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ANTIOXIDANT EFFECT OF CAMEL MILK ON ACUTE ALCOHOLIC LIVER INJURY IN MICE

Bule QI[1], Dandan Wu[1], Xiaoyun Wu[1], Naqin[1], Shiqi Hao[1], Rimutu Ji[1,2] and Liang Ming[1,2]

[1]Key Laboratory of Dairy Biotechnology and Bioengineering, Ministry of Education, College of Food Science and Engineering, Inner Mongolia Agricultural University, 010018, Hohhot, China

[2]Camel Research Institute of Inner Mongolia, 737300, Alashan, China

ABSTRACT

This study aims to explore the protective effect of camel milk (CM) on oxidative stress in mice and analyse its mechanism of action. The experimental animals were randomly divided into four groups: NC (normal diet), ET (normal diet, then ethanol), CM (normal diet and CM) and ET+CM (normal diet and CM, then ethanol). Then by measuring serum and liver tissue superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activity, glutathione (GSH), malondialdehyde (MDA) and other indicators to explore its antioxidant capacity. The results showed that CM attenuated ethanol-induced hepatotoxicity by reducing elevated liver enzyme, decreasing ethanol-induced reactive oxygen species (ROS) generation, inhibiting malonaldehyde (MDA) levels and reversing depletion of antioxidative defense system in mice liver. Furthermore, CM ameliorated ethanol-induced oxidative stress by down-regulating the expression of cytochrome P4502 E1 (CYP2E1) enzyme. These data suggest that CM, a health-promoting antioxidant food, exhibits hepatoprotective effects. The hepatoprotective mechanisms of CM were associated with alleviation of ethanol-induced oxidative stress through inhibiting CYP2E1 enzyme.

Key words: Alcoholic liver injury, Camel milk, CYP2E1, Oxidative damage

Liver is the main organ for alcohol metabolism, more than 80% of the ingested alcohol is metabolised in the liver (Stornetta *et al*, 2018). The course of Alcoholic liver disease (ALD) usually manifests as alcoholic fatty liver (AFL) at the beginning and turns into alcoholic steatohepatitis (ASH), alcoholic liver fibrosis and alcoholic cirrhosis. Excessive drinking can also induce extensive hepatocyte necrosis or even liver failure during severe hepatocellular cancer (HCC) (Banerjee *et al*, 2013; Liu *et al*, 2017). Alcohol metabolism will produce reactive oxygen species (ROS) and oxidative stress, which is the main mechanism implicated in the pathogenesis of ALD (Cai *et al*, 2018; Leung and Nieto, 2013).

Camel milk is considered one of the most valuable food sources for nomadic people in arid and semi-arid areas and has been consumed for centuries due to its nutritional values and medicinal properties (Dowelmadina *et al*, 2014; Yadav *et al*, 2015; Kaskous, 2016; Kula and Dechasa, 2016). It has high quality of composition and various bioactive ingredients, showing special properties that make it distinct and unique compared to other species milk (Wernery, 2007; Smits *et al*, 2011; Hamed *et al*, 2012; Yadav *et al*, 2015). The past decade, researchers have found a dramatic increase and growing interest in the use of camel milk for its medicinal values; while bioactive ingredients of camel milk have gained significant attention to scientists all over the world to study their potential health benefits (Rasheed *et al*, 2016; Gader and Alhaider, 2016; Mullaicharam, 2014). Scientifically, it has been proved that camel milk ingredients are excellent for nutritional view point as it contains high levels of lacto-peroxidase, immunoglobulin G (IgG), secretory immunoglobulin A (SIgA), copper, iron and vitamin C which giving it superior antioxidant and antimicrobial activities (Haj and Kanhal, 2010; Hailu *et al*, 2016). Camel milk can reduce liver damage caused by alcohol and protect liver tissue from alcoholism by improving liver tissue malondialdehyde, total antioxidant activity, tumour necrosis factor and caspase activity in alcoholic liver disease rats (Darwish *et al*, 2012).

In preent study, ICR mice were used to establish an animal model of acute alcohol liver injury to explore the protective effect of camel milk on oxidative stress in mice and to analyse its mechanism of action.

Materials and Methods

Preparation of vacuum freeze-dried skimmed milk powder

After the camel milk was collected, it was quickly cooled to 4°C and the upper fat was removed

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by centrifugation at 3500rpm/min for 40min. After heating in a 60°C water bath for 30min, it was vacuum freeze-dried. The obtained camel milk powder was stored in a refrigerator at -20°C and sealed.

Animals and experimental design

Male ICR mice (25±1g) were obtained from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. (China; License Number SCXK 2016 -0006). The mice were housed in ventilated polypropylene cages (3 mice per cage) maintained at 22 ± 2°C with 50 ± 5% humidity and subjected to a 12h light/dark cycle. After 1 week of acclimation, all the mice were randomly separated into 4 groups as follows: (I) normal group (NC, n = 10); (II) camel milk group (CM, n = 10); (III) ethanol group (ET, n = 10); (IV) ethanol and camel milk group (ET+CM, n = 10). The initial body weights were controlled similar, and there were no significant differences among the groups. CM and ET+CM groups were subjected to oral camel milk treatments twice daily for 14 days via oral gavage, NC and ET groups only received 0.3mL of distilled water. The mice fasted for 6h after the last medical treatment for inducing hepatotoxicity by administering 50% alcohol (7.3 g/kg bw) to groups ET and ET+CM via oral gavage in 3 equal doses administered at 1h intervals. After 6h, the mice were anaesthetised with ethylether for sampling blood from eyeball extirpation. After incubating the blood samples at 37°C for 45min, serum was collected by centrifugation at 3000rpm for 20min at 4°C. The liver was excised and weighed after mouse were dissected and stored at -80°C.

Analysis of AST and ALT

The Blood samples were centrifuged at 3000rpm (20min, 4°C), and then detected alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum via the kits (C010-2-1 and C009-2-1). All operating steps were carried out in strict accordance with the instructions and then the absorbance was measured via using a microplate reader.

Determination of serum ROS production

According to the manufacturer's instructions (Shanghai Enzyme-linked Biotechnology Co, Shanghai, China; ml009876-1) and as previously described, ROS antibody was precoated on an ELISA plate. The serum/liver homogenate samples were added into ELISA plate wells and incubated for 60min at 37°C. Detecting HER labeling polyclonal antibody was then added into ELISA plate. Next, the avidin-peroxidase conjugates were added into ELISA plate and incubated for 15min. Finally, TMB substrates for coloring were added into ELISA plate for 15min. Absorbance of serum samples at 450nm was measured using a plate reader and serum ROS was obtained.

Determination of biochemistry

The levels of Malondialdehyde (MDA), Glutathione Peroxidase (GSH-Px), NADPH oxidase, Glutathione (GSH) and Superoxide Dismutase (SOD) were detected using kits (ml114150, ml058194, ml214840, ml063305 and ml001998) in accordance with the protocols from the manufacturer.

Quantitative RT-PCR analysis

Total liver RNA was extracted using RNAiso Plus (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions and reverse-transcribed into cDNA with the PrimeScript RT reagent kit (TaKaRa Bio, Otsu, Japan). cDNA was amplified by real-time quantitative polymerase chain reaction (RT-qPCR) using the SYBR Premix Ex TaqII (TaKaRa Bio, Otsu, Japan) and the appropriate primers shown in Table 1. The thermocycler conditions used were 95°C for 30s, followed by 40 cycles of 95°C for 5s and 60°C for 30s. The relative mRNA expression of carnitine cytochrome P4502E1 (CYP2E1) was calculated by the $2^{-\Delta\Delta Ct}$ method; β-actin genes were used as internal controls.

Data analysis

All data were presented as means ± standard error of the mean (SEM) and analysed by GraphPad prism 5.03 (GraphPad Software Inc., San Diego, CA, USA). Comparisons among all groups were performed with one-way analysis of variance (ANOVA) test.

Results and Discussion

Effect of camel milk on the activity of serum enzymes

ALT and AST are commonly used as indicators of liver function and health status, which can reflect

Table 1. Oligonucleotide sequence of primers for RT-qPCR.

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
β-Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
CYP2E1	AGGGGACATTCCTGTGTTCC	TTACCCTGTTTCCCCATTCC

the liver damage and health status of animals. To further confirmed the protective effects of camel milk, the degree of cellular injury caused by ethanol was estimated by the leakage of ALT and AST enzymes from the hepatocytes. Table 2 shows that the effects of CM on serum biochemical values in mice with acute alcoholic liver injury. As compared with the NC group, the serum ALT and AST activities of the ET group were significantly increased, indicating that the ALD mice were successfully modeled. The activities of the two enzymes in serum were decreased to various extents by intragastric administration of CM. Collectively, these results indicate that CM suppresses ethanol-induced liver damage in mice and has protective effect on alcoholic liver injury.

Effect of camel milk on liver index and oxidative stress in the liver

The liver index of mice can partly reflect the degree of liver injury. Ethanol causes an increased liver index in experimental animal models including rats and mice (Luo *et al*, 2013; Liu *et al*, 2015). Ethanol causes liver enlargement, hyperplasia, hyperemia, oedema and other diseases. After intragastric administration of alcohol, the liver index (liver weight/body weight) of the ET group increased significantly as compared with the NC group (Table 3). There was no significant difference in liver index between the mice in the ET+CM and NC group. Compared with the ET group, the liver index of the ET+CM group was significantly lower (P<0.001), indicating that camel milk can alleviate liver damage in mice.

Ethanol-induced liver injury is a common occurrence in liver disease, which has been considered to be a global health problem (Koch *et al*, 2004). Ethanol-induced oxidative stress is widely considered to play a major role in mechanisms of ethanol-induced liver injury (Cederbaum *et al*, 2009). Therefore, using appropriate antioxidant agents to inhibit oxidative stress is an attractive approach for prevention and treatment of ethanol-induced liver injury. SOD is an endogenous antioxidant enzyme that can catalyse the disproportionation of superoxide to generate H_2O_2, which is then decomposed into water under the action of catalase in the cytoplasm (Kalyanaraman, 2013; Wang *et al*, 2018). MDA is known as an end product of lipid peroxidation and is a significant marker of the increased oxidative stress. GSH is a major nonprotein thiol antioxidant, which plays a central role in coordinating antioxidant defense in the living organisms. It is involved in the maintenance of normal cell structure and function, probably through its redox and detoxification reactions (Hu *et al*, 2015).

Thus several indicators for hepatic oxidative stress including GSH, GSH-Px, MDA and SOD were assayed to examine whether camel milk could ameliorate liver oxidative stresses induced by alcohol-caused injuries. Excessive drinking could cause a significant increase in hepatic MDA level (Fig 1C) and sharp decline of hepatic GSH activities, GSH-Px activities and SOD contents (Fig 1A, B and D) in comparison with the NC groups. This indicated that the oxidative stress level in the liver of ALD mice was significantly increased. Compared with the ET group, the MDA level of the ET+CM group was significantly reduced, and the GSH, GSH-Px and SOD were significantly increased. Taken together, these data suggest that CM can alleviate ethanol-induced oxidative stress through inhibition of MDA levels and elevation of antioxidative defense system.

Recent studies have demonstrated that excess ethanol triggers the increase of ROS generation in liver tissues (Ye *et al*, 2015; Sid *et al*, 2013). As a vital marker of oxidative stress, ROS production plays an important role in the pathogenesis of various liver

Tabel 2. Effects of camel milk on serum ALT and AST in mice.

Parameters	NC	ET	CM	ET+CM
ALT(U/L)	15.91±1.01	30.65±3.625$^{\#\#}$	7.497±0.415$^{\#**}$	18.96±0.799**
AST(U/L)	22.18±0.667	40.61±4.311$^{\#\#}$	14.36±0.551**	26.17±1.104**

Data are expressed as mean ± SEM of 10 animals. Significantly different from the normal group at $^{\#}p< 0.01$, $^{\#\#}p< 0.001$. Significantly different from the ethanol-treated group at $^{*}p< 0.01$, $^{**}p< 0.001$. NC: normal group, ET: ethanol group, CM: camel milk group, ET+CM: ethanol and camel milk group.

Table 3. Effects of camel milk on liver index in ALD mice.

Group	NC	ET	CM	ET+CM
Liver index± SEM	3.77±0.051	4.0±0.038$^{\#\#}$	3.766±0.058**	3.782±0.046**

Liver index (%) = Liver weight/Body weight × 100%.Significantly different from the control group at $^{\#\#}P< 0.001$; significantly different from the model group at $^{**}P< 0.001$. NC: control group, ET: ethanol group, CM: camel milk group, ET+CM: ethanol and camel milk group.

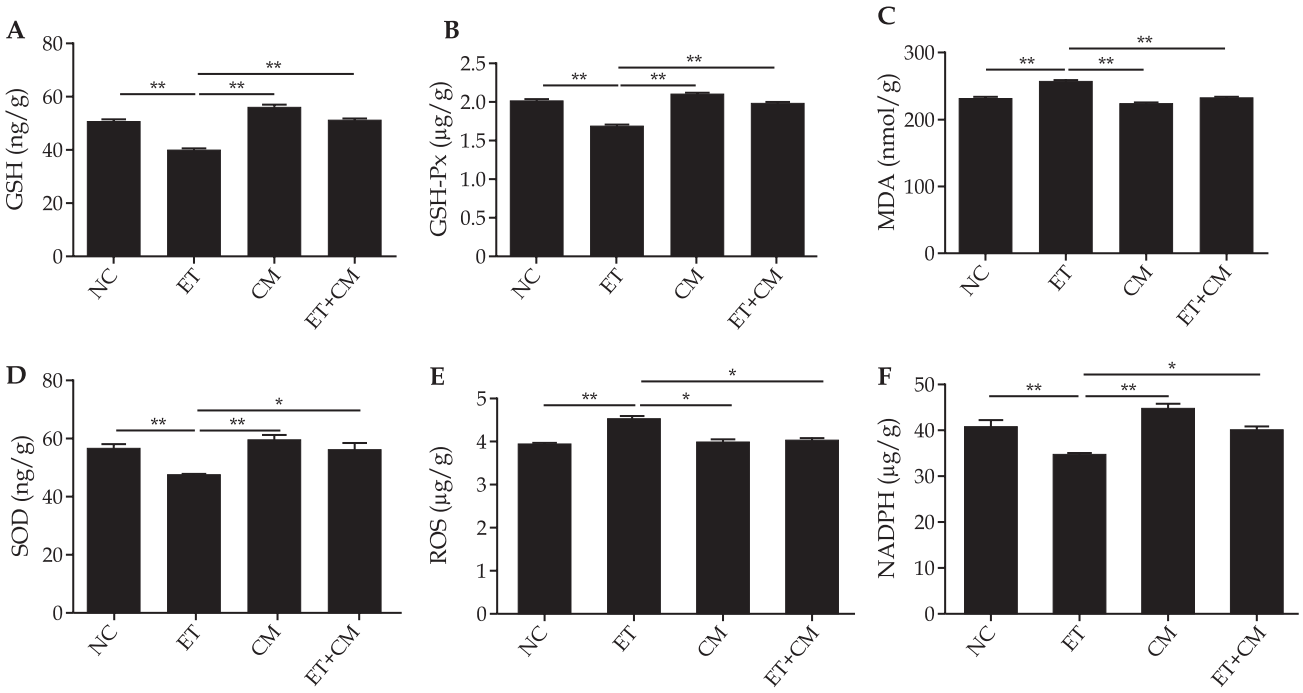

Fig 1. Effects of camel milk on the antioxidant capacity of the liver tissues in ALD mice. (A) GSH level, (B) MDA level, (C) GSH-Px level, (D) SOD level, (E) ROS level and (F) NADPH level were measured with microplate reader. Data are expressed as mean ± S.E.M. (n = 10) for each group. *P < 0.01, **P < 0.001. NC: normal group, ET: ethanol group, CM: camel milk group, ET+CM: ethanol and camel milk group.

disorders including alcoholic liver disease (Jiang *et al*, 2014). To test the effects of CM on oxidative stress induced by alcohol, the production of ROS was further measured. As shown in Figure 1E, ethanol treatment significantly increased ROS level compared with the control group, whereas CM pretreatment markedly reduced ROS generation in ethanol-treated mice. Accordingly, our findings indicate that CM exhibits hepatoprotective capability and suppresses ethanol-induced liver damage via inhibition of oxidative stress.

NADPH oxidase in hepatic Kupffer cells plays a predominant role in the pathogenesis of early alcohol-induced liver injury by stimulating the generation of ROS and proinflammatory cytokines (Mochel *et al*, 2010; Lee and Yang, 2012). To further elucidate the mechanism involved in the protective effect of CM on ethanol-induced liver injury, activation of NADPH oxidase was analysed. As shown in Figure 1F, ethanol treatment significantly decreased NADPH level compared with the NC group, whereas CM pretreatment markedly increased the production of NADPH in serum and liver homogenate of ethanol-treated mice. The results imply that CM improves the level of NADPH oxidase, which can alleviate ethanol-induced liver injury.

Effect of camel milk on oxidative stress in the serum

It can be seen from Fig 2 that at a dose of 7.3g/kgbw of 50% ethanol solution, the serum levels of GSH, GSH-Px, MDA, SOD, ROS and NADPH in the ET group were significantly different from those in the NC group (P<0.01, P<0.001), indicating that acute alcohol induction mice produce oxidative damage, which lead to a decrease or increase in the level of antioxidant substances in the mice serum. Compared with the ET group, the serum GSH (P<0.001), GSH-Px (P<0.001), MDA (P<0.001), SOD (P<0.01) ROS (P<0.01) and NADPH (P<0.001) level of mice in the ET+CM group was significantly reduced or increased. It shows that camel milk has the effect of regulating the level of antioxidants in acute alcohol mice.

Effects of camel milk on mRNA expression of CYP2E1 genes in the liver

CYP2E1, a central functional enzyme in alcohol metabolism, has been generally considered as a major contributor to the over-production of ROS during its catalytic circle (Leung and Nieto, 2013). Long-term and large amounts of alcohol consumption activate the expression of hepatic CYP2E1 and trigger oxidative stress, leading to tissue inflammation and injuries (Lu and Cederbaum, 2008).

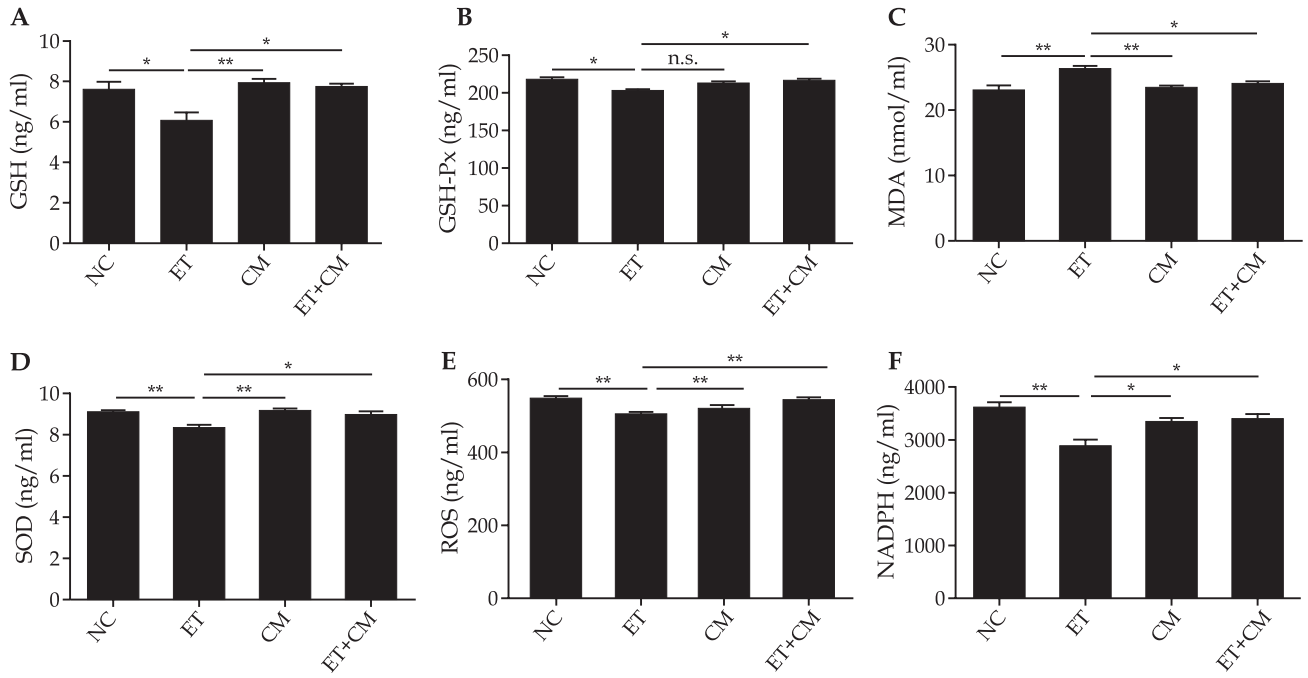

Fig 2. Effects of camel milk on the antioxidant capacity of the liver in ALD mice. (A) GSH level, (B) MDA level, (C) GSH-Px level, (D) SOD level, (E) ROS level and (F) NADPH level were measured with microplate reader. Data are expressed as mean ± S.E.M. (n = 10) for each group. **P* < 0.01, ***P* < 0.001. NC: normal group, ET: ethanol group, CM: camel milk group, ET+CM: ethanol and camel milk group.

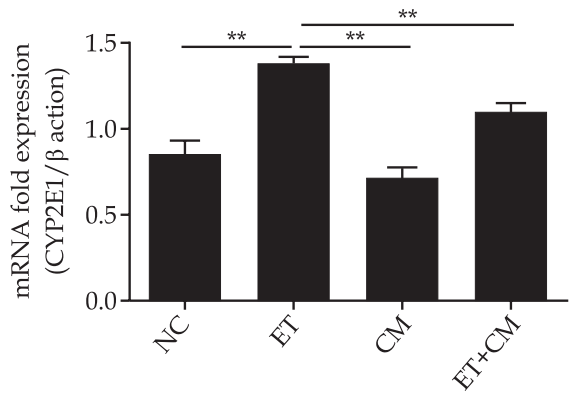

Fig 3. Effect of camel milk on the expression of CYP2E1 in ethanol-induced liver injury in mice. *P < 0.05, **P < 0.01. NC: normal group, ET: ethanol group, CM: camel milk group, ET+CM: ethanol and camel milk group.

To illuminate the mechanisms underlying the protective effects of CM on ethanol-induced oxidative stress, CYP2E1 was analysed by RT-qPCR. As shown in Fig 3, after ethanol exposure, the expression level of CYP2E1 in livers was significantly increased compared with the NC group. However, CM pretreatment markedly suppressed the increase of ethanol-induced CYP2E1 expression. Taken together, the results demonstrated that CM attenuates ethanol-induced oxidative stress by inhibiting CYP2E1 expression.

Conclusions

In conclusion, the present study demonstrated that feeding CM to alcohol-treated mice can prevent ethanol-induced liver damage. The protective effects of CM were associated with alleviation of ethanol-induced oxidative stress via inhibiting CYP2E1 and NADPH oxidase activities. Our findings provide new insights into hepatoprotective mechanisms of CM and a promising antioxidant-rich food for prevention of alcoholic liver injury.

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Conflicts of Interest

The authors declare no conflict of interest.

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