Effect of Dietary Conjugated Linoleic Acid on Lipid Peroxidation and Histological Change in Rat Liver Tissues

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The effect of dietary conjugated linoleic acid (CLA) on hepatic lipid parameters in Sprague−Dawley rats was examined. When rats were fed a diet containing CLA at 0 (control), 1, or 2% of the weight of the amount of food given for 3 weeks, the liver weight exhibited a slight increase in the CLA-fed groups, although the difference was not significant. Lipid accumulation in the hepatocytes of CLA-fed rats was also demonstrated by electron microscopic observation. In addition, the liver thiobarbituric acid reactive substances levels were significantly higher in the 2 wt % CLA group than in the other two dietary groups, and the levels of phosphatidylcholine hydroperoxide were higher in CLA-fed groups when compared to that of the control group. On the other hand, the serum lipid peroxide levels were comparable among all three dietary groups. Levels of triglycerides in the white adipose tissue (WAT) and serum nonesterified fatty acid (NEFA) were reduced in a CLA-dose-dependent manner. CLA was shown to accumulate in the WAT much more than in the serum or liver. These results suggest that CLA accelerates the decomposition of storage lipids in WAT and the clearance of serum NEFA levels, resulting in lipid peroxidation and a morphological change in the liver.

Keywords: *Conjugated linoleic acid; liver histology; phosphatidylcholine hydroperoxide; thiobarbituric acid reactive substances*

INTRODUCTION

Conjugated linoleic acid (CLA) is a generic term for the geometric and positional isomers of octadecadienoic acid. It has been reported that CLA has diverse physiological functions such as anticarcinogenic (Ip et al., 1995, 1996; Thompson et al., 1997; Visonneau et al., 1997), antidiabetic (Houseknecht et al., 1998), antiplatelet (Truitt et al., 1999), and antiallergic (Sugano et al., 1998) activities. It has been also reported that CLA has antioxidant properties as well. For example, dietary CLA can reduce the thiobarbituric acid reactive substance (TBARS) level in the mammary gland (Ip et al., 1991), and CLA can act as a more powerful antioxidant than butylated hydroxytoluene (BHT) and α-tocopherol (Ha et al., 1990). On the other hand, it has been reported that CLA does not act as an effective radical scavenger and metal chelator (Van den Berg et al., 1995). When the liver homogenate of rats that were fed diets containing extra virgin olive oil (EVO) or EVO and 4% CLA was oxidated by adenosine 5′-diphosphate-Fe or *tert*-butyl hydroperoxide, lipid peroxidation occurred, generating conjugated diene fatty acid hydroperoxide. These results suggest that CLA dose not have any antioxidant properties (Banni et al., 1998). Moreover, free CLA and CLA methyl ester accelerated oxygen uptake upon heating at 90 °C, and CLA-containing triglyceride (TG) did not affect the oxidative process (Chen et al., 1997). In oxidatively challenged liver cells, CLA could behave as a prooxidant by decreasing the activity of catalase (CAT) and superoxide dismutase (SOD) at 20 ppm (Cantwell et al., 1998).

The effect of CLA on the liver was studied by some researchers, and it was reported that CLA can regulate the expression of acyl-CoA oxidase (ACO), cytochrome P450A1, and liver fatty acid binding protein (Belury et al., 1997). The peroxisomal β-oxidation pathway is adopted mainly for the decomposition of long-chain fatty acids, thus producing hydrogen peroxide, so it is thought that disorder of this pathway can induce lipid accumulation and lipid peroxidation. In addition, it has been reported that CLA can activate the peroxisome proliferator-activated receptor (PPAR) γ in white adipose tissue (WAT) in the Zucker diabetic rat (Houseknecht et al., 1998). It has also been reported that dietary CLA reduced the WAT weight (Sugano et al., 1998); however, hepatic lipid accumulation was also induced by dietary CLA (Belury et al., 1997). Thus, the effect of dietary CLA on liver function needs to be researched in detail. In the present paper, the effect of a high CLA diet on lipid peroxidation and liver morphology is studied.

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Table 1. Fatty Acid Composition of the Experimental Diet

	CLA dose		
fatty acid (wt %)	0%	1%	2%
16:0	7.5	7.4	7.3
18:0	2.5	2.5	2.5
18:1 (*n*−9)	14.2	14.3	14.5
18:2 (*n*−6)	73.7	63.3	52.8
CLA	ND[a]	10.6	21.2
18:3 (*n*-3)	0.7	0.6	0.5
others	1.4	1.3	1.2

[a] ND, not detected.

EXPERIMENTAL PROCEDURES

Experimental Animals and Diets. Dietary CLA and safflower oil were prepared by Rinol Oil Mills Co., Ltd. (Nagoya, Japan). Male, 4-week-old Sprague−Dawley rats (Seiwa Experimental Animals, Fukuoka, Japan) were fed a nonpurified diet and were provided water ad libitum for a week after arrival. After acclimation, the rats were separated into three groups of seven rats each. Experimental diets were prepared according to AIN-93G diets as shown in Table 1, and body weight and food intake were measured every other day. After 3 weeks of feeding, rats were sacrificed by withdrawing blood from the abdominal aorta under light anesthesia using diethyl ether, and then tissues were excised and weighed. Serum was prepared immediately after blood collection. Tissues and serum were kept at −30 °C before analysis. This experiment was carried out under the guidelines stated for animal experiments in Faculty of Agriculture and the Graduate Course, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese government.

Histological Studies. After excision, tissues were minced and prefixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 3.4% sucrose and 3 mM calcium chloride. The post fixation for electron microscopy (EM) was performed with 1% osmic acid dissolved in the 0.1 M cacodylate buffer on ice for 1.5 h. Afterward, samples were dehydrated in graded ethanol and then replaced with propylene oxide and embedded in Epon 812 resin/dodecenyl succinic anhydride/methyl nadic anhydride/2,4,6-tri(dimethylaminomethyl phenol) = 23:15.5:11.7:0.75. All of the reagents for embedding were purchased from TAAB (Berkshire, U.K.). Ultrathin sections were doubly stained with 2% uranyl acetate for 60 min and 0.4% lead citrate for 10 min and then analyzed on an electron microscope (JEM 2000EX JEOL, Tokyo, Japan) at 80 kV.

Lipid Peroxidation Assay. As an index of lipid peroxidation, TBARS and phosphatidylcholine hydroperoxide (PCOOH) levels in the liver and serum were measured. TBARS levels were measured using a commercial kit (Wako Pure Chemicals, Osaka, Japan). PCOOH levels were measured using a method described by Miyazawa et al. (1987, 1992). Namely, total lipids in the liver and serum were extracted by chloroform/methanol = 2:1 containing 0.002% BHT (Folch et al., 1957). The extraction procedure was performed two times, and the extracted solution was evaporated under a nitrogen stream in a dark condition. PCOOH was measured by high-performance liquid chromatography (Waters LC Module I, Tokyo, Japan) with a chemiluminescence detector (JASCO CL-925, Tokyo, Japan) equipped with a Finepak SIL NH2-5 (JASCO). The mobile phase consisted of a mixture of 2-propanol, methanol, and distilled water (130:45:25), and the solution for the luminescence reaction contained 10 mg of cytochrome *c* (Sigma, Tokyo, Japan) and 2 mg of luminol (Wako) in 1 L of borate buffer (pH 9.6). To prepare the PCOOH standard, a solution containing 2 mg of PC and 0.4 mg of Rose Bengal (Wako) in 2 mL of methanol was irradiated using a spectrofluorophotometer (Shimadzu RF-1500, Kyoto, Japan) scanning from 220 to 800 nm for 1 h. To remove Rose Bengal, Sep-Pak Accell Plus QMA cartridges (Milford, MA) were used after the irradiation, and the concentration of the PCOOH standard was measured by using the xylenol-orange assay using cumene hydroperoxide as a standard (Jiang et al., 1991).

Table 2. Growth Parameters and Liver Weight of Rats Fed the Experimental Diets[a]

	CLA dose		
	0%	1%	2%
initial body wt (g)	148 ± 4	148 ± 3	148 ± 2
final body wt (g)	274 ± 8	267 ± 6	262 ± 4
food intake (g/day)	18.7 ± 0.4	18.4 ± 0.3	17.4 ± 0.4
liver wt (g/100 g of body wt)	3.3 ± 0.1	3.8 ± 0.1	3.7 ± 0.2

[a] Values are means ± SE for seven rats.

TG levels in WAT and serum NEFA level were measured using commercial kits (Wako).

Measurement of Fatty Acid Composition. Lipid extraction from the liver, serum, and WAT was performed according to the method described by Folch et al. (1957). The solution containing total fatty acids from the liver was dissolved in 2 mL of 0.87% sulfuric acid in methanol and 1 mL of dimethyl sulfoxide to methylate the sample. The analysis of fatty acid composition was performed by gas−liquid chromatography (Shimadzu GC-17A) using a Supelcowax 10 column (0.32 mm × 60 m, 0.25 mm film thickness; Supelco Inc., Bellefonte, PA). The column temperature was raised from 150 to 220 °C at the rate of 4 °C/min, and the detector and injector temperatures were set at 250 °C. Identification of the CLA peaks was performed by equivalent chain length (Ha et al., 1989) using gas chromatography−mass spectrometry (JEOL Auto MS 50).

Statistical Analysis. Data were analyzed by Duncan's new multiple-range test to evaluate the significance of difference.

RESULTS

Growth Parameter. As shown in Table 2, there were no significant differences among the three dietary groups in terms of initial body weight, final body weight, and food intake. However, the liver weight was heavier in the two CLA-fed groups than in the control group, although this difference was not significant (Table 2). There was also no significant difference among the three groups in terms of weights of kidney, spleen, lung, heart, WAT, and brown adipose tissue (data not shown).

Liver Histology. Electron micrographs of the liver samples are shown in Figure 1. In the 2 wt % CLA group, the intrahepatocytic lipid accumulation is easily identifiable. On the other hand, there were much fewer lipid droplets in the control group. In the 1 wt % CLA group, the degree of lipid accumulation was between the control and 2 wt % CLA groups (data not shown). Differences in the number or morphology of other organelle-like peroxisome and mitochondria were not seen among the three dietary groups.

Hepatic Lipid Peroxidation. The effects of dietary CLA on serum and liver peroxide levels are summarized in Figure 2. During the process of lipid peroxidation, hydroperoxide is produced as the first stable product in both the radical and nonradical reactions, and TBARS are the secondary products. Serum TBARS levels were comparable among the three dietary groups. Serum PCOOH levels in the control and 2 wt % CLA groups were similar, but the 1 wt % CLA was lower than the other two groups, although this difference was not significant. Hepatic TBARS levels were approximately the same for the control and 1 wt % CLA groups, but the 2 wt % group was almost twice as high. Hepatic PCOOH levels for both CLA-fed groups were ∼3-fold higher than that of the control group.

Lipid Parameters in Serum and Adipose Tissue. Levels of TG in WAT and serum NEFA are shown in Table 3. WAT TG levels (micromoles per gram of tissue)

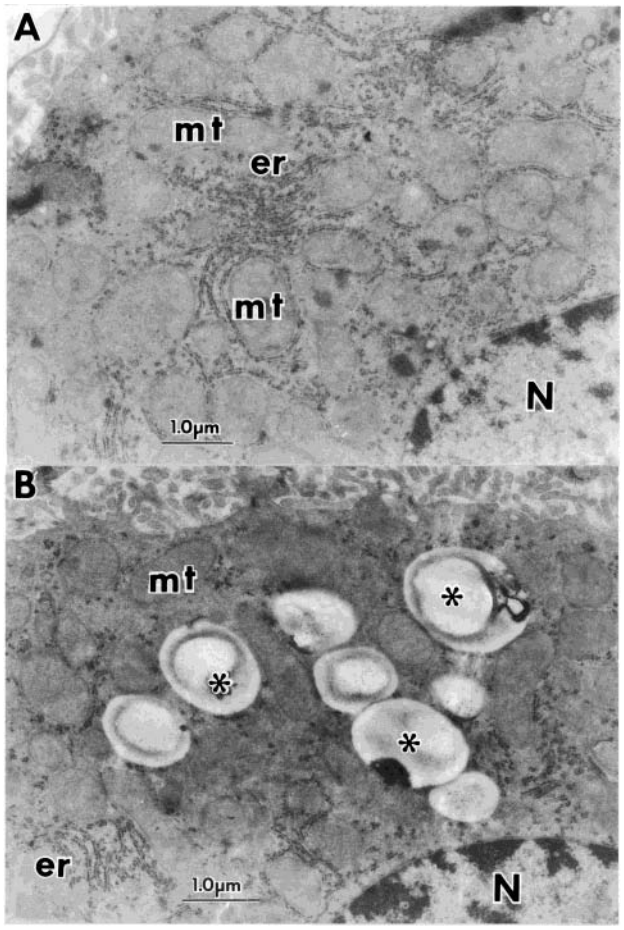

Figure 1. Electron micrographs of livers of rats fed with either (A) 0% CLA or (B) 2% CLA. *, lipid droplets; N, nucleus; mt, mitochondria; er, endoplasmic reticulum.

Table 3. Effect of Dietary CLA on WAT TG and Serum NEFA Level[a]

	CLA dose		
	0%	1%	2%
serum NEFA (mequiv/mL)	1.1 ± 0.3[a]	0.7 ± 0.1[b]	0.4 ± 0.1[c]
WAT TG (μmol/g of tissue)	393 ± 17[a]	322 ± 36[ab]	252 ± 35[b]

[a] Values are means ± SE for five or six rats. Values not sharing a common superscript letter are significantly different at $p < 0.05$.

decreased with the increase of CLA dose. The values for the 1 and 2 wt % CLA groups are 81 and 63% that of the control group, respectively, showing a significant drop. When we calculated TG level as milligrams per gram of lipids, the same tendency was observed (data not shown). Serum NEFA, which is supplied from WAT TG as an enzymatic decomposition product, decreased in a CLA-dose-dependent manner.

Table 4. Effect of Dietary CLA on Total Fatty Acid Composition of Liver[a]

	CLA dose		
fatty acid (wt %)	0%	1%	2%
14:0	0.4 ± 0.1	0.6 ± 0.5	0.5 ± 0.0
16:0	20.0 ± 1.5	19.2 ± 0.9	19.6 ± 0.6
16:1	0.6 ± 0.4	1.1 ± 0.2	0.7 ± 0.1
18:0	15.8 ± 2.1	13.5 ± 1.1	17.2 ± 1.2
18:1 (*n*−9)	8.5 ± 1.1	8.3 ± 1.3	8.0 ± 2.1
18:2 (*n*−6)	20.3 ± 1.3	17.6 ± 1.7	17.5 ± 2.3
CLA	ND[a]	1.2 ± 0.2[b]	2.6 ± 0.6[c]
18:3 (*n*−6)	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.1
20:3 (*n*−6)	0.3 ± 0.1	0.5 ± 0.3	0.3 ± 0.0
20:4 (*n*−6)	21.6 ± 1.6	17.7 ± 1.8	20.6 ± 1.3
22:4 (*n*−6)	0.8 ± 0.0	0.9 ± 0.1	0.8 ± 0.1
22:5 (*n*−6)	1.9 ± 0.5	2.7 ± 0.4	2.6 ± 0.3
saturated	36.1 ± 2.3	33.2 ± 2.0	37.3 ± 1.4
monounsaturated	9.1 ± 0.8	9.4 ± 0.9	8.6 ± 1.3
polyunsaturated	45.3 ± 2.1	40.8 ± 3.7	44.7 ± 2.2

[a] Values are means ± SE for five rats. Values not sharing a common superscript letter are significantly different at $p < 0.05$. ND, not detected.

Fatty Acid Composition. Total fatty acid composition in the liver, serum, and WAT are shown in Tables 4−6. Accumulation of CLA was shown to occur in a dose-dependent manner. In the CLA-fed groups, CLA content in WAT was ∼5-fold higher than in serum and liver. CLA was not detected in the control group. As shown in Tables 4−6, linoleic acid demonstrated a tendency to decrease in the CLA-fed group, but the difference was not significant in the liver and serum. Linoleic acid content of WAT in the 2 wt % CLA group was significantly lower than in the other dietary groups. Dietary CLA did not affect the composition of other major fatty acids in the liver, serum, and WAT. Total PUFA, saturated fatty acids (SA), and monounsaturated fatty acids level were all comparable among the three dietary groups.

DISCUSSION

In this paper, we measured TBARS and PCOOH levels in the serum and liver. Serum TBARS and PCOOH levels did not show much difference among the three groups; however, hepatic TBARS and PCOOH levels were significantly higher in the CLA groups than

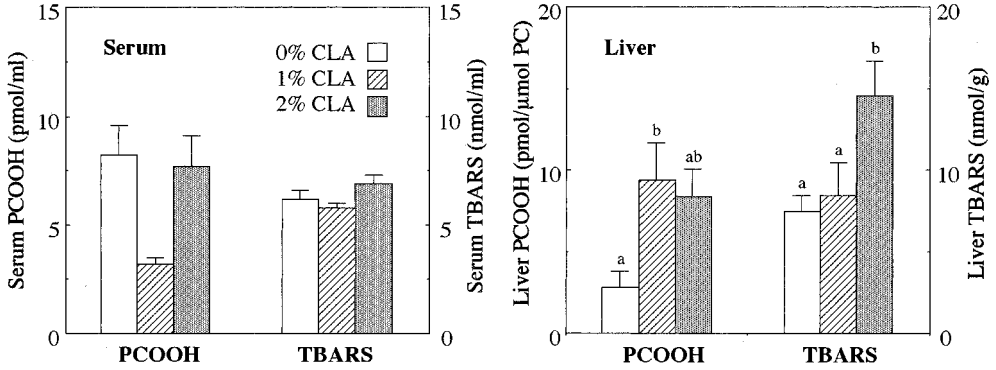

Figure 2. Effect dietary CLA on serum and liver lipid peroxidation levels in rats: 0% CLA (white bars); 1% CLA (slashed bars); 2% CLA (shaded bars). Data are means ± SE for five samples. Values not sharing a common letter (a, b) are significantly different at $p < 0.05$.

Table 5. Effect of Dietary CLA on Total Fatty Acid Composition of Serum[a]

	CLA dose		
fatty acid (wt %)	0%	1%	2%
14:0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
16:0	18.4 ± 1.8	18.9 ± 0.8	19.0 ± 1.8
16:1	1.4 ± 0.3	2.9 ± 2.0	1.1 ± 0.2
18:0	10.9 ± 0.6	10.8 ± 0.4	12.2 ± 1.5
18:1 (*n*−9)	8.5 ± 1.6	6.9 ± 0.4	8.1 ± 1.0
18:2 (*n*−6)	20.8 ± 1.6	20.1 ± 0.7	18.2 ± 1.0
CLA	ND[a]	1.0 ± 0.2[b]	2.4 ± 0.3[c]
18:3 (*n*−6)	ND	ND	ND
20:3 (*n*−6)	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.0
20:4 (*n*−6)	22.4 ± 2.2	29.0 ± 2.1	25.0 ± 3.0
22:4 (*n*−6)	0.7 ± 0.1	0.7 ± 0.0	1.1 ± 0.7
22:5 (*n*−6)	1.7 ± 0.3	2.3 ± 0.1	1.8 ± 0.2
saturated	29.6 ± 2.1	30.0 ± 1.2	31.6 ± 3.3
monounsaturated	9.9 ± 1.6	7.8 ± 0.5	9.1 ± 1.1
polyunsaturated	46.1 ± 2.0	53.5 ± 1.9	48.3 ± 3.9

[a] Values are means ± SE for five rats. Values not sharing a common superscript letter are significantly different at $p < 0.05$. ND, not detected.

Table 6. Effect of Dietary CLA on Total Fatty Acid Composition of WAT[a]

	CLA dose		
fatty acid (wt %)	0%	1%	2%
14:0	1.4 ± 0.1	1.8 ± 0.1	2.0 ± 0.1
16:0	26.1 ± 1.0	28.0 ± 0.7	29.7 ± 0.7
16:1	3.9 ± 0.4	3.0 ± 0.2	2.5 ± 0.3
18:0	6.7 ± 2.1	3.9 ± 0.1	4.3 ± 0.1
18:1 (*n*−9)	23.4 ± 0.2	22.2 ± 0.3	21.3 ± 0.8
18:1 (*n*−7)	2.3 ± 0.3	1.9 ± 0.3	1.8 ± 0.2
18:2 (*n*−6)	38.4 ± 2.6[a]	36.4 ± 0.6[a]	29.7 ± 0.4[b]
CLA	ND[a]	5.0 ± 0.1[b]	10.5 ± 0.4[c]
18:3 (*n*−6)	ND	ND	ND
20:3 (*n*−6)	ND	ND	ND
20:4 (*n*−6)	ND	ND	ND
22:4 (*n*−6)	ND	ND	ND
22:5 (*n*−6)	ND	ND	ND
saturated	34.3 ± 2.7	33.5 ± 0.8	35.9 ± 0.9
monounsaturated	27.3 ± 0.5	25.1 ± 0.3	23.8 ± 0.8
polyunsaturated	38.4 ± 2.6	41.4 ± 0.7	40.2 ± 0.5

[a] Values are means ± SE for five rats. Values not sharing a common superscript letter are significantly different at $p < 0.05$. ND, not detected.

in the control group. PCOOH is a stable primary product of the peroxidation process of PC in the radical and nonradical reactions. It has been reported that the liver PCOOH level in rats being fed a 10% fish oil diet is significantly higher than that of rats fed a 10% safflower oil (Miyazawa et al., 1992). Thus, the amount of liver PCOOH can be easily regulated by varying the dietary fatty acid composition. TBARS is a lipid peroxidation secondary product. As shown in Figure 2, the liver PCOOH levels were similar between the two CLA-fed groups. However, the liver TBARS level for the 2 wt % CLA group was significantly higher than control but the 1 wt % CLA group was not. Therefore, hepatic lipid peroxidation in the 2 wt % CLA group was accelerated as compared to the 1 wt % group. Among the three dietary groups, levels of PUFA and the major fatty acids determined from the samples taken from the liver, serum, and WAT were shown to have no significant differences. These results suggest that lipid peroxidation induced by dietary CLA did not contribute to the change of fatty acid composition.

It has been reported that CLA reduces catalase, superoxide dismutase, and glutathione peroxidase but does not induce lipid peroxidation in rat hepatocytes (Cantwell et al., 1999). It has also been reported that dietary CLA reduces glutatione-*S*-transferase activity in Sprague−Dawley rats, but not significantly (Ip et al., 1991). Ip's results suggest that the liver TBARS level tended to be lower in the CLA-fed group, so in this condition CLA could not act as prooxidant. It has been reported that CLA suppresses the growth of human breast and colon cancer cell lines accompanied with the elevation of lipid peroxidation (O'Shea et al., 1999). In human studies, CLA administration is shown to cause nonenzymatic and enzymatic lipid peroxidation (Basu et al., 2000). In this paper, CLA was administrated at 2 wt %, and this amount is higher than was used by the cited researchers in various studies; therefore, excess CLA treatment might trigger hepatic lipid peroxidation, and CLA should be administrated with an antioxidant to avoid hepatic lipid peroxidation.

WAT stores excess energy as TG, and when energy is needed, TG is decomposed to glycerol and NEFA by lipoprotein lipase (LPL). NEFA is then transported into various tissues via the bloodstream. We show that CLA decreases the serum NEFA level, as previously demonstrated in the Zucker diabetic fatty rats that were fed CLA (Houseknecht et al., 1998). It has been reported that CLA reduces not only body fat level (Park et al., 1997; West et al., 1998) but also the weight of WAT (Sugano et al., 1998). CLA has also been shown to regulate the LPL activity of 3T3-L1 adipocytes in vitro (Park et al., 1997). Thus, the reduction of the TG level in WAT may be a result of LPL activity. However, the reduction of the serum NEFA level seems to be inconsistent with the above-mentioned result because NEFA is supplied from WAT as a decomposition product of TG. Serum NEFA is mainly incorporated into the muscle and liver, so CLA could accelerate the incorporation of NEFA into muscle and liver, thus reducing serum NEFA.

In conclusion, dietary CLA induces lipid peroxidation and triggers deposition of lipids into the rat liver. The data presented here suggest that administration of oral CLA can be used to inhibit the lipid peroxidation process. However, CLA reduces both WAT TG and serum NEFA levels so it is hypothesized that serum NEFA derived from WAT TG is incorporated into the liver excessively. Whether or not this effect is limited to short-term feeding is unknown. Nonetheless, the effects of long-term feeding of CLA may cause problems; therefore, attention must be paid to avoid high doses of CLA so as not to cause liver damage.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; TBARS, thiobarbituric acid reactive substances; PCOOH, phosphatidylcholine hydroperoxide; TG, triglyceride; WAT, white adipose tissue; NEFA, nonesterified fatty acid; BHT, butylated hydroxytoluene; EVO, extra virgin olive oil; CAT, catalase; ACO, acyl-CoA oxidase; PUFA, polyunsaturated fatty acid; LPL, lipoprotein lipase.

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