Treatment With Bovine Gallstones Exacerbates Liver Damage, but Enhances Hepatoprotection by Bear Gall Powder in Carbon Tetrachloride-Intoxicated Rats

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ABSTRACT—The interactions between bovine gallstones (Goou) and bear gall powder (Yutan) in decreases in serum transaminase levels were investigated in rats intoxicated with carbon tetrachloride (CCl_4). The p.o. administration of Goou significantly increased both serum transaminase levels and hepatic lipid peroxidation following i.p. administration of CCl_4 . Concomitant administration of both Goou and Yutan resulted in decreases of serum transaminase levels and hepatic lipid peroxidation, which were more remarkable than with administration of Yutan alone. Goou significantly increased the estimated hepatic blood flow in the indocyanine green clearance test and enhanced the delivery of CCl_4 to the liver from the peritoneal cavity. These findings suggest that Goou exacerbates CCl_4 -induced hepatic damage because of the accelerated delivery of CCl_4 to the liver and that Goou might have a hemodynamic drug interaction with Yutan in the liver, possibly enhancing the hepatoprotective effect of Yutan.

Keywords: Animal crude drug, Bovine gallstone, Bear gall powder, Carbon tetrachloride, Liver

Chinese animal crude drugs such as bovine gallstones (BEZOAR BOVIS, “Goou” in Japanese) and bear gall powder (FEL URSI, “Yutan” in Japanese) have long been said to be effective for liver diseases in the Orient (1, 2). Goou consists mainly of bilirubin, cholesterol and cholic acid as a bile acid (3). Yutan consists mainly of ursodeoxycholic acid (UDCA) as a bile acid and taurine as an amino acid (4). We published a preliminary clinical report on the effectiveness of Goou and Yutan for the treatment of chronic liver diseases (5). This report revealed for the first time that the concomitant administration of both Goou and Yutan remarkably decreased the transaminase levels of hepatitis patients, but the actual mechanisms of the pharmacological effects of Goou and Yutan have not been clarified.

In this study, we tried to determine the effects of Goou and Yutan on carbon tetrachloride (CCl_4)-intoxicated rats livers and to discuss Goou’s hemodynamic interaction with CCl_4 or Yutan.

MATERIALS AND METHODS

Chemicals

Goou and Yutan were obtained from Itoh Kampo Co.

(Osaka). They had been importing them from a Hong Kong trader, who obtained Goou from Australian and North American cows and Yutan from Southeast Asian and Himalayan bears, long before the so-called Washington convention (Convention on International Trade in Endangered Species of Wild Fauna and Flora) to protect wild animals came into effect in January 1988.

Reagents used were obtained from the following commercial sources: CCl_4 , 2-thiobarbituric acid (TBA), tetraethoxypropane and α, α -diphenyl- β -picrylhydrazyl (DPPH) (Tokyo Kasei Co., Tokyo); NADPH (Sigma Chemical Co., St. Louis, MO, USA); $^{14}\text{CCl}_4$ (New England Nuclear Co., Boston, MA, USA); and indocyanine green (ICG) (Daiichi Pure Chemicals Co., Ltd., Tokyo). Other chemicals used were guaranteed grade and were obtained locally.

Animals

Male Wistar rats weighing 200 g were used in all experiments. Animals received a single dose of CCl_4 (2 ml/kg, b.w., i.p.) in olive oil and simultaneously given once an aqueous solution of Goou (10, 50 or 100 mg/kg, b.w.) and/or Yutan (10, 50 or 100 mg/kg, b.w.) through a gastric tube. Control animals were received olive oil instead of

CCl_4 , and 0.9% sodium chloride instead of Gooou or Yutan solution. Maintenance of animals and experimental procedures were carried out in accordance with the guidelines of The Japanese Pharmacological Society.

Serum transaminase levels and ICG clearance

The blood was collected from the portal vein of the rats anesthetized by pentobarbital (50 mg/kg, i.p.), and serum transaminase levels were measured using a GOT-UV Test kit and a GPT-UV Test kit (Wako Pure Chemicals Industries, Ltd., Osaka).

The ICG concentration in serum was measured spectrophotometrically (UV-2100S spectrophotometer; Shimadzu, Kyoto) from the absorbance at 805 nm in the anesthetized rats 15 min after administration of 0.5 mg/kg of ICG through the tail vein (6).

Lipid peroxidation and antioxidants

The lipid peroxides formed in the liver were quantitated according to the method of Ohkawa et al. (7). Briefly, tissue homogenate was mixed with sodium dodecyl sulfate, acetate buffer (adjusted to pH 3.5 with NaOH) and an aqueous solution of TBA. After heating at 95°C for 60 min in a water-bath, the red color formed was extracted with a *n*-butanol-pyridine (15 : 1) mixture, and the intensity of the color was estimated spectrophotometrically from the absorbance at 532 nm. Tetraethoxypropane was used as a standard for the assay, and lipid peroxide contents was expressed as nmoles of malonic dialdehyde (MDA), namely, TBA-reactive substance (TBA-RS), formed per mg protein. Protein was determined by the method of Lowry et al. (8) with bovine serum albumin as the standard.

The levels of water-soluble and fat-soluble antioxidants in the liver were determined by using a stable free radical, DPPH, according to the procedure of Glavind (9).

Cytochrome P_{450} and drug metabolism

Microsomes were prepared from 0.25 M sucrose homogenates of the liver from rats according to the procedure of Hogeboom (10) as follows: The homogenate was centrifuged at $24,000 \times g$ for 10 min, and the supernatant thus obtained was further centrifuged at $105,000 \times g$ for 60 min to obtain the microsomal fraction. The microsomal pellets were suspended in 0.15 M KCl, kept in an ice-cold tube and used within 8 hr for each determination.

Hepatic microsomes, which were prepared from rats 24 hr after dosing with Gooou and/or Yutan (50 mg/kg, b.w., p.o.), were used to study drug metabolism. The content of cytochrome P_{450} was determined by the method of Omura and Sato (11). The hexobarbital oxidation and aniline hydroxylation were assayed spectrophotometrical-

ly by the methods of Cooper and Brodie (12) and Kato and Gillette (13), respectively.

Lipid peroxidation in microsomes

The extent of CCl_4 -induced lipid peroxidation in microsomes in vitro was estimated by measuring TBA-RS formed in the incubation media (14, 15). Briefly, liver microsomes (2 mg protein) were preincubated with Gooou and/or Yutan (0.1 mg) at 37°C for 2 min with 0.2 M potassium phosphate buffer (pH 7.5) containing 400 μM NADPH in a final volume of 2 ml. After the addition of 2 μmol of CCl_4 , they were incubated for 30 min in tightly capped test tubes. The reaction was stopped by adding 0.2 ml of 8.1% sodium dodecyl sulfate and 1.5 ml of 20% acetic acid (pH 3.5) in an ice cold water bath. Under the same experimental conditions, the blank level of TBA-RS formation was measured in the absence of NADPH and was subtracted from each experimental value.

$^{14}\text{CCl}_4$ metabolism in microsomes

Liver microsomes (2 mg protein) were preincubated with Gooou and/or Yutan (0.1 mg) and then further incubated at 37°C for 2.5, 5, 15 and 30 min with 0.2 M potassium phosphate buffer (pH 7.5) containing 0.8 μmol of CCl_4 with $^{14}\text{CCl}_4$, 200 μM NADPH and 3 mM EDTA in a final volume of 2 ml. A part of the reaction mixture was placed in 10 ml of ice cold toluene and stored at 4°C for 3 days with occasional shaking. An aliquot of the toluene layer was mixed with toluene scintillator containing 0.5% (W/V) PPO and 0.03% (W/V) POPOP, and then the radioactivity extracted into the toluene layer was measured by a liquid scintillation spectrometer (16–18). The specific activity of the $^{14}\text{CCl}_4$ used in present experiment was 3.8 $\mu\text{Ci}/\text{mmole}$.

Distribution of $^{14}\text{CCl}_4$ in the liver

At 0.5, 1, 2 and 5 hr after injecting CCl_4 (2 ml/kg, b.w.) containing $^{14}\text{CCl}_4$ in olive oil, intraperitoneally, the rats were sacrificed and liver specimens were taken. The liver specimens were rapidly weighed, minced and put into 10 ml ice cold toluene and stored at 4°C for 3 days with occasional shaking. The radioactivity extracted into the toluene was measured as described above.

Statistics

Statistical analysis was performed by ANOVA. Differences were accepted as statistically significant at P values < 0.05 .

RESULTS

Serum transaminase levels and hepatic lipid peroxidation

Serum GOT and GPT significantly increased 24 hr after

Fig. 1. Effects of Goou and Yutan on serum transaminase levels of the CCl₄-treated rats. In the Goou, Yutan or Goou + Yutan group, Goou and/or Yutan (10 (▨), 50 (□) or 100 (▩) mg/kg b.w., p.o.) were given simultaneously with CCl₄ (2 ml/kg b.w., i.p.). The rats in the CCl₄ group were given saline instead of these drugs. Serum GOT and GPT were measured 24 hr later. Each column represents the mean $\pm$ S.E. obtained from four or five separate experiments. Control values were 45 ± 9 for GOT and 35 ± 10 for GPT ($n=5$, mean $\pm$ S.E.). The value obtained with CCl₄ was significantly higher than the control value ($P < 0.001$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to the value obtained with CCl₄.

Fig. 2. Effects of Goou and Yutan on hepatic lipid peroxidation in CCl₄-treated rats. The treatment with CCl₄, Goou and/or Yutan was the same as described in the Fig. 1 legend. Each column represents the mean $\pm$ S.E. obtained from four or five separate experiments. The control value was 118 ± 20 ($n=5$, mean $\pm$ S.E.). The value obtained with CCl₄ was significantly higher than the control value ($P < 0.001$). * $P < 0.05$, ** $P < 0.01$, compared to the value obtained with CCl₄.

the administration of CCl₄ (Fig. 1). The GOT and GPT of the CCl₄-administered rats were further significantly increased by the simultaneous administration of Goou (50

or 100 mg/kg). In contrast, GOT and GPT were found to be decreased by the administration of Yutan (50 or 100 mg/kg). The rats given both Goou and Yutan (50 or 100

mg/kg) showed statistically significant improvements in GOT and GPT, exceeding those of rats given Yutan alone.

As shown in Fig. 2, TBA-RS in the liver increased approximately three fold at 24 hr after the administration of CCl_4 . This hepatic TBA-RS was further increased by the administration of Goou (50 or 100 mg/kg), but was significantly decreased by the administration of Yutan (50 or 100 mg/kg). The concomitant administration of Goou and Yutan (50 or 100 mg/kg) markedly decreased the hepatic TBA-RS of CCl_4 -administered rats.

Cytochrome P_{450} , hexobarbital oxidation, aniline hydroxylation and hepatic antioxidant levels

The content of cytochrome P_{450} (nmol/mg protein) was 0.34 ± 0.02 , and oxidation of hexobarbital (nmol/30 min/mg protein) and hydroxylation of aniline (nmol/20 min/mg protein) were 5.2 ± 0.4 and 0.34 ± 0.05 , respectively, in control rats ($n=5$, mean $\pm$ S.E.). Neither Goou nor Yutan affected the content of cytochrome P_{450} , oxidation of hexobarbital or hydroxylation of aniline in hepatic microsomes (data not shown).

Hepatic antioxidants ($\mu\text{equiv/g}$ liver) were found to be mostly of the water-soluble type (19.8 ± 2.5), and only

small amounts of fat-soluble antioxidants (0.08 ± 0.02) were detected in control rats ($n=4$, mean $\pm$ S.E.). Goou and Yutan had no marked effect on the contents of these antioxidants (data not shown).

CCl_4 -induced lipid peroxidation in hepatic microsomes

As shown in Fig. 3, a marked increase in TBA-RS formation was noticed in the media containing CCl_4 . When the media were incubated with Goou and/or Yutan, the CCl_4 -induced lipid peroxidation was significantly reduced.

$^{14}\text{CCl}_4$ metabolism in hepatic microsomes

The metabolic degradation of $^{14}\text{CCl}_4$ by hepatic microsomes was significantly reduced by the addition of Goou; in contrast, the addition of Yutan did not affect the metabolism of $^{14}\text{CCl}_4$ (Fig. 4).

ICG clearance

The ICG clearance test was performed in the control and the Goou and/or Yutan-treated (for 1 day) rats. As shown in Fig. 5, both drugs, especially Goou, significantly augmented ICG clearance.

Fig. 3. Effects of Goou and Yutan on CCl_4 -induced lipid peroxidation in hepatic microsomes. Hepatic microsomes (2 mg protein) were incubated with Goou and/or Yutan (0.1 mg) with or without 2 μmol of CCl_4 . Control (□), Goou (○), Yutan (△), Goou + Yutan (◇), CCl_4 (■), CCl_4 + Goou (●), CCl_4 + Yutan (▲) and CCl_4 + Goou + Yutan (◆). Each point represents the mean $\pm$ S.E. obtained from four separate experiments. In the CCl_4 , CCl_4 + Goou, CCl_4 + Yutan and CCl_4 + Goou + Yutan group, the values obtained at 10 and 30 min after the addition of CCl_4 were significantly higher than each initial (0 min) value ($P < 0.001$). * $P < 0.05$, ** $P < 0.001$, compared to the value obtained with CCl_4 .

Fig. 4. Effects of Goou and Yutan on the metabolism of CCl_4 in hepatic microsomes. The metabolism of CCl_4 was estimated by the disappearance of radioactivity of the added $^{14}\text{CCl}_4$ in an incubation mixture containing microsomes in vitro. $^{14}\text{CCl}_4$ is converted to $^{14}\text{CCl}_3$ and the ^{14}C of CCl_3 is then metabolized to $^{14}\text{CO}_2$ via the microsomal mixed function oxidase system. The $^{14}\text{CO}_2$ produced ultimately evaporates (17). Hepatic microsomes (2 mg protein) were incubated with Goou and/or Yutan (0.1 mg) with 2 μmol of CCl_4 containing $^{14}\text{CCl}_4$. Control (○), Goou (●), Yutan (▲) and Goou + Yutan (◆). The points represent the mean value or the mean $\pm$ S.E. obtained from two or four separate experiments. * $P < 0.01$, compared with the Control value.

Fig. 5. Effects of Goou and Yutan on ICG clearance in rats. Rats in the test groups were given Goou and/or Yutan (50 mg/kg b.w., p.o.), and control rats were given saline instead of these drugs. Twenty-four hours later, the concentration of ICG in serum was measured 15 min after the administration of ICG (0.5 mg/kg b.w.). Each column represents the mean $\pm$ S.E. obtained from four separate experiments. * $P < 0.05$, ** $P < 0.001$, compared with the Control value.

Fig. 6. Effects of Goou and Yutan on the distribution of CCl₄ in the livers of rats. The amount of radioactivity in hepatic tissue was measured after the intraperitoneal administration of CCl₄ (2 ml/kg b.w.) containing ¹⁴CCl₄. Rats were given Goou and/or Yutan (50 mg/kg b.w.) or saline as a control 1 hr before the administration of CCl₄. Control (CCl₄ alone) (○), Goou (●), Yutan (▲) and Goou + Yutan (◆). The points represent the mean value or the mean $\pm$ S.E. obtained from two or four separate experiments. * $P < 0.05$, ** $P < 0.01$, compared with the Control value.

Distribution of ¹⁴CCl₄ in the liver

After 2 hr of dosing with CCl₄ containing ¹⁴CCl₄, there was greater uptake of ¹⁴CCl₄ in the livers of rats simultaneously given Goou or Goou + Yutan as compared to animals given CCl₄ (¹⁴CCl₄) alone (Fig. 6).

DISCUSSION

Our previous report (5) clearly demonstrated that the concomitant administration of both Goou and Yutan resulted in marked improvement of GOT and GPT in all hepatitis patients within one month. The administration of Goou alone, however, improved neither GOT nor GPT. In the present study, we have endeavored to elucidate the interaction of Goou and Yutan on the improvement of GOT and GPT using experimental injury of the rat liver.

Serum transaminase levels of CCl₄-treated rats were further increased by the administration of Goou and decreased by the concomitant administration of Goou and Yutan more than the administration of Yutan alone. As hepatic lipid peroxidation is a major factor in the induction of hepatocellular necrosis following CCl₄ administration (19), hepatic lipid peroxidation was then examined: the hepatic lipid peroxidation was increased by the administration of Goou and decreased by the administration of Yutan, both significantly. The concomitant administration of Goou and Yutan decreased lipid peroxidation more than the administration of Yutan alone. These findings suggest that the concomitant administration of Goou and Yutan may exert a protective action against CCl₄-induced hepatocellular damage by reducing the formation of lipid peroxides in the liver, and they indicate that there may be some synergistic interaction between Goou and Yutan in decreasing hepatic lipid peroxide formation in CCl₄-administered rats.

It has been well-established that CCl₄ is cleaved, yielding a CCl₃ radical, by the mixed function oxidase system in liver microsomes, and that the CCl₃ radical reacts with unsaturated fatty acids to form lipid peroxides in the liver (19). As to these observations, the effects of Goou and Yutan administration on the activity of the microsomal mixed function oxidase system were investigated. As a result, Goou and Yutan did not influence the content of cytochrome P₄₅₀ and the metabolism of drugs such as hexobarbital and aniline in hepatic microsomes. On the other hand, antioxidants can function as free radical scavengers that may decrease lipid peroxidation. Treatment with Goou and/or Yutan, however, had no effects on the antioxidant contents of the in vivo liver. These results did not explain why the administration of Goou enhanced and the concomitant administration of Goou and Yutan suppressed lipid peroxidation in the liver of

CCl₄-administered rats.

The effects of Goou and Yutan on CCl₄-induced lipid peroxidation, which were demonstrated *in vivo*, were further investigated *in vitro* using hepatic microsomes. Contrary to expectation, it was found that Goou had a direct suppressive effect on CCl₄-induced lipid peroxidation in hepatic microsomes. This finding suggested that Goou might prevent the metabolism of CCl₄ to the CCl₃ radical via the mixed function oxidase system and/or that Goou might have an antioxidative effect and thereby terminate the radical reactions. To assess these possibilities, first, the effect of Goou on the metabolic degradation of CCl₄ was investigated *in vitro* in hepatic microsomes: the degradation of ¹⁴CCl₄ by hepatic microsomes was significantly reduced by the addition of Goou. Furthermore, Goou decolorized the DPPH in the methanol solution (data not shown), indicating that Goou had antioxidant properties. The anti-oxidative effect of bilirubin, the principal ingredient of Goou, is well-known (20). The effects of Goou on CCl₄-induced lipid peroxidation *in vivo*, therefore, cannot be explained by these effects on microsomes *in vitro*.

As Goou has been reported to dilate vessels and improve blood flow in organs (3), we investigated the effect of Goou on ICG clearance, which is an estimate of hepatic blood flow (21): Goou significantly accelerated hepatic blood flow. This finding suggested that Goou might enhance the transport of CCl₄ from the peritoneal cavity to the liver by increasing hepatic blood flow, possibly resulting in the enhancement of CCl₄-induced lipid peroxidation in the liver. In fact, it was confirmed that the hepatic distribution of ¹⁴CCl₄ following intraperitoneal injection of ¹⁴CCl₄ was enhanced by simultaneous treatment with Goou. A few previous reports have suggested the possibility that some bile acids increase blood flow in internal organs (22, 23), although the component of Goou contributing to the increased hepatic blood flow and the actual mechanisms remain to be elucidated.

On the other hand, the suppressive effect of Yutan on CCl₄-induced lipid peroxidation was recognized not only *in vivo* in the liver but also *in vitro* in hepatic microsomes. Yutan did not suppress the metabolic degradation of CCl₄ in microsomes. From these results, it can be surmised that Yutan may have a direct effect on the microsomal membrane, possibly decreasing the susceptibility of the membrane to lipid peroxide formation induced by CCl₄. The main ingredient of Yutan is UDCA which has recently been used to treat chronic liver diseases such as primary biliary cirrhosis (24) and chronic hepatitis C (25). Because UDCA is a hydrophilic bile acid that is less toxic than hydrophobic bile acids such as chenodeoxycholic acid (26), it is believed that administered UDCA protects the cellular component against

the toxicity of hydrophobic bile acids (27). Taurine, which is also present in Yutan, has been recognized to be a membrane stabilizer (18) and to improve GOT and GPT in liver damage (28). Our previous study (14) revealed that the suppressive effect of taurine against CCl₄-induced lipid peroxidation occurs both *in vivo* and *in vitro*.

One of the interesting findings obtained in the present study is that Goou might enhance the suppressive effect of Yutan on hepatic lipid peroxidation induced by CCl₄. Considering the interaction of Goou and CCl₄ mentioned above, it is speculated that Goou may enhance the transport of Yutan from the intestinal cavity to the liver, resulting in the enhancement of the hepatoprotective effect of Yutan. Under these circumstances, the suppressive effect of Yutan on hepatic lipid peroxidation might be superior to the accelerating effect of Goou on hepatic lipid peroxidation. The possibility that Goou has a hemodynamic interaction with other drugs adequately explains the observation that Goou is generally used as a so-called "compound medicine"—a drug that enhances the effectiveness of other drugs—in traditional oriental medicine. For example, Rokushingan (in Japanese) contains Goou and Yutan or deer sexual glands (MOSCHUS), and Henshikou (in Japanese) contains Goou and snake bile: these drugs are also effective for liver diseases (29).

Although the present findings are based on crude extracts of heterogeneous natural products, major constituents of Goou and Yutan are readily available. Therefore, it would be desirable to factor out which constituents are responsible for the intriguing effects observed. Experimental *in vivo* and *in vitro* studies based on this viewpoint are proceeding in our laboratory.

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