Journal of Animal and Veterinary Advances 9 (22): 2854-2858, 2010
ISSN: 1680-5593

Functions of CLA and ARA for Prevention of CCl_4-Induced Fatty Liver in Mice

[1]Daichi Oikawa, [2]Yoriko Akimoto, [2]Yurika Mizobe,
[2]Shoichiro Tsuyama and [2]Mitsuhiro Furuse
[1]Laboratory of Food Science, Faculty of Education,
Nagasaki University, Nagasaki 852-8521, Japan
[2]Laboratory of Advanced Animal and Marine Bioresources,
Graduate School of Bioresources and Bioenvironmental Sciences,
Kyushu University, Fukuoka 812-8581, Japan

Abstract: Oral administration of carbon tetrachloride (CCl_4) 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 CCl_4 was investigated in mice. Male mice (age, 8 week) were given commercial diet and combinations of paraffin or CCl_4 solution with or without CLA or ARA oil were orally administered. Food intake and body weight were significantly reduced in the CCl_4 group. Although, no significant changes in liver weight were observed, liver triacylglycerol contents in the CCl_4+CLA and CCl_4+ARA groups were markedly higher than those in groups other than the CCl_4 group. Plasma AST and ALT levels were unusually elevated after CCl_4 administration irrespective of oil treatment. CCl_4+ARA treatment greatly increased PGE_2 content in the liver and followed by CCl_4+CLA treatment. In conclusion, co-administration of CLA and ARA enhanced liver PGE_2 levels after CCl_4 treatment but acute hepatitis was not attenuated.

Key words: Conjugated linoleic acid, arachidonic acid, mice, carbon tetrachloride, hepatitis, prostaglandin E_2

INTRODUCTION

Carbon tetrachloride (CCl_4) 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 CCl_4. Furthermore, chronic administration of CCl_4 induces increases in fibers in the liver which results in hepatic cirrhosis. CCl_4 forms the radical $\bullet CCl_3$ 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 E_1 (PGE_1) and prostaglandin E_2 (PGE_2), respectively. Prostaglandins (PGs) are known to have various functions (Gurr *et al*., 2002). For example, alcoholic liver injury can be attenuated by increasing of PGE_2 levels (Lukivskaya *et al*., 2001) and PGE_1 or PGE_2 injections into rat liver prevent toxicity induced by CCl_4 (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 PGE_2 content in the liver (Nakanishi *et al*., 2004; Tsuboyama-Kasaoka *et al*., 2000). In contrast, acute CLA supplementation increased PGE_2 content as compared to

Corresponding Author: Daichi Oikawa, Laboratory of Food Science, Faculty of Education, Nagasaki University, Nagasaki 852-8521, Japan

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 CCl_4 through hepatic PGE_2 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, CCl_4, paraffin+CLA, paraffin+ARA, CCl_4+CLA and CCl_4+ARA, respectively. CLA oil (triacylglycerol-type) provided by Nisshin Oillio 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 Ccl_4 were purchased from Sigma-Aldrich (St. Louis, USA). CLA or ARA oil (6.25 μL g^{-1} body) and liquid paraffin or CCl_4 (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 aminotransferase (ALT) concentrations were analyzed using a commercial kit (transaminase CII-test, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

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

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.

PGE_1 and PGE_2 concentrations: PGE_1 and PGE_2 were extracted using the methods recommended by GE Health Care Ltd. (Little Chalfont, UK). PGE_1 assay was performed with a PGE_1 EIA kit (Assay designs Inc, Ann Arbor, USA) and PGE_2 assay was performed with a PGE_2 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 Ccl_4-treated groups was ignificantly ($F(6,41) = 59.8$; $p<0.0001$) less than in other groups. Similarly, body weight gain in CCl_4 groups was significantly ($F(6,41) = 20.5$; $p<0.0001$) lower than in the intact and paraffin groups and was lowest in the CCl_4+ARA group. No significant ($F(6,41) = 0.96$; $p = 0.47$) influences on liver weight were observed among the treatments. However, CCl_4-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 CCl_4+CLA and CCl_4+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 CCl_4 administration, but supplementation with CLA or ARA did not reduce the elevated AST and ALT levels induced by Ccl_4. With regard to plasma lipids, administered CCl_4 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 CCl_4 alone, combination of CLA or ARA with CCl_4 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 PGE_2 content is shown in Fig. 1. CCl_4+ARA treatment significantly ($F(6,36) = 4.06$; $p = 0.003$) increased hepatic PGE_2 content when compared with other treatments, except for Ccl_4+CLA. PGE_2 content in the CCl_4+CLA group tended to be higher than that in the intact group, the CCl_4 alone group and the three paraffin groups. The effect on liver PGE_1 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; CCl_4: 36.4±15.2; paraffin+CLA: 3.0±0.6; Ccl_4+CLA: 30.4±10.7; paraffin+ARA: 7.1±0.4 and Ccl_4+ARA: 34.7±12.2). Single administration of CCl_4 alone or with fatty acids reduced both food intake and body weight in mice (Table 2). Mice were freely given commercial diet in thepresent experiment. Okamoto and Okabe (2000) similarly reported that combination of CCl_4 with olive oil suppressed food intake and increased body weight gain in rats, both with and without food restriction. Single administration of CCl_4 or oils did not induce hepatic enlargement (Table 2).

However, liver triacylglycerol content was higher in CCl_4 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 CCl_4 tended to increase with CLA or ARA but did not differ between paraffin and paraffin with CLA or ARA treatment.

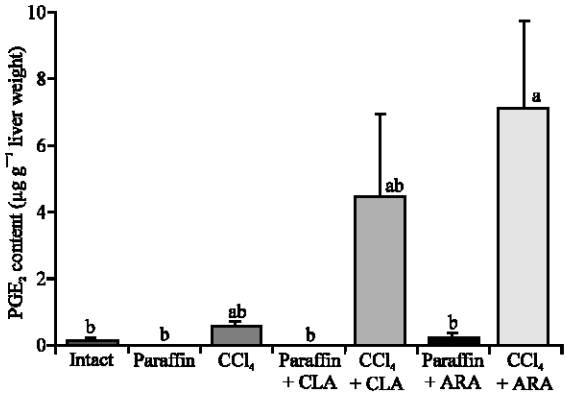

Fig. 1: Hepatic PGE_2 content in mice given combinations of paraffin or CCl_4 with CLA or ARA. Number of mice used in the intact, paraffin, CCl_4, paraffin+CLA, paraffin+ARA, CCl4+CLA and CCl_4+ARA groups were 6, 6, 5, 6, 6, 7 and 7, respectively. Values are means±S.E.M. Groups labeled with different letters are significantly different ($p<0.05$)

Table 2: The effect of combinations of paraffin or CCl_4 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^{abc}	168±15^{a}	93±10^{c}	162±11^{ab}	101±18^{bc}	131±19^{abc}	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 CCl_4 suppresses degradation of fatty acids (Neubert and Maibauer, 1959; Recknagel and Lombardi, 1961; Smuckler and Beneditt, 1965). On the other hand, CCl_4 was reported to increase hepatic triacylglycerol content, as damage to endoplasmic reticulum by CCl_4 inhibits production and secretion of very low density lipoprotein (Recknagel *et al*., 1960).

Indeed, single CCl_4 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 CCl_4 administration with CLA or ARA because co-administration of CLA or ARA tended to prevent the reductions in plasma total cholesterol by CCl_4 treatment. The hepatotoxicity of CCl_4 was very potent and CCl_4 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} CCl_4 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 CCl_4 thus, single injection of CLA or ARA was unable to inhibit Ccl_4-induced hepatitis. In contrast, hepatic PGE_2 content was high in the CCl_4+ARA followed by CCl_4+CLA groups (Fig. 1). It was assumed that PGE_2 levels were transiently elevated to prevent hepatotoxicity induced by Ccl_4. ARA is a precursor of PGE_2 and CCl_4 stimulated PGE_2 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 Ccl_4 treated rats. Analogs of PGE_2, such as 16,16-dimethyl PGE_2 have been confirmed to exhibit hepatoprotection against CCl_4 (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 PGE_2 (Nakanishi *et al*., 2004), long term ARA results in elevated hepatic PGE which in hibits 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 CCl_4 induce isoprostane D_2 and E_2 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 PGE_2 than with ARA injection. In addition, CCl_4 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 CCl_4 in mice while these fatty acids were able to enhance production of PGE_2 in the liver.

ACKNOWLEDGEMENTS

This study was supported by a research fellowship of the Japan Society for the promotion of science for young scientists (No. 09551) and a Nagasaki University President's Fund Grant. Mice were housed at the Biotron Institute of Kyushu University.

REFERENCES

Bligh, E.G. and W.J. Dyer, 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37: 911-917.

Closa, D., M. Torres, G. Hotter, G. Bioque, O.S. Leon, E. Gelpi and J. Roselli-Catafau, 1998. Prostanoids and free radicals in Cl4C induced hepatotoxicity in rats: Effect of astilbin. Prostaglandins Leukot. Essent. Fatty Acids, 56: 331-334.

Eulitz, K., M.P. Yurawecz, N. Sehat, J. Fritsche and J.A. Roach *et al*., 1999. Preparation, separation and confirmation of the eight geometrical cis/trans conjugated linoleic acid isomers 8,10-through 11,13-18:2. Lipids, 34: 873-877.

Gonzalez-Periz, A., A. Planaguma, K. Gronert, R. Miquel and M. Lopez-Parra *et al*., 2006. Docosahexaenoic acid (DHA) blunts liver injury by conversion to protective lipid mediators: Protectin D1 and 17S-hydroxy-DHA. FASEB. J., 20: 2537-2539.

Gurr, M.I., J.L. Harwood and K.N. Frayn, 2002. Lipid Biochemistry: An Introduction. 5th Edn., Blackwell Science Ltd., Oxford UK., pp: 77-83.

Kawamoto, N., A. Murai, J.I. Okumura and M. Furuse, 1996. Effect of enprostil, prostaglandin E2 analogue, on liver triacylglycerol concentration in mice treated with or without carbon tetrachloride. Anim. Sci. Technol., 67: 104-105.

Kepler, C.R. and S.B. Tove, 1967. Biohydrogenation of unsaturated fatty acids. J. Biol. Chem., 242: 5686-5692.

Kepler, C.R., K.P. Hirons, J.J. McNeill, and S.B. Tove, 1966. Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. J. Biol. Chem., 241: 1350-1354.

Kim, S.J., Y.S. Jung, M.Y. Yoon and Y.C. Kim, 2007. Comparative effects of dimethylsulfoxide on metabolism and toxicity of carbon tetrachloride and dichloromethane. J. Applied Toxicol., 27: 25-31.

Lukivskaya, O.Y., A.A. Maskevich and V.U. Buko, 2001. Effect of ursodeoxycholic acid on prostaglandin metabolism and microsomal membranes in alcoholic fatty liver. Alcohol, 25: 99-105.

Masaki, N., Y. Ohta, H. Shirataki, I. Ogata and S. Hayashi *et al*., 1992. Hepatocyte membrane stabilization by prostaglandin E1 and E2: Favorable effects on rat liver injury. Gastroenterology, 102: 572-576.

Masuda, Y., 2006. Learning toxicology from carbon tetrachloride-induced hepatotoxicity. J. Pharma. Soc. Jap., 126: 885-899.

Morrow, J.D., T.A. Minton, C.R. Mukundan, M.D. Campbell and W.E. Zackert *et al*., 1994. Free radical-induced generation of isoprostanes *in vivo*. Evidence for the formation of D-ring and E-ring isoprostanes. J. Biol. Chem., 269: 4317-4326.

Nakanishi, T., D. Oikawa, T. Koutoku, H. Hirakawa, Y. Kido, T. Tachibana and M. Furuse, 2004. γ-linolenic acid prevents conjugated linoleic acid-induced fatty liver in mice. Nutr., 20: 390-393.

Neubert, D. and D. Maibauer, 1959. Comparative examination of the oxidative functions of the mitochondria and microsomes in experimental liver injuries. Naunyn. Schmiedebergs Arch. Exp. Pathol. Pharmakol., 235: 291-300.

Oikawa, D., T. Nakanishi, Y. Nakamura, Y. Takahashi and T. Yamamoto *et al*., 2003. CLA and DHA modify skin properties in mice. Lipids, 38: 609-614.

Okamoto, T. and S. Okabe, 2000. Carbon tetrachloride treatment induces anorexia independently of hepatitis in rats. Int. J. Mol. Med., 6: 181-183.

Pariza, M.W., Y. Park and M.E. Cook, 2001. The biologically active isomers of conjugated linoleic acid. Prog. Lipid Res., 40: 283-298.

Quiroga, J. and J. Prieto, 1993. Liver cytoprotection by prostaglandins. Pharmacol. Ther., 58: 67-91.

Recknagel, R.O. and A.K. Ghoshal, 1966. Lipoperoxidation as a vector in carbon tetrachloride hepatotoxicity. Lab. Invest., 15: 132-148.

Recknagel, R.O. and B. Lombardi, 1961. Studies of biochemical changes in subcellular particles of rat liver and their relationship to a new hypothesis regarding the pathogenesis of carbon tetrachloride fat accumulation. J. Biol. Chem., 236: 564-569.

Recknagel, R.O., B. Lombardi and M.C. Schotz, 1960. A new insight into pathogenesis of carbon tetrachloride fat infiltration. Proc. Soc. Exp. Biol. Med., 104: 608-610.

Rush, B., M.V. Merritt, M. Kaluzny, T.V. Schoick, M.N. Brunden and M. Ruwart, 1986. Studies on the mechanism of the protective action of 16,16-dimethyl PGE2 in carbon tetrachloride induced acute hepatic injury in the rat. Prostaglandins, 32: 439-455.

Smuckler, E.A. and E.P. Beneditt, 1965. Studies on carbon tetrachloride intoxication. III. A subcellular defect in protein synthesis. Biochem., 4: 671-679.

Tsuboyama-Kasaoka, N., M. Takahashi, K. Tanemura, H.J. Kim and T. Tange *et al*., 2000. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. Diabetes, 49: 1534-1542.