). Chronic alcohol models increased circulating concentrations of lipopolysaccharide (LPS) compared with the PF group and the severity of hepatic injury was correlated to serum level of LPS (p>0.05) (Fig 3A). The level of LPS was significantly decreased from the CM group of mice compared with the ET group (p<0.05). The above results indicated that gavage of camel milk can protect the liver of mice from the damage to ethanol and reduced the content of LPS in serum.

The production of TNF-α is one of the earliest events in many types of liver injury, triggering the production of hepatocyte, other inflammation and apoptosis (Takahashi *et al*, 2012; Marks *et al*, 1990). Chronic alcohol models increased liver tissue TNF-α (Fig 3B) and several TNF-α inducible cytokines (Parkin *et al*, 2019), such as interleukin-1β (Fig 3C) and interleukin-6 (Fig 3D) are also increased compared with the PF group (p<0.01). Compared with the ET group, we found that IL-6 in the liver of CM group mice showed a downward trend, while TNF-α and IL-1β levels were significantly decreased (p<0.01). In the NM group, liver TNF-α level was significantly decreased (p<0.05) and IL-6 and IL-1β levels were not significantly different. Through the above results, camel milk by inhibiting the formation of TNF-α to reduce the effect of inflammatory response is more obvious than cow milk.

Hepatocyte apoptosis is an important pathologic feature of human ALD. Apoptosis results from multiple mechanisms, including ethanol mediated hepatotoxicity, induction of oxidative stress, inhibition of survival genes (c-Met) and induction of proapoptotic signaling molecules (TNF-a and Fas ligand) (Takahashi *et al*, 2012). It could be postulated that the inflammation of liver cells, produced upon ethanol intoxication, might lead to apoptosis of hepatocytes *via* caspase-3 activation. Osawa *et al* (2001) had demonstrated the involvement of caspase cascade activation in TNF-α induced hepatocytes apoptosis (Osawa *et al*, 2001). The activity of Caspase-3 was significantly increased to the livers of the mice from ET group compared with the PF group (p<0.01) (Fig 4) and the level of Caspase-3 was decreased to the livers of the mice from CM group compared with the ET group (p>0.05) (Fig 4). We found that our results were consistent with previous studies. Gavage of camel milk can protect the liver of mice from the damage to ethanol, again explains hepatoprotective action of camel milk.

These results revealed that chronic ethanol feeding plus a single binge (the NIAAA model) can cause chronic liver damage (Stice *et al*, 2015), which was manifested by significantly increased serum ALT, AST and LPS levels in mice (Bertola *et al*, 2013). Camel milk treatment could protect the liver by decreasing the levels of ALT, AST, LPS, MDA and TG, increasing SOD and GSH activity as well as decrease levels of TNF-α, IL-1β and IL-6 content and Caspase-3 activity. Thus, regular consumption of camel milk could increase the antioxidant capacity of the liver and regulate inflammation and apoptosis, in this way camel milk can prevent the liver from being damaged. Camel milk is a potential liver-protective food without any side effects.

This study indicated the protective effect of camel milk in mice. It could be postulated that

camel milk exerted its effect of ethanol-induced hepatotoxicity via modulating the extent of lipid peroxidation, augmenting the antioxidant defense system decrease LPS. Inhibition of ROS and LPS generation might account for the observed decrease in TNF-α, IL-1β and IL-6 levels and caspase-3 activity following camel milk treat. Thus, it is likely that oxidative stress and LPS are a common signal transducer for a diverse cell death-inducing stimulate. Camel milk through enhancing the antioxidant ability and decreasing the LPS level of decreased various toxic substance-induced oxidative stresses in the liver. The protective effect of cow milk on mice liver is much worse than camel milk. Therefore, it is of great research value to further study the individual active compounds present in camel milk. This will enable us to understand the exact mechanisms responsible for liver protection.

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