The Recovering Effect of Betaine on Carbon Tetrachloride-Induced Liver Injury

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(Received October 21, 1997)

Summary The recovering effect of betaine (derivative of choline) on carbon tetrachloride (CCl₄)-injured liver was investigated by oral and intraperitoneal administration of betaine at 24 h after acute CCl₄ intoxication. The effect of betaine was estimated by the activity of alanine aminotransferase (ALT) released into the serum, histological score, and the incorporation of S-phase indicator (5-bromo-2-deoxyuridine/5-fluoro-2'-deoxyuridine; BrdU/FdU). A significant decrease in the activity of serum ALT was observed by the intraperitoneal (3 mg/kg body) or oral (15 mg/kg body) administration of betaine. Furthermore, an increase in the uptake of BrdU into the hepatocyte nuclear DNA and a reduction in liver necrosis after the oral treatment of betaine (15 mg/kg body) was observed. From these results, the administration of betaine showed a significant effect in recovering CCl₄-injured liver.

Key Words liver injury, CCl₄, betaine, BrdU, recovery

Intraperitoneal injection of CCl₄ leads to the disruption of hepatocyte membrane and related enzymatic reaction due to the free radical products provided by liver drug-metabolizing enzymes (1). Since cell membranes are composed of phospholipids, these reactions, occurring during liver injury, are responsible for all membrane damage. Contrarily, it has been well demonstrated (2) that hepatocytes under the regeneration cycle have a higher tolerance to stress as compared to the resting condition. Therefore, the progression of these reactions in the liver is dependent on hepatocellular regeneration and hepatolobular restoration.

By the catalyzed reaction of betaine-homocysteine methyltransferase, methyl groups are transferred from betaine to homocysteine, and also form methionine that produces further conversion to S-adenosylmethionine (SAM) (3). The synthesizing processes of methionine and SAM through the methylation of

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homocysteine are two of the most important biochemical hepatic pathways (4–6). Therefore, the administration of betaine would contribute to the preservation of the liver homeostasis.

In this report, we demonstrated the positive effect of betaine on acute CCl₄ liver injury.

MATERIALS AND METHODS

Experimental liver injury by CCl₄. All mice were six-week-old ddy males weighing 28–30 g. They were kept under a daily controlled 12-h light/dark lighting cycle at 23°C for one week prior to the experiments, and fed ad libitum during the acclimation period. CCl₄ was dissolved in Panacete 810 (P 810: synthetic middle-chain saturated triacylglycerol; Nichiyu Liposome Co. Ltd., Tokyo, Japan) at a concentration of 10 (v/v)%.. After deprivation of food, mice were intraperitoneally injected with 1 mL/kg body weight of CCl₄. During the experimental period, all animals were handled under the Guidelines for Experimental Animals of Fujita Health University.

Administration of betaine and preparations of serum and liver samples. Twenty-four hours after the CCl₄ injection, several groups of mice were treated with respective doses of betaine (purified from beta vulgaris subsp. vulgaris) intraperitoneally or orally and the others were injected with P 810 and saline only as controls. Blood was drawn from ether-anesthetized mice through heart puncture and the serum was separated by centrifugation (7,000 × g, 4°C, 10 min). Alanine aminotransferase (ALT) activities of the sera were immediately assayed by an automatic analyzer COBAS MIRA (Japan Roche, Tokyo, Japan).

Analysis of de novo DNA synthesis. In order to elucidate the effects of betaine on the rate of liver regeneration after the treatment of CCl₄, 1 mL/100 g body weight of 5-bromo-2'-deoxyuridine/5-fluoro-2'-deoxyuridine (BrdU/FdU; RPN 210, Amersham International Co. Ltd., England) was injected at 2 h before sacrifice. Liver samples were fixed with methanol for 24 h at 4°C, embedded in paraffin, and sectioned. All immunohistochemical procedures for BrdU/FdU incorporation assay were carried out according to the attached instructions using mouse monoclonal anti-BrdU antibody as a primary antibody following secondary peroxidase-conjugated anti-mouse IgG2a antibody. All immunological reactions were carried out under room temperature, and sample slides were washed with PBS three times between each reaction. Anti-BrdU antibody-reacted nuclear granules were stained with 3,3'-diaminobenzidine tetrahydrochloride (RPN 210, Amersham International Co., Ltd.). The number of stained nuclear granules in a field were counted using optical microscopy at a magnification of 400. Three to 5 samples were obtained from each group; 10 random fields were counted from each sample and the mean values of 10 fields of each sample were calculated.

Liver histology. The tissue was fixed with 10% buffered formalin for a week, processed routinely and then embedded in paraffin, sectioned and stained with

J Nutr Sci Vitaminol
Recovering Effect of Betaine on Liver Injury

hematoxylin/eosin for light microscopic examination. The areas representing
necrosis were estimated measuring the two dimensions of maximal length under
light microscopy. The necrosis areas in a field were counted using optical microscopy
at a magnification of 400. Five samples were obtained from each group; 8 random
fields were counted from each sample and the mean values of 8 fields of each sample
were calculated.

Statistical analysis. The values of serum aminotransferase activity, DNA
synthesis and histological scores were all expressed as mean ± standard deviation
(SD). For all analyses, p < 0.05 was the minimal requirement for statistically
significant difference in ANOVA testing.

RESULTS AND DISCUSSION

The toxicity of CCl₄ is generally confirmed as its metabolic activation into the
trichloromethyl radical in the cytochrome P450 system. This radical attacks the
cell membrane that leads to lipid peroxidation (7), necrosis of centrilobular cells
(8), disruption of the endoplasmic reticulum (9), and the failure of energy production
through glycolysis by mitochondria (10). In this study, several doses of betaine
(intraperitoneal, 1–10 mg/kg body; oral, 10–30 mg/kg body) were administered to

![ALT graph]

Fig. 1. The effects of betaine on mouse liver injured by CCl₄. The mice were
intraperitoneally injected with 10 μl/g body weight of 10% CCl₄ at 24h before
betaine administration. Aminotransferase activities (ALT) at 24h after intra-
peritoneal (1–10 mg/kg body) and oral (10–30 mg/kg body) administration of betaine
were estimated using an automatic analyzer, COBAS MIRA. The results represent
the mean ± SD of ten mice. An asterisk indicates significant differences between
betaine-treated group and only CCl₄-treated group (*p < 0.05). Control, non-CCl₄
-treated; P 810, Panacete 810 plus saline; CCl₄, carbon tetrachloride plus saline.

Vol 44, No 2, 1998
Table 1. Histological analysis of betaine treatment for CCl₄-injured liver.

<table>
<thead>
<tr>
<th></th>
<th>Intraperitoneal administration (mean ± SD; 10⁻⁴ mm²)</th>
<th>Oral administration (mean ± SD; 10⁻⁴ mm²)</th>
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</thead>
<tbody>
<tr>
<td>CCl₄</td>
<td>306.8 ± 226.1</td>
<td>361.7 ± 178.8</td>
</tr>
<tr>
<td>Betaine</td>
<td>226.1 ± 115.5</td>
<td>189.8 ± 77.6*</td>
</tr>
</tbody>
</table>

The areas of hepatocyte necrosis were estimated by measuring two dimensional lengths under microscopy. Liver samples were prepared 24h after betaine administration (intraperitoneal, 3 mg/kg body weight; oral, 15 mg/kg body weight). The tissue was fixed with 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxyline/eosin. The results represent the mean ± SD of 8 randomly selected fields of 5 samples in one group. Asterisk indicates a significant difference as compared with the CCl₄-treated group (*p<0.05).

Table 2. 5-Bromo-2-deoxyuridine/5-fluoro-2'-deoxyuridine (BrdU/FdU) incorporation into the hepatic nucleus at 24 h after the administration of betaine.

<table>
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<tr>
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<th>BrdU/FdU incorporation (Counts/microscopic field)</th>
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<tbody>
<tr>
<td></td>
<td>Intraperitoneal administration</td>
</tr>
<tr>
<td>CCl₄</td>
<td>113.3 ± 2.2</td>
</tr>
<tr>
<td>Betaine</td>
<td>99.4 ± 25.2</td>
</tr>
</tbody>
</table>

BrdU was injected at 2 h before sacrificing. The results represent the mean ± SD of 10 randomly selected fields of 5 mice in one group. Asterisks indicates a significant difference as compared with the CCl₄-treated group (**p<0.01). One microscopic field corresponded at an area of 2.74 mm², and the number of anti-BrdU antibody-positive cells was counted under a magnification of 400. Betaine administered: intraperitoneal, 3 mg/kg body weight; oral, 15 mg/kg body weight.

the mice 24 h after the CCl₄ injection, since the maximal peak of serum ALT activity released was observed at 24 h after CCl₄ treatment as reported in our previous study (11). As shown in Fig. 1, the levels of ALT activity released in the sera were restrained to about half of that in CCl₄-treated mice, for both oral (15 mg/kg body weight) and intraperitoneal (3 mg/kg body weight) administration. The results of Fig. 1 suggested that the optimal concentrations of betaine under our experimental conditions are 3 mg/kg body weight for intraperitoneal administration and 15 mg/kg body weight for oral administration. Furthermore, oral administration (15 mg/kg body weight) of betaine drastically decreases the level of liver necrosis (Table 1) and increases the incorporation of BrdU/FdU into hepatocellular nuclear DNA (Table 2). Betaine intraperitoneally administered at 3 mg/kg body weight tended to decrease liver necrosis but did not induce the uptake of S-phase indicator (Tables 1 and 2).
Fig. 2. Immunohistochemical photographs of the hepatonuclear uptake of BrdU. Experimental procedure is described in the text (×400). Control, non-CCl4 treated. P 810, Panacete 810 plus saline; CCl4, carbon tetrachloride plus saline; betaine (oral or intraperitoneal administration), carbon tetrachloride plus 3 mg/kg body or 15 mg/kg body of betaine.
Figure 2 shows the typical features of nuclear BrdU uptake in the control, P 810-treated, CCl₄-injected, and CCl₄ with betaine-administered mice. The positive nuclear granules were very rare in the CCl₄-injected mice (Fig. 2c, g). Contrarily, the oral administration (15 mg/kg body weight) of betaine drastically increased the positive nuclear granules and number of double nuclei cells. Therefore, the oral administration of betaine to post-CCl₄ intoxicated mice may enhance hepatocellular proliferation. However, we could not elucidate a fundamental difference in the two administration methods in this report.

It is widely accepted that orally or intraperitoneally administered betaine distributes into the liver and muscle within 12 h (12) and plays an important role for SAM production, which will lower hepatic collagen and prolyl hydroxylase activity (13). The concentration of SAM in mouse liver was raised within 10 min after the oral administration of betaine (increase rate of SAM at 10 min after treatment; betaine/control: 2.35±0.83) (unpublished data). Therefore, it can be assumed that the administration of betaine to a CCl₄-injured mouse may reduce liver fibrosis and change the amount of phosphatidylcholine (PC), since a substantial amount of PC in the liver is synthesized by phosphatidylethanolamine methyltransferase using SAM as a methyl donor (14). Consequently, an alteration of cell membrane circumstances may induce hepatocellular proliferation.

In conclusion, the administration of betaine using either method (oral or intraperitoneal) lessens the activity of serum ALT. Furthermore, the oral administration of betaine modulates the rate of proliferation in regenerating liver. However, more detailed investigation is required for an understanding of the mechanism of in vivo influence of betaine.

REFERENCES


