Short Communication

A triglyceride-lowering effect of cattle bile is associated with elevation of cholesterol levels and liver injury in mice

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Abstract

Since cholic acid (CA) has been demonstrated to suppress triglyceride (TG) synthesis, cattle bile (CB) constituted mainly of CA may exert a TG-lowering effect. However, harmful effects of CB such as elevation of cholesterol (Cho) levels and hepatotoxicity are also assumed to be induced. In this study, we demonstrated that diets containing CB at 0.5 and 1.0 % (w/w) reduced TG levels in blood and liver of mice, which was associated with the elevation of Cho levels in blood and liver and liver injury. Our results suggest that practical use of CB as a TG-lowering agent is not recommended.

Key words animal bile, digestive medicine, cholic acid, cholesterylesters, transaminase.

Abbreviations ALT, alanine aminotransferase; AST, aspartic aminotransferase; CA, cholic acid; CB, cattle bile; Cho, cholesterol; DCA, deoxycholic acid; FXR, farnesoid X receptor; SREBP, sterol regulatory element binding protein; TG, triglyceride.

Introduction

Bile preparations harvested from bear or domestic animals such as cattle and pig have been utilized as components of digestive medicines in Japan. Bile acids are the major components of animal bile and are known to facilitate fat emulsification and digestion of ingested fats. These properties of animal bile can explain its use as a component of digestive medicines. However, the availability of bear bile in Japan is now limited because bear is a CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora)-listed animal species and the export and import of bear as well as its parts are tightly regulated. In Japan, cattle bile (CB) is used more extensively than bear bile as a component of digestive medicines. It is known that glycine- and taurine-conjugates of cholic acid (CA) are major components (>50 %, w/w) of CB. We have already demonstrated that the efficacy of CB to facilitate fat digestion by pancreatic lipase is indistinguishable from that of bear bile.

Recent studies have demonstrated that administration of CA can suppress lipogenesis leading to the reduction of triglyceride (TG) levels in blood and liver of mice. It was shown that CA downregulates the expression of sterol regulatory element binding protein (SREBP)-1c expression through the activation of farnesoid X receptor (FXR), a nuclear receptor for bile acids. Several enzymes involved in TG and fatty acid synthesis are upregulated by SREBP-1c.

There are several reports indicating that cholesterol
(Cho) levels in blood and liver are elevated in the experimental animals administered with CA.\textsuperscript{3,6} It has also been demonstrated that CA administration enhances absorption of Cho from the small intestine,\textsuperscript{7} downregulates low-density lipoprotein receptors\textsuperscript{8} and reduces the expression level of \( \tau \)-hydroxylase (CYP7A1) leading to a reduction of the utilization of Cho for bile acid synthesis.\textsuperscript{9,10} Furthermore, it is well known that CA exerts hepatotoxicity in the experimental animals,\textsuperscript{11,12} which is possibly due to its direct cytotoxicity for liver cells.\textsuperscript{13} There is a report showing that hepatotoxicity coincides with the elevation of hepatic Cho levels in the rats fed a CA-supplemented diet,\textsuperscript{14} suggesting that accumulated Cho may play a role in the hepatotoxicity of CA.

Since the reduction of TG levels by CA has been demonstrated,\textsuperscript{3,4} CB containing CA is expected to exert a TG-lowering effect. However, it is also possible that CB, like CA,\textsuperscript{3,6} elevates Cho levels and induces hepatotoxicity.\textsuperscript{15} We therefore examined whether supplementation of the two different levels of CB in diets modulates TG and Cho levels in blood and liver of mice and induces hepatotoxicity. If CB reduces TG levels in the absence of the elevation of Cho levels and hepatotoxicity, CB may be considered as a TG-lowering agent. In contrast, if a TG-lowering effect is associated with the elevation of Cho levels or hepatotoxicity, practical use of CB would be limited.

**Materials and Methods**

**Diets and animals:** A powdered chow (Quick fat, Clea, Tokyo, Japan) was used as a control diet and basal diet for the preparation of test diets. The basal diet is specially designed to produce hyperlipidemia and hypoglycemia in rodents. This diet contained 13.6 % (w/w) beef tallow (30.0 % en) and its total energy content was 408 kcal/100g. The test diets were prepared by supplementing the powdered diet with CB (Lot 237K1119) purchased from Mitsubishi Pharmaceutical Co., Ltd., (Nara, Japan) in house at 1 and 0.5 % (w/w), each of them was separately compared with the control diet in Exp. 1 and Exp. 2, respectively. Bile acid composition in CB was determined as described previously\textsuperscript{23} and is shown in Table 1. In both Exp. 1 and 2, 12 female ICR mice at 6 weeks of age (SLC, Shizuoka, Japan) were divided into two groups composed of 6 mice/group, which were assigned for control and CB diets. The protocols for this experiment were approved by the Committee for Animal Care and Experiments of the University of Toyama.

<table>
<thead>
<tr>
<th>Bile acid</th>
<th>Content (% w/w)</th>
</tr>
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<tbody>
<tr>
<td>Taurocholic acid (TCA)</td>
<td>19.5</td>
</tr>
<tr>
<td>Glycocholic acid (GCA)</td>
<td>14</td>
</tr>
<tr>
<td>Glycodeoxycholic acid (GDCA)</td>
<td>1.7</td>
</tr>
<tr>
<td>Taurodeoxycholic acid (TDCA)</td>
<td>4.9</td>
</tr>
</tbody>
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**Sampling of blood and liver:** Blood (-50 \( \mu \)L) was obtained by puncturing the retro-orbital plexus with heparinized capillaries 2, 4 and 6 weeks after the start of feeding. The heparinized blood was centrifuged to obtain plasma, which was stored at -80 °C until analysis. At the end of the feeding periods (6 wks), mice were fasted overnight and killed by cervical dislocation to obtain blood for the preparation of serum. The liver was washed in cold saline and frozen quickly in liquid nitrogen. Serum and liver samples were stored at -80 °C until analysis. A portion of the liver was fixed in 10% formalin for histological assessment.

**Blood analysis:** TG and total Cho concentration in plasma and alanine aminotransferase (ALT) and aspartic aminotransferase (AST) activities in serum were determined using commercial clinical assay kits (Wako Pure Chem, Osaka, Japan).

**Silica gel thin-layer chromatography of liver total lipids:** Total lipids of liver were extracted according to Blight and Dyer methods.\textsuperscript{16} Total lipids were dissolved in CHCl\(_3\) and total lipids derived from 1 mg liver tissue/5 \( \mu \)L CHCl\(_3\) were spotted onto high-performance thin-layer chromatography (HPTLC) plates (Merck KGaA, Darmstadt, Germany) in approximately 5-mm-wide lanes. The plates were developed with a mixture of petroleum ether/diethyl ether/acetic acid (80/30/1, v/v/v). The plates were air-dried and then sprayed with 3% cupric acetate-8% phosphoric acid in water. Lipid spots were visualized by heating the plates on hot plates at 150 °C for 20 min. Increasing amounts (0.8, 2, 4, 6
and 8 µg/lane) of cholesteryl oleate, triolein, oleic acid and cholesterol (TLC mix, Larodan AB, Malmo, Sweden) were spotted in parallel with the samples to estimate the linearity of spot intensities obtained by these lipid standards. The images of plates were incorporated by a computer scanner.

**Histological assessment for liver:** A portion of liver specimens fixed in formalin was used to prepare paraffin sections for hematoxylin-eosin staining. Another portion of liver specimens was frozen to prepare sections for Sudan stain to detect lipid deposition in liver.

**Statistical analysis:** Statistical significance of the difference between the control and CB diet groups was estimated by unpaired Student’s t-test.

**Results**

**Body weight:** In both Exp. 1 and Exp. 2, there was no significant difference in body weight of the mice fed control and CB diets at any time points of measurement (Fig. 1).

**Effect of 1.0% CB diet on plasma and hepatic lipid levels and serum transaminase activities (Exp. 1):** Plasma TG levels measured at 2 and 4 wks were not significantly different between the control and 1.0% CB diet groups (p>0.05) but were significantly lower in the 1.0% CB diet group than in the control group at 6 wks (p<0.05) (Fig. 2a). In contrast, plasma total Cho levels at 6 wks of feeding were significantly higher in the 1.0% CB diet group than in the control group (p<0.01) (Fig. 2b). These levels at 2 and 4 wks of feeding were not significantly different between the two dietary groups (p>0.05). Similar to TG levels in blood, these levels in liver lipids were also lower in the 1.0% CB diet group than in the control group. Liver lipids of control mice contained only a trace amount of cholesteryl esters (ChoE), although this level was markedly greater in those of the 1.0% CB diet group (Fig. 2c). Cho levels were not significantly different between the two dietary groups. Serum ALT activity was significantly higher in the 1.0% CB diet group than in the control group (p<0.001) (Fig. 2d), although the difference was not greater than two fold between the two groups (28 ± 2.9 vs. 54 ± 4.5 karmen unit, respectively). AST was not different between the two dietary groups.

**Effect of 0.5% CB diet on plasma and hepatic lipids and serum transaminase activities (Exp. 2):** We next examined whether a lower level of CB (0.5%, w/w) added to the diet exerts similar effects to those induced by the 1.0% CB diet. Reduction of plasma TG levels was also shown in the mice fed the 0.5% CB diet at 2 and 6 wks of feeding compared with the control group (p<0.05 and p<0.01, respectively) (Fig. 3a). Similarly, plasma total Cho levels were elevated in the 0.5% CB diet group at 2 and 4 wks of feeding compared with the

![Exp. 1](image1.png)  ![Exp. 2](image2.png)

**Fig. 1** Changes in body weight
The 1% CB (Exp. 1) and 0.5% CB (Exp. 2) diets did not significantly affect body weight of mice compared with the control diet.
control group (\(p<0.05\) and \(p<0.01\), respectively) (Fig. 3b). The effects of the 0.5% CB diet on the levels of liver TG and ChoE were similar to those induced by the 1.0% CB diet (Fig. 3c). Serum ALT activity was significantly higher in the 0.5% CB diet group than in the control group (\(p<0.05\)) (Fig. 3d), although this difference was only 1.5-fold (24 ± 1.3 vs. 37 ± 4.0 karmen unit) and was smaller than that shown between the control and 1.0% CB diet groups (Fig. 2d). AST was not significantly different between the two dietary groups.

**Light microscopic assessments of liver injury:** The sections of liver from mice fed the 1.0% CB diet revealed the occurrences of necrotized hepatocytes (Fig. 4). In addition, infiltrating leukocytes composed mainly of neutrophils were also observed in liver parenchyma. In addition, Sudan staining clarified the occurrence of fatty droplets stained as reddish particles in liver parenchyma in the 1.0% CB diet group. Sections of liver from the control mice did not show any pathological changes.

**Discussion**

Glycine and taurine conjugates of CA were the major forms found in CB and their content was 33.5% (w/w) (Table 1). Therefore, the CA contents in the 0.5 and 1.0% CB diets were calculated to be 0.17 and 0.34% (w/w), respectively. It has been shown that reduction of TG levels\(^{3,4}\) and elevation of Cho levels\(^{6}\) as well as hepatotoxicity\(^{11,12}\) were induced in the rodents fed diets supplemented with CA ranging from 0.4 to 0.5% (w/w). The effects of the CB diets were moderate because significant changes in these lipid levels were

![Fig. 2](image_url)

*Fig. 2* Effect of 1.0% CB diet on plasma and liver lipids and serum transaminase activity

Plasma TG (a) and total Cho (b) levels were determined at 2, 4 and 6 wks of feeding. Total lipids extracted from liver were separated on thin-layer chromatography plates and visualized with copper sulfate/phosphoric acid solution. Increasing amounts of ChoE, TG, free fatty acid and Cho were separated in parallel. Liver lipid samples from 3 mice fed control (lanes 6, 7 and 8) and CB (lanes 9, 10 and 11) diets were analyzed (c). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in serum were measured at the end of feeding of control and CB diets (d). Statistically significant difference between control and 1.0% CB diet groups was analyzed by unpaired Student’s *t*-test.
shown at limited time points of measurements and the elevation of ALT activity was less than two-fold that of the control levels (Figs. 2 and 3). Therefore, the relatively low levels of CA contained in CB would be sufficient to account for the effects on lipid levels and hepatotoxicity. CB also contained smaller but not negligible amounts of taurine and glycine conjugates of deoxycholic acid (DCA) at 6.6 % (w/w) (Table 1), which is more hydrophobic and cytotoxic than CA.\textsuperscript{15,16} DCA conjugates might also play a role in hepatotoxicity caused by CB. It was demonstrated that the potencies of DCA to elevate blood and liver Cho levels were weaker than that of CA in rats,\textsuperscript{5} although the effect of DCA on TG levels is unknown. These results suggest that different bile acid species contained in CB play different roles in the regulation of TG and Cho levels and hepatotoxicity.

In addition, since the diets containing two different levels of CB (0.5 and 1.0 %, w/w) induced inconsistent changes in plasma TG and Cho levels over the time points of measurements (Figs. 2 and 3), suggesting that factor(s) other than CA or DCA in CB might confound dose-relating effects of CB on these plasma lipid levels. Further detailed analyses are necessary to clarify the role of bile acids in the differential effects of CB on TG and Cho levels.

Watanabe et al.\textsuperscript{41} showed that blood total Cho was reduced in obese KK mice fed a diet containing CA at 0.5% (w/w). In this study, obese KK mice were maintained on a high fat diet in the absence of Cho, although the basal diet used in our study contains Cho at 0.24 % (w/w). It is possible that the elevation of Cho levels is due to the presence of Cho in the diet because CA can enhance absorption of Cho from the intestines.\textsuperscript{7} However, the report by Watanabe et al.\textsuperscript{41} demonstrated...
a marked elevation of hepatic ChoE levels, which was also shown in our study using CB (Figs. 2 and 3). In addition, Ikemoto et al. showed that dietary CA did not lower TG levels in high-fat diet-fed female C57BL/6 mice, but a marked reduction of TG levels was shown when CA was combined with Cho (1%, w/w). This result suggests that the reduction of TG levels may be a secondary response followed by the elevation of Cho levels in blood and liver. As shown in our study, the major form of Cho accumulated in the liver of mice fed CB was ChoE (Figs. 2 and 3). Occurrence of the lipid droplets found in liver sections (Fig. 4) would be due to the accumulation of ChoE. Taken together, it was suggested that ChoE accumulation plays a causal role in the reduction of TG as well as hepatotoxicity in CB fed mice. TG accumulation is generally accepted as a pathogenic factor in steatohepatitis. However, no pathogenic role of hepatic ChoE in liver diseases has been well clarified until now.

The average range of CB supplemented in regular digestive medicines used in Japan is 50 to 100 mg/day. Within this range of CB ingestion, it is not necessary to consider the possibilities of Cho accumulation and hepatotoxicity. However, animal bile preparations including CB are allowed to be ingested at up to 500 mg/day as a component of digestive medicines in Japan. Further studies are necessary to estimate the safety of higher doses of CB from the viewpoints of Cho metabolism and hepatotoxicity. In addition, our data, at the very least, indicate that CB is not suitable for practical use as a safe TG-lowering agent.

References


