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 Towe, 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 PGE₁ 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 hepatic toxicity induced by CCl₄ through hepatic PGE₂ production in mice.

MATERIALS AND METHODS

**Animals and treatments:** Male mice (age, 8 weeks; 
Sae-12 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 (triaclyglycerol-type) provided by Nissien Oilio Group, Ltd. (Tokyo, Japan). CLA oil contained 83.0% CLA (40.5% 9-cis, 11-trans-CLA and 39.6% 10-trans, 12-cis-CLA). ARA oil (SUNGA40S; triacylglycerol-type) provided by Suntray 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 body) and liquid paraffin or CCl₄ (1.25 μL/g 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 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.

**PGE₅ and PGE₂ concentrations:** PGE₅ and PGE₂ were extracted using the methods recommended by GE Health Care Ltd. (Little Chalfont, UK). PGE₅ assay was performed with a PGE₅ 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: 6.4±1.5; paraffin+CLA: 3.0±0.6; CCl4+CLA: 3.0±1.0; paraffin+ARA: 7.1±0.4 and CCl4+ARA: 3.4±1.2). 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)]](image)

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.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Food intake (g day-1)</th>
<th>Body weight gain (g)</th>
<th>Weight (g)</th>
<th>Triacylglycerol (mg g-1 liver)</th>
<th>Total cholesterol (mg g-1 liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>5.9±0.25*</td>
<td>0.04±0.04*</td>
<td>1.96±0.10</td>
<td>6.69±1.88*</td>
<td>2.65±0.24*</td>
</tr>
<tr>
<td>Paraffin</td>
<td>5.87±0.19*</td>
<td>0.20±0.32*</td>
<td>2.00±1.16</td>
<td>6.17±0.69*</td>
<td>2.82±0.23*</td>
</tr>
<tr>
<td>CCl4</td>
<td>1.5±0.30*</td>
<td>-4.4±1.04*</td>
<td>2.02±0.62</td>
<td>26.7±6.21*</td>
<td>2.82±0.53*</td>
</tr>
<tr>
<td>Paraffin+CLA</td>
<td>4.5±0.47*</td>
<td>0.27±0.52*</td>
<td>1.59±0.08</td>
<td>8.63±2.01*</td>
<td>2.74±0.14*</td>
</tr>
<tr>
<td>CCl4+CLA</td>
<td>1.8±0.12*</td>
<td>-5.3±0.72*</td>
<td>2.06±0.09</td>
<td>45.3±7.80*</td>
<td>3.89±0.30*</td>
</tr>
<tr>
<td>Paraffin+ARA</td>
<td>3.89±0.30*</td>
<td>1.20±0.38*</td>
<td>1.79±0.08</td>
<td>4.88±0.53*</td>
<td>3.89±0.30*</td>
</tr>
<tr>
<td>CCl4+ARA</td>
<td>0.8±0.19*</td>
<td>-7.8±0.89*</td>
<td>1.91±0.06</td>
<td>42.3±4.69*</td>
<td>2.98±0.42*</td>
</tr>
</tbody>
</table>

Plasma

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (Kammen U)</th>
<th>ALT (Kammen U)</th>
<th>Triacylglycerol (mg dl-1)</th>
<th>Total cholesterol (mg dl-1)</th>
<th>Free fatty acid (mmol L-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>303±59*</td>
<td>53±9*</td>
<td>170±28</td>
<td>118±8*</td>
<td>1.03±0.09*</td>
</tr>
<tr>
<td>Paraffin</td>
<td>266±72*</td>
<td>47±11*</td>
<td>180±51</td>
<td>168±15*</td>
<td>1.12±0.19*</td>
</tr>
<tr>
<td>CCl4</td>
<td>492±17*</td>
<td>783±4*</td>
<td>120±14</td>
<td>93±10*</td>
<td>1.32±0.10*</td>
</tr>
<tr>
<td>Paraffin+CLA</td>
<td>372±51*</td>
<td>64±8*</td>
<td>165±19</td>
<td>162±11*</td>
<td>1.04±0.08*</td>
</tr>
<tr>
<td>CCl4+CLA</td>
<td>492±22*</td>
<td>784±4*</td>
<td>90±16</td>
<td>101±18*</td>
<td>1.31±0.12*</td>
</tr>
<tr>
<td>Paraffin+ARA</td>
<td>369±62*</td>
<td>6310±4*</td>
<td>115±25</td>
<td>131±19*</td>
<td>1.30±0.07*</td>
</tr>
<tr>
<td>CCl4+ARA</td>
<td>492±20*</td>
<td>785±42*</td>
<td>106±11</td>
<td>120±15*</td>
<td>1.29±0.04*</td>
</tr>
</tbody>
</table>

ALT, Alanine aminotransferase;ARA, Arachidonic acid;AST, Aspartate aminotransferase; CLA, Conjugated Linoleic Acid. Values are means±S.E.M. 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 Mairbauer, 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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