

Functions of CLA and ARA for Prevention of CCl₄-Induced Fatty Liver in Mice

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Abstract: Oral administration of carbon tetrachloride (CCl₄) induces acute hepatitis while Prostaglandins (PGs) are proposed to attenuate liver injury. Conjugated Linoleic Acid (CLA) influences the synthesis of PGs and Arachidonic Acid (ARA) is a precursor of PGs. Therefore, whether CLA or ARA attenuates the hepatitis induced by CCl₄ was investigated in mice. Male mice (age, 8 week) were given commercial diet and combinations of paraffin or CCl₄ solution with or without CLA or ARA oil were orally administered. Food intake and body weight were significantly reduced in the CCl₄ group. Although, no significant changes in liver weight were observed, liver triacylglycerol contents in the CCl₄+CLA and CCl₄+ARA groups were markedly higher than those in groups other than the CCl₄ group. Plasma AST and ALT levels were unusually elevated after CCl₄ administration irrespective of oil treatment. CCl₄+ARA treatment greatly increased PGE₂ content in the liver and followed by CCl₄+CLA treatment. In conclusion, co-administration of CLA and ARA enhanced liver PGE₂ levels after CCl₄ treatment but acute hepatitis was not attenuated.

Key words: Conjugated linoleic acid, arachidonic acid, mice, carbon tetrachloride, hepatitis, prostaglandin E₂

INTRODUCTION

Carbon tetrachloride (CCl₄) induces liver injury after oral administration. Liver color changes reddish violet and lipids accumulate in the liver within 24 h of single oral administration (Masuda, 2006). Necrosis of hepatocytes in surrounding of central venous, damage to lysosomes and mitochondria in hepatic cells are induced by CCl₄. Furthermore, chronic administration of CCl₄ induces increases in fibers in the liver which results in hepatic cirrhosis. CCl₄ forms the radical •CCl₃ which then promotes lipoperoxidation and reduces enzymatic activity in microsome (Recknagel and Ghoshal, 1966). These phenomena are known to be related to decreases in cytochrome P450 which is mainly associated with energy and drug metabolism.

Long chain n-6 Polyunsaturated Fatty Acids (PUFAs) are considered to be essential fatty acids, and are metabolized by elongases and desaturases. In n-6 PUFAs, Linoleic Acid (LA) is metabolized to γ-Linolenic Acid (GLA), Dihomo-γ-Linolenic Acid (DGLA) and Arachidonic Acid (ARA). DGLA and ARA are then metabolized to Prostaglandin E₁ (PGE₁) and prostaglandin E₂ (PGE₂), respectively. Prostaglandins (PGs) are known to have various functions (Gurr *et al.*, 2002). For example,

alcoholic liver injury can be attenuated by increasing of PGE₂ levels (Lukivskaya *et al.*, 2001) and PGE₁ or PGE₂ injections into rat liver prevent toxicity induced by CCl₄ (Masaki *et al.*, 1992). However, previous studies have largely used PG pharmacologically. No information is available on attenuation by nutrients. Conjugated Linoleic Acid (CLA) is a generic term referring to the positional and geometrical isomers of LA which are largely present in food items produced from ruminant animals (Kepler *et al.*, 1966; Kepler and Tove, 1967). The double bond structures in CLA are different from those in LA, although CLA and LA have the same carbon chain length and number of double bonds (Eulitz *et al.*, 1999). The double bonds in CLA form conjugated diene structures, and numerous CLA isomers have been discovered. CLA has been found to have several functions, including anti-obese effects, anti-carcinogenesis and anti-atherogenesis effects (Nakanishi *et al.*, 2004; Oikawa *et al.*, 2003; Pariza *et al.*, 2001).

However, feeding with CLA diet for 4 week was found to induce liver enlargement with high lipid content in mice, as chronic CLA reduces PGE₂ content in the liver (Nakanishi *et al.*, 2004; Tsuboyama-Kasaoka *et al.*, 2000). In contrast, acute CLA supplementation increased PGE₂ content as compared to

the LA group (Nakanishi *et al.*, 2004). Therefore, the purpose of the present study was to investigate whether CLA or ARA attenuates hepatotoxicity induced by CCl₄ through hepatic PGE₂ production in mice.

MATERIALS AND METHODS

Animals and treatments: Male mice (age, 8 weeks; Sea:ddY strain; purchased from CLEA Japan, Inc., Tokyo, Japan) were kept at 25°C on a 12 h light-dark cycle (8:00-20:00), housed individually, and had free access to a commercial diet (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) and water. The chemical composition (%) of the commercial diet was: moisture, 7.7; crude protein, 23.6; crude fat, 5.3; crude ash, 6.1; crude fiber, 2.9 and nitrogen-free extract, 54.4. Mice were divided into seven groups of 7 mice each according to body weight, after acclimation for 6 days. The 7 groups were intact (commercial diet alone), paraffin, CCl₄, paraffin+CLA, paraffin+ARA, CCl₄+CLA and CCl₄+ARA, respectively. CLA oil (triacylglycerol-type) provided by Nisshin Oilio Group, Ltd. (Tokyo, Japan) was used. CLA oil contained 83.0% CLA (40.5% 9-cis, 11-trans-CLA and 39.6% 10-trans, 12-cis-CLA). ARA oil (SUNTGA40S: triacylglycerol-type) provided by Suntory Ltd. (Osaka, Japan) was used and ARA content was 45.3%. The fatty acid composition of these oils is shown in Table 1. On the other hand, liquid paraffin and CCl₄ were purchased from Sigma-Aldrich (St. Louis, USA). CLA or ARA oil (6.25 µL g⁻¹ body) and liquid paraffin or CCl₄ (1.25 µL g⁻¹ body) were orally administered once using 1 mL syringes attached to tubes. Mice were given *ad libitum* access to commercial diet and food intake and body weight were individually determined for 1 day. Mice were killed by cervical dislocation and decapitation at one day after administration. Experimental procedures conformed to the guidelines for Animal Experiments of the Faculty of Agriculture and of the Graduate Course of Kyushu University, as well as the Laws (No.105) and Notifications (No.6) of the Japanese Government.

Sample collection: Blood was collected from the carotid artery into tubes with heparin when mice were decapitated. Blood was centrifuged for 10 min at 690×g and the collected plasma was stored at -30°C until assay for lipids and hepatitis parameters. Livers were then removed and weighed. Liver tissue was stored at -80°C until analyses for lipids.

Plasma assay: Triacylglycerol, cholesterol and free fatty acid contents were analyzed using commercial kits (Triglyceride E-test, Cholesterol E-test and NEFA C-test, respectively, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Aspartate aminotransferase (AST) and Alanine

Table 1: Compositions (% of total fatty acids) of experimental oils

Composition	CLA	ARA
C16:0	5.3	8.6
C18:0	1.4	6.5
C18:1	9.7	6.3
C18:2 (n-6)	0.6	9.4
CLA	83.0	-
9 cis, 11 trans-	40.5	-
10 trans, 12 cis-	39.6	-
cis, cis-	1.8	-
trans, trans-	1.1	-
C18:3 (n-6)	-	2.3
C20:3 (n-6)	-	3.2
C20:4 (n-6)	-	45.3
Others	-	18.4

CLA, Conjugated Linoleic Acid; ARA, Arachidonic Acid

aminotransferase (ALT) concentrations were analyzed using a commercial kit (transaminase CII-test, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Liver analyses

Triacylglycerol and cholesterol contents: Total lipids in the liver were extracted using the method of Bligh and Dyer (1959) with some modification. An acetic acid (0.1 mol L⁻¹)/chloroform/methanol (1:2.5:1.25, vol/vol/vol) solution was added to the liver sample, followed by homogenization. The mixture was left for 10 min at room temperature. Chloroform and distilled water were added and the mixture was stirred. The solution was centrifuged for 10 min at 890 × g in order to separate it into two distinct phases and the lower phase was collected. Chloroform was again added into the upper phase followed by mixing, centrifugation and collection of the lower phase. Both fractions were filtered and mixed with distilled water/methanol/chloroform (1:1:0.1, vol/vol/vol) solution. Samples were centrifuged at 890×g for 10 min. The lower phase was dried with a centrifuge evaporator and the dried extract was diluted with isopropanol followed by assay using triacylglycerol and cholesterol determination kits.

PGE₁ and PGE₂ concentrations: PGE₁ and PGE₂ were extracted using the methods recommended by GE Health Care Ltd. (Little Chalfont, UK). PGE₁ assay was performed with a PGE₁ EIA kit (Assay designs Inc, Ann Arbor, USA) and PGE₂ assay was performed with a PGE₂ EIA kit (GE health care Ltd, Little Chalfont, UK).

Statistical methods: Data were statistically analyzed by one-way analysis of variance. When significant effects were seen, the seven dietary groups were compared by Tukey-Kramer's. Statistical significance was set at p<0.05. Results are shown as the means±SM. Statistical outliers were omitted by the Thompson rejection test at p<0.01.

RESULTS AND DISCUSSION

Food intake, body weight gain, tissue weight and plasma constituents are shown in Table 2. Daily food intake in CCl₄-treated groups was significantly ($F(6,41) = 59.8; p < 0.0001$) less than in other groups. Similarly, body weight gain in CCl₄ groups was significantly ($F(6,41) = 20.5; p < 0.0001$) lower than in the intact and paraffin groups and was lowest in the CCl₄+ARA group. No significant ($F(6,41) = 0.96; p = 0.47$) influences on liver weight were observed among the treatments. However, CCl₄-treated groups showed significantly ($F(6,41) = 16.6; p < 0.0001$) elevated triacylglycerol content in the liver as compared to the intact and paraffin+ARA groups while the triacylglycerol contents in CCl₄+CLA and CCl₄+ARA were 4.6-9.3 times higher than in the intact and paraffin groups, respectively. On the other hand, total cholesterol content in the liver was not significantly ($F(6,41) = 1.39; p = 0.24$) different among the groups. Plasma AST and ALT levels were significantly ($F(6,40) = 2737; p < 0.0001$ and $F(6,40) = 2737; p < 0.0001$) elevated after CCl₄ administration, but supplementation with CLA or ARA did not reduce the elevated AST and ALT levels induced by CCl₄. With regard to plasma lipids, administered CCl₄ significantly ($F(6,40) = 3.93; p = 0.004$) lowered total cholesterol concentration when compared to the paraffin and paraffin+CLA groups.

In contrast to CCl₄ alone, combination of CLA or ARA with CCl₄ tended to promote the release of cholesterol into blood while triacylglycerol and free fatty acid in plasma did not significantly ($F(6,40) = 1.96; p = 0.10$ and $F(6,40) = 1.73; p = 0.14$) differ among the groups. Liver PGE₂ content is shown in Fig. 1. CCl₄+ARA treatment significantly ($F(6,36) = 4.06; p = 0.003$) increased hepatic PGE₂ content when compared with other treatments, except for CCl₄+CLA. PGE₂ content in the CCl₄+CLA group tended to be higher than that in the intact group, the CCl₄ alone group and the three paraffin groups. The effect on liver PGE₂ content (ng g⁻¹ liver weight) was significant ($F(6,36) = 3.42; p = 0.009$) different among the groups (intact: 8.1±1.4; paraffin:

4.5±0.6; CCl₄: 36.4±15.2; paraffin+CLA: 3.0±0.6; CCl₄+CLA: 30.4±10.7; paraffin+ARA: 7.1±0.4 and CCl₄+ARA: 34.7±12.2). Single administration of CCl₄ alone or with fatty acids reduced both food intake and body weight in mice (Table 2). Mice were freely given commercial diet in the present experiment. Okamoto and Okabe (2000) similarly reported that combination of CCl₄ with olive oil suppressed food intake and increased body weight gain in rats, both with and without food restriction. Single administration of CCl₄ or oils did not induce hepatic enlargement (Table 2).

However, liver triacylglycerol content was higher in CCl₄ groups than in other groups. Data were similar to the results obtained by Kawamoto *et al.* (1996). Furthermore, triacylglycerol accumulation in the liver induced by CCl₄ tended to increase with CLA or ARA but did not differ between paraffin and paraffin with CLA or ARA treatment.

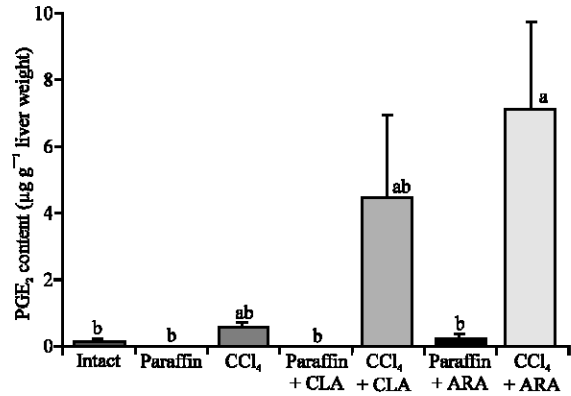


Fig. 1: Hepatic PGE₂ content in mice given combinations of paraffin or CCl₄ with CLA or ARA. Number of mice used in the intact, paraffin, CCl₄, paraffin+CLA, paraffin+ARA, CCl₄+CLA and CCl₄+ARA groups were 6, 6, 5, 6, 6, 7 and 7, respectively. Values are means±S.E.M. Groups labeled with different letters are significantly different ($p < 0.05$)

Table 2: The effect of combinations of paraffin or CCl₄ with CLA or ARA treatments on food intake, body weight gain, liver and plasma constituents

Groups	Intact	Paraffin	CCl ₄	Paraffin+CLA	CCl ₄ +CLA	Paraffin+ARA	CCl ₄ +ARA
Food intake (g day ⁻¹)	5.94±0.25 ^a	5.87±0.19 ^a	1.51±0.30 ^c	4.54±0.47 ^b	1.08±0.12 ^c	3.89±0.30 ^b	0.86±0.19 ^c
Body weight gain (g)	0.04±0.34 ^a	0.20±0.32 ^a	-4.41±0.49 ^{bc}	0.27±0.52 ^a	-3.30±0.72 ^b	1.20±0.38 ^a	-5.76±0.80 ^c
Liver							
Weight (g)	1.96 ± 0.10	2.00±0.16	2.02±0.02	1.99±0.08	2.06±0.09	1.79±0.08	1.91±0.06
Triacylglycerol (mg g ⁻¹ liver)	6.69±1.88 ^c	9.17±2.60 ^{bc}	26.7±6.21 ^{ab}	8.63±2.01 ^{bc}	45.3±7.80 ^a	4.88±0.93 ^c	42.3±4.69 ^a
Total cholesterol (mg g ⁻¹ liver)	2.65±0.24	2.82±0.23	2.82±0.53	2.74±0.14	3.55±0.09	2.23±0.34	2.98±0.42
Plasma							
AST (Karmen U)	303±55 ^b	266±72 ^b	4921±17 ^a	372±51 ^b	4928±22 ^a	369±62 ^b	4920±20 ^a
ALT (Karmen U)	53±9 ^b	47±11 ^b	783±3 ^a	64±8 ^b	784±4 ^a	63±10 ^b	785±2 ^a
Triacylglycerol (mg dL ⁻¹)	170±28	180±51	120±14	165±19	90±16	115±23	106±11
Total cholesterol (mg dL ⁻¹)	118±8 ^{bc}	168±15 ^a	93±10 ^c	162±11 ^{ab}	101±18 ^{bc}	131±19 ^{bc}	120±15 ^{abc}
Free fatty acid (mEq L ⁻¹)	1.03±0.09	1.12±0.19	1.32±0.10	1.04±0.08	1.31±0.12	1.00±0.07	1.20±0.04

ALT, Alanine aminotransferase; ARA, Arachidonic acid; AST, Aspartate aminotransferase; CLA, Conjugated Linoleic Acid, Values are means±SEM., groups with different letters are significantly different ($p < 0.05$)

Co-administered lipids were predicted to accumulate in the liver as impairment of mitochondria by CCl₄ suppresses degradation of fatty acids (Neubert and Maibauer, 1959; Recknagel and Lombardi, 1961; Smuckler and Beneditt, 1965). On the other hand, CCl₄ was reported to increase hepatic triacylglycerol content, as damage to endoplasmic reticulum by CCl₄ inhibits production and secretion of very low density lipoprotein (Recknagel *et al.*, 1960).

Indeed, single CCl₄ administration decreased total cholesterol released into the blood in the present study (Table 2). However, damage to the endoplasmic reticulum was not believed to induce further fatty liver in CCl₄ administration with CLA or ARA because co-administration of CLA or ARA tended to prevent the reductions in plasma total cholesterol by CCl₄ treatment. The hepatotoxicity of CCl₄ was very potent and CCl₄ increased AST and ALT activities by 16 and 14 fold over the intact group. Kim *et al.* (2007) reported that 0.1 or 0.2 mmol kg⁻¹ CCl₄ administered into the peritoneal cavity, respectively increased serum AST and ALT levels in ICR mice by 5 or 65 fold and 17 or 378 fold as compared to saline group. CLA or ARA co-treatment did not attenuate the elevation of AST and ALT levels induced by CCl₄ thus, single injection of CLA or ARA was unable to inhibit CCl₄-induced hepatitis. In contrast, hepatic PGE₂ content was high in the CCl₄+ARA followed by CCl₄+CLA groups (Fig. 1). It was assumed that PGE₂ levels were transiently elevated to prevent hepatotoxicity induced by CCl₄. ARA is a precursor of PGE₂ and CCl₄ stimulated PGE₂ production from ARA when compared to the paraffin+ARA group. Closa *et al.* (1998) reported that PG contents of the liver were significantly reduced in CCl₄ treated rats. Analogs of PGE₂, such as 16,16-dimethyl PGE₂ have been confirmed to exhibit hepatoprotection against CCl₄ (Quiroga and Prieto, 1993). Thus, the administration period for the oils may be too short in the present study. Although chronic administration of CLA induces fatty liver due to reductions in hepatic PGE₂ (Nakanishi *et al.*, 2004), long term ARA results in elevated hepatic PGE which inhibits hepatotoxicity. On the other hand, Gonzalez-Periz *et al.* (2006) reported that docosahexaenoic acid given for 1 week prevents damage against DNA and oxidative stress in mouse liver. Oxidative stress and free radicals derived from CCl₄ induce isoprostane D₂ and E₂ and lipid peroxide (Morrow *et al.*, 1994).

Hence, ARA treatment was thought to generate PG isomers and the protective effects against CCl₄-induced hepatitis may be better with direct injection of PGE₂ than with ARA injection. In addition, CCl₄ suppresses cytochrome P450 activity which is related to the electron transfer system and drug metabolism (Rush *et al.*, 1986). Single treatments of CLA or ARA were considered to be

unable to enhance cytochrome P450 activity in the present study. Therefore, the relationship between cytochrome P450 and nutritional fatty acids remains to be clarified.

CONCLUSION

In this study, single co-administration of CLA or ARA was unable to attenuate acute hepatitis induced by CCl₄ in mice while these fatty acids were able to enhance production of PGE₂ in the liver.

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