〈Original article〉

Effect of three major nutrients on carbon tetrachloride-induced hepatotoxicity in mice

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Summary In this study, the effect of nutrients on hepatotoxicity induced by carbon tetrachloride (CCl_4) in mice was examined. The mice were divided into control, fasted, fed, starch, albumin and olive oil groups, and those groups, except for the control group, were injected intraperitoneally with CCl_4. The fed and starch groups showed significantly decreased activities of serum aminotransferase, concentrations of hepatic thiobarbituric acid reactive substances and triglycerides (TG) when compared with the fasted group. The hepatic glycogen concentrations were significantly increased in the fed and starch groups when compared with the fasted group. There was a significant exponential negative correlation between the increase in glycogen concentration and the decrease in alanine aminotransferase (ALT) activity, whereas the increase in TG concentration had a significant positive correlation with the increase in ALT activity. The sucrose intake was also similar to that of the starch intake. In conclusion, these results indicate that glucide intake protects mice from CCl_4-induced hepatotoxicity in comparison to protein or lipid intake. Furthermore, high lipid intake aggravates CCl_4-induced hepatotoxicity.

Key words: Carbon tetrachloride (CCl_4), Three major nutrients, Thiobarbituric acid reactive substances (TBARS), Triglyceride, Glycogen

1. Introduction

Carbon tetrachloride (CCl_4) has been used to induce hepatotoxicity in many experimental animals[1]. In general, it is known that CCl_4 is metabolized to the trichloromethyl radical ($.CCl_3$) by the liver microsomal cytochrome P-450 as the drug-metabolizing enzyme[2, 3]. $.CCl_3$ formed from CCl_4 in hepatocytes reacts rapidly with oxygen to yield a trichloromethyl peroxy radical. These radicals attack the unsaturated fatty acids in the cell membrane, leading to lipid peroxidation and necrosis. Furthermore, the accumu-

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lation of lipid peroxide in hepatocytes enhances hepatotoxicity. In general, it is also already known that resistance to CCl_4-induced liver injury is influenced by the nutritional condition[4]. Gomez et al. reported that there is a significant difference in the response of the liver to CCl_4 between starved and fed rats[5]. McLean et al. reported that giving a high-protein diet to rats worsens the CCl_4-induced liver injury[6]. Nakajima et al. reported that rats maintained on a high carbohydrate (sucrose) diet are protected from this liver injury[7]. However, this experiment provided two or more of the nutrients and did not provide one single nutrient. In the present study, we investigated the protective effect of three major nutrients against CCl_4-induced hepatotoxicity in mice.

2. Materials and methods

1. Animals

Male ddY strain mice, 6 weeks old, were purchased from Japan SLC, Inc. (Shizuoka). They were maintained with commercial laboratory chow, Oriental MF (Oriental Yeast Co., Tokyo, Japan), and water *ad libitum* for 1 week prior to experiments on a 12 h dark/12 h light cycle. All experiments were performed under the guideline of the Animal Care and Use Committee of Fujita Health University.

2. Experimental designs

The effect of nutrients on CCl_4-induced liver injury was examined in three experiments. In experiment 1, the mice were divided into eight groups (seven mice per group) and fasted for 24 hours before the experiment. Thereafter, four groups were provided with Oriental MF, and the other four groups were fasted. After 24 hours, the fed and fasted groups were injected intraperitoneally with CCl_4 (0, 1.0, 5.0 and 10.0 mmol/kg body weight) dissolved in Panacete 810 (synthetic middle-chain saturated triacylglycerol, NOF Co., Tokyo, Japan). In experiment 2, the mice were divided into six groups (seven mice per group) and fasted for 24 hours before the experiment. Thereafter, the groups 1 and 3 were fed Oriental MF, group 2 was fasted again, and groups 4, 5 and 6 were provided with starch as a glucide diet, albumin as a protein diet and olive oil as a lipid diet, respectively. After 24 hours, groups 2-6 were injected intraperitoneally with CCl_4 (1.0 mmol/kg BW) dissolved in Panacete 810, and group 1 as the control group was injected with Panacete 810. In experiment 3, the mice were divided into four groups (seven mice per group) and fasted for 24 hours before the experiment. Thereafter, groups 1 and 3 were fed Oriental MF, group 2 was fasted again, and group 4 was provided with a sucrose solution *ad libitum*. 24 hours after the administered Oriental MF and sucrose solution, groups 2-4 were injected intraperitoneally with CCl_4 (1.0 mmol/kg BW) dissolved in Panacete 810, and group 1 as the control group was injected with Panacete 810. In all the experiments, at 24 hours after CCl_4 injection, blood samples were drawn from Somnopentyl (Pentobarbital Sodium; Kyoritsu Seiyaku Corporation, Tokyo, Japan) anesthetized mice through a heart puncture for the enzyme analysis, and liver tissues were immediately isolated for the biochemical analysis.

3. Sample Preparation

Blood samples were centrifuged at 7,000 × g for 10 min at 4℃, and the sera were carefully separated. The separated sera and isolated livers were stored at -80℃ until use. Liver samples were homogenized for 30 sec using a Polytron homogenizer (KINEMATICA AG, Lucerne, Switzerland) with nine volumes (per gram of tissue) of ice-cold 0.14 M KCl including 1 % Triton X-100, the homogenates were sonicated on ice for 30 sec and were centrifuged at 7,000 × g for 10 min at 4℃.

4. Biochemical analyses

The activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed using an UV-kinetic method by an automatic analyzer COBAS MIRA (Roche Diagnostics GmbH, Mannheim, Germany)[8]. The following parameters in the liver homogenate were determined: the contents of Thiobarbituric acid reactive substances (TBARS) as a measure of lipid peroxide, triglycerides (TG), and glycogen. The concentrations of TBARS, TG, and glycogen were measured by the thiobarbituric acid

method, acetylacetone method, and anthrone method, respectively[9-11]. The amounts of TBARS, TG and glycogen were expressed as that of malondialdehyde (MDA), glycerol trioleate and glucose, respectively. The reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) and KATAYAMA CHEMICAL INDUSTRIES Co., Ltd. (Osaka, Japan).

5. Statistical analysis

The obtained data are expressed as the mean ± standard deviation (SD). Statistical calculations for this study were carried out using a software package "Prism 4" purchased from GraphPad Software, Inc. (San Diego CA). The data were statistically evaluated using one-way ANOVA. For all analyses, probability P values less than 0.05 were considered as significant difference.

3. Results

1. Effect of the nutritional condition and volume of injected CCl_4 on CCl_4-induced hepatotoxicity (exper-

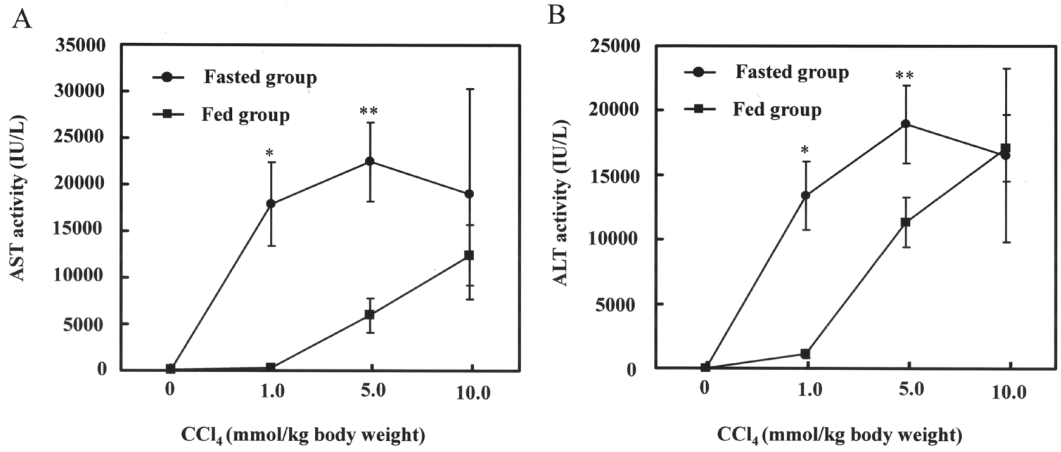

Fig. 1 Effect of nutritional condition and injection volume of CCl4 on (A) serum AST activity and (B) ALT activity in the CCl_4-treated mice (experiment 1).
Values are mean ± SD, n = 7. *,** These values differed significantly ($P < 0.001$, $P < 0.01$) from the fed group at the same concentration of CCl_4 volume.

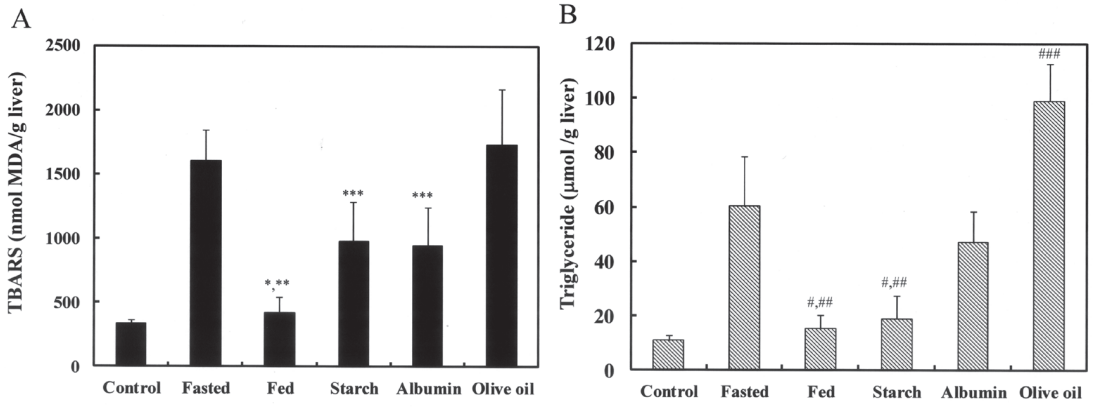

Fig. 2 Effects of three major nutrients on (A) TBARS and (B) Triglyceride concentrations in the CCl_4-treated mice (experiment 2).
Values are mean ± SD, n = 7. * These values differed significantly ($P < 0.001$) from the Fasted and Olive oil groups, ** these values differed significantly ($P < 0.05$) from the Starch and Albumin groups, *** these values differed signif icantly ($P < 0.01$) from the Fasted and Olive oil groups, # these values differed significantly ($P < 0.001$) from the Fasted and Olive oil groups, ## these values differed significantly ($P < 0.01$) from the Albumin group, and ### these values differed significantly ($P < 0.001$) from the fasted and Albumin groups.

iment 1)

The activities of serum AST and ALT in serum, as marker enzymes of liver damage, were significantly decreased in the 1.0 and 5.0 mmol/kg BW CCl_4-treated fed groups when compared with the fasted groups (Fig. 1). The 1.0 mmol/kg BW CCl_4-treated fed group hardly developed any liver damage. However, the 10.0 mmol/kg BW CCl_4-treated fed group developed liver damage, and there was no significant difference in aminotransferase activities between the 10.0 mmol/kg BW CCl_4-treated fed and fasted groups.

3.2. Protective effect of three major nutrients against CCl_4-induced liver injury (experiment 2)

The weight change and nutrient intake of mice in this experiment period are shown in Table 1. The body weight of control and fed groups increased, and that of the other groups decreased. The olive oil group took in the highest energy among the three kinds of nutrient groups, this group and the fed group were almost the same for calorie content. The aminotransferase activities were significantly decreased in the fed and starch groups when compared with the fasted, albumin, and olive oil groups (Table 2). No significant difference in serum aminotransferase activities was observed between the fasted and albumin groups. However, the level of aminotransferase activities in the olive oil group was significantly higher than in the fasted group. The fed, starch and albumin groups significantly reduced the TBARS concentration in the liver when compared with the fasted group, and the TBARS concentration in the fed group was significantly lower than that in the starch and albumin groups (Fig. 2A). There was no significant difference in the TBARS concentration between the fasted and olive oil groups. The fed and starch groups significantly reduced the TG concentration in the liver when compared with the fasted, albumin and olive oil groups, and the TG concentration in the olive oil group was significantly higher than that in the fasted and albumin groups (Fig. 2B).

Table 1 Effects of three major nutrients on body weight and energy intake in the CCl_4-treated mice (experiment 2)

Experimental condition	Panacete810	Carbon Tetrachloride				
	Control (Fed)	Fasted	Fed	Starch	Albumin	Olive oil
Final body weight, g	36.9 ± 2.9	28.8 ± 1.5	36.3 ± 3.8	27.1 ± 1.8	30.3 ± 1.7	28.0 ± 1.8
Body weight gain, g/2 days	1.3 ± 1.0 [a]	-6.2 ± 0.7	3.8 ± 0.7 [b]	-2.2 ± 0.5	-0.9 ± 0.8	-1.5 ± 1.2
Energy intake, kcal / mouse	16.8	0	20.4	12.7	8.2	20.3

Values are means ± S.D., n = 7. [a]These values differed significantly ($P < 0.001$) from the fasted, Starch, Albumin and Olive oil groups, and [b]these values differed significantly ($P < 0.001$) from all the other groups.

Table 2 Effects of three major nutrients on serum aminotransferase activities in the CCl_4-treated mice (experiment 2)

Experimental condition	Panacete810	Carbon Tetrachloride				
	Control (Fed)	Fasted	Fed	Starch	Albumin	Olive oil
AST activity (IU/L)	67 ± 25	19104 ± 7593 [a]	1067 ± 784 [b]	2439 ± 1251 [b]	18865 ± 5185 [a]	37769 ± 4530
ALT activity (IU/L)	18 ± 5	17623 ± 6496 [a]	2275 ± 1427 [b]	3954 ± 2020 [b]	16795 ± 4163 [a]	23223 ± 2107

Values are means ± S.D., n = 7. [a]These values differed significantly ($P < 0.05$) from the Olive oil group, and [b]these values differed significantly ($P < 0.001$) from the fasted, Albumin and Olive oil groups.

The glycogen concentration in the liver was significantly increased in the fed, starch and albumin groups when compared with the fasted and olive oil groups (Table 3). In addition, the glycogen concentration in the liver in the fed and starch groups was markedly higher than the albumin group. The liver glycogen level had a significant negative correlation with serum ALT activity (r = -0.863, $p < 0.001$, Fig. 3A), whereas the liver TG level had a significant positive correlation with serum ALT activity (r = 0.894, p < 0.001, Fig. 3B).

3. Protective effect of sucrose against CCl_4-induced hepatotoxicity (experiment 3)

The average calorie intakes of the control and

Table 3 Effects of three major nutrients on hepatic glycogen concentration in the CCl_4-treated mice (experiment 2)

Experimental condition	Panacete810	Carbon Tetrachloride				
	Control (Fed)	Fasted	Fed	Starch	Albumin	Olive oil
Glycogen (μmol glucosyl residues/g liver)	380 ± 68	11 ± 6	283 ± 72 [a]	235 ± 185 [b]	41 ± 15 [c]	4 ± 3

Values are means ± S.D., n = 7. [a]These values differed significantly ($P < 0.001$) from the Fasted, Albumin and Olive oil groups, [b]these values differed significantly ($P < 0.01$) from the Fasted, Albumin and Olive oil groups, and , [c]these values differed significantly ($P < 0.05$) from the Fasted and Olive oil groups.

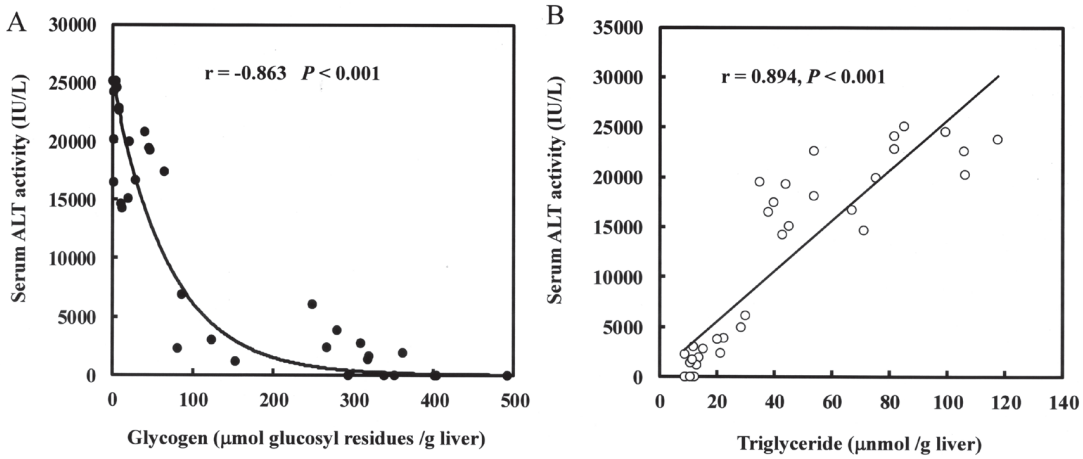

Fig. 3 Relationship between serum ALT activity and hepatic levels of triglyceride(A) or glycogen (B) (experiment 2).

Table 4 Effects of sucrose on serum aminotransferase activities in the CCl_4-treated mice (experiment 3)

Experimental condition	Panacete810	Carbon Tetrachloride		
	Control (Fed)	Fasted	Fed	Sucrose
AST activity (IU/L)	50 ± 14	30689 ± 5343	2383 ± 1325 [a]	312 ± 158 [b,c]
ALT activity (IU/L)	34 ± 15	23213 ± 2444	6179 ± 2930 [a]	772 ± 445 [b,c]

Values are mean ± SD, n = 7. [a]These values differed significantly ($P < 0.05$) from the Fasted group, [b]these values differed significantly ($P < 0.01$) from the Fasted group, [c]these values differed significantly ($P < 0.05$) from the Fed group.

fed groups in the experiment period were 18.8 kcal and 19.2 kcal, and the quantities of glucide intake were 11.0 kcal and 11.2 kcal, respectively. The average calorie intake of the sucrose group in this period was 12.0 kcal, and this result was equal to the quantity of glucide intake of the control and fed groups. The aminotransferase activities of the fed and sucrose groups were significantly decreased when compared with the fasted group, and the aminotransferase activities of the sucrose group was the lowest among all the groups (Table 4). The fed and sucrose groups significantly reduced the TBARS and TG concentrations in the liver when compared with the fasted group, there was no significant difference in the TBARS and TG concentrations between the fed and sucrose groups (Fig. 4). The glycogen concentration in the liver was significantly decreased in the fasted group when compared with the fed and sucrose groups (Table 5).

4. Discussion

In this study, we examined the protective effect of nutrients to CCl_4-induced liver injury by focusing on the changes in CCl_4 concentration, nutritional condition and the types of nutrients. The result of experiment 1 showed that feeding protected mice against CCl_4-induced liver injury. However, feeding showed no protective effect against hepatotoxicity when CCl_4 at a high concentration (10.0 mmol/kg BW) was administered to mice. Therefore, it was revealed from this result that both the CCl_4 concentration and the nutritional condition influence the degree of liver injury.

As the 1.0 mmol/kg BW CCl_4-treated fed group hardly developed any liver injury, this concentration was used for the next experiment. In experiment 2, when the glucide (starch), protein (albumin) and a lipid (olive oil) were administered to mice independently, we examined whether the three major nutrients

Table 5 Effects of sucrose on hepatic glycogen concentration in the CCl_4-treated mice (experiment 3)

Experimental condition	Panacete810	Carbon Tetrachloride		
	Control (Fed)	Fasted	Fed	Sucrose
Glycogen (μmol glucosyl residues/g liver)	182 ± 71	12 ± 8	188 ± 79 [a]	188 ± 122 [a]

Values are mean ± SD, n = 7. [a]These values differed significantly ($P < 0.01$) from the Fasted group.

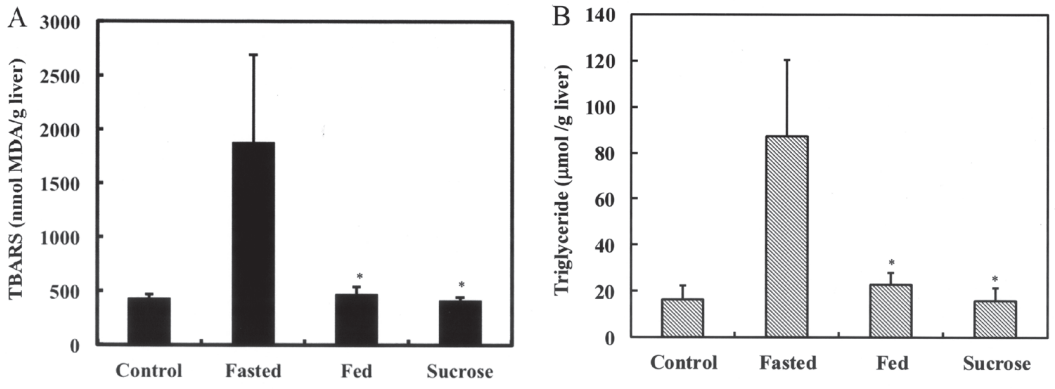

Fig. 4 Effects of sucrose on (A) TBARS and (B) Triglyceride concentrations in the CCl_4-treated mice (experiment 3). Values are mean ± SD, n = 7. *These values differed significantly ($P < 0.001$) from the Fasted group.

have the protection effect in CCl_4-induced liver injury. The most effective nutrient was starch (12.7 kcal), which took in less energy than the olive oil group (20.3 kcal). This result suggests that glucide intake protects the liver from CCl_4-induced liver injury. Furthermore, it was also demonstrated that the onset of this liver injury could be suppressed by the content of glycogen in liver tissue because there was a significant negative correlation between the amount of glycogen in liver tissue and the ALT activity in serum. Rahman et al. reported that glycogen is mobilized during the disposal of peroxides by the cultured astroglial cells from the rat brain[12]. The cultured cell needs a glutathione redox cycling system for the disposal of peroxides. The glutathione redox cycling system is very important in cellular peroxide detoxification. The peroxide generated in the body is metabolized by glutathione peroxidase (GPx) and reduced glutathione (GSH) as the substrate of GPx to the corresponding alcohols. The generated glutathione disulfide (GSSG) is then reproduced by glutathione reductase (GR) to GSH. GR needs the reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a coenzyme when GSSG is reproduced to GSH. The oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) generated by this reaction is regenerated by the pentose phosphate pathway (PPP) to NADPH. Glucose 6-phosphoric acid, which is the first substrate of PPP, is then required. Therefore, the aggravation of CCl_4-induced liver injury is suppressed by the increase of the glycogen in liver tissue because glucose-6-phosphate is supplied by glycogen. Although the olive oil group took in the most energy among the three kinds of nutrient groups, olive oil intake aggravated this liver injury. These results suggest that the TBARS level in liver tissue is increased by the free radicals derived from CCl_4 that oxidize the TG accumulated in the liver. Hence, we infer the following from these results. The mitochondrial swelling and cristae disappearance of cristae are induced by the various free radicals which arise from the metabolism of CCl_4. The function of mitochondrial ß-oxidation that converts lipid into energy is reduced. Because the administered lipid is not converted into energy, a lot of that is accumulated in the liver, and it is suggested that the administered lipid becomes the supply source of TBARS. Amino acid that is ingested in the body as protein is partly used for a gluconeogenesis. In case of mitochondrial dysfunction by CCl_4, the generation of glucose, which has a protective effect on CCl_4-induced liver injury, from amino acid is inhibited. Furthermore, since all amino acid is not changed into glucose, we inferred that the CCl_4-induced liver injury had not been improved. In experiment 3, we investigated the effect of sucrose on CCl_4-induced liver injury. As a result, the administration of sucrose also increased significantly the glycogen concentration in liver and prevented the onset of this liver injury. This result agrees with the previously reported study of Nakajima et al[7].

In conclusion, in order to prevent the onset of CCl_4-induced liver injury, calorie intake is not necessarily important, but the types of administered nutrients are very important. Glucide is the most important nutrient among the three major nutrients in terms of this liver injury. In addition, it became clear that ingestion of a lipid at the time of this liver injury onset worsened the symptoms.

References

1. Chopra P, Roy S, Ramalingaswami V, Nayak NC: Mechanism of carbon tetrachloride hepatotoxicity. An in vivo study of its molecular basis in rats and monkeys. Lab Invest, 26: 716-727, 1972.
2. Brattin WJ, Glende EA Jr, Recknagel RO: Pathological mechanisms in carbon tetrachloride hepatotoxicity. J Free Radic Biol Med, 1: 27-38, 1985.
3. Recknagel RO, Glende EA Jr, Dolak JA: Mechanisms of carbon tetrachloride toxicity. Pharmacol Ther, 43: 139-154, 1989.
4. Krishnan N, Stenger RJ: Effects of starvation on the hepatotoxicity of carbon tetrachloride. A light and electron microscopic study. Am J Pathol, 49: 239-255, 1966.
5. Gomez MI, de Castro CR, de Ferreyra EC, Acosta ND, de Fenos OM, Castro JA: Mechanistic studies on carbon tetrachloride hepatoxicity in fasted and fed rats. Toxicol Appl Pharmacol, 32: 101-108, 1975.
6. McLean AE, McLean E: Diet and toxicity. Br med Bull, 25: 278-281, 1969.
7. Nakajima T, Koyama Y, Sato A: Dietary modification of metabolism and toxicity of chemical substances－

with special reference to carbohydrate. Biochem Pharmacol, 31: 1005-1011, 1982.

8. Karmen A: A note on the spectrometric assay of glutamic-oxalacetic transaminase in human blood serum. J Clin lnvest, 34: 131-133, 1955.

9. Mihara M, Uchiyama M: Effects of antioxidants on the TBA reaction of various rat liver homogenates. Biochem Med, 30: 131-134, 1983.

10. Fletcher MJ: A colorimetric method for estimating serum triglycerides. Clin Chim Acta, 22: 393-397, 1968.

11. Roe Jh, Dailey R: Determination of glycogen with the anthrone reagent. Anal Biochem, 15: 245-250, 1966.

12. Rahman B, Kussmaul L, Hamprecht B, Dringen R: Glycogen is mobilized during the disposal of peroxides by cultured astroglial cells from rat brain. Neurosci Lett, 290: 169-172, 2000.