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The Effect of Olive Leaf Extract on Hepatic Fat Accumulation in Sprague-Dawley Rats Fed a High-fat Diet

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Oleuropein, the active constituent of olive leaf extract, possesses anti-oxidant, hypoglycemic, and hypolipidemic activities. We aimed to assess whether the effect of olive leaf extract on hepatic fat accumulation is preventive or therapeutic. Sprague-Dawley (SD) rats were fed a high-fat diet with (ODOD group) or without (HDHD group) olive leaf extract (1,000 mg/kg diet) for 38 weeks. Another group of rats were fed a high-fat diet for 23 weeks, followed by a high-fat diet with olive leaf extract (1,000 mg/kg diet) for 15 weeks (HDOD group). Serology, histopathology, anti-oxidative activity, and liver fatty acid synthesis were compared to those fed a standard diet (LDLD group) at 26 and 41 weeks of age. The serum levels of total cholesterol, triglyceride and aspartate aminotransferase tended to be lower in the ODOD group as compared to the HDHD and HDOD groups, although there were no significant differences. Histopathologically, hepatic steatosis tended to be less evident in the HDOD and ODOD groups as compared to the HDHD group, and lobular inflammation was not observed in the ODOD group at 26 weeks of age. Hepatic thioredoxin-1 staining tended to be less evident in the ODOD group than in the HDHD and HDOD groups at 41 weeks of age. There were no significant differences in hepatic lipogenic enzyme activities between the ODOD group and HDHD/HDOD groups. Our data suggest that olive leaf extract had a preventive, rather than therapeutic, effect on hepatic steatohepatitis in SD rats fed a high-fat diet.

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Keywords: Non-alcoholic fatty liver disease, Olive leaf extract, Oleuropein, High-fat diet, Sprague-Dawley (SD) rats

Introduction

Nonalcoholic fatty liver disease (NAFLD) is recognized as the most common cause of chronic liver disease in many countries. NAFLD does not progress to more severe liver disease in most cases, but the liver occasionally exhibits fibrosis and necroinflammation, indicating the presence of nonalcoholic steatohepatitis (NASH).[1,2] The recent increase in the number of patients with NAFLD has necessitated the study of treatment options which include dietary interventions. However, to date, no standardized treatments have been approved.[3-5] Based on the "two-hit" model of NASH pathogenesis,[6,7] the strategies to inhibit NAFLD or NASH progression consist of adipose tissue and oxidative stress reduction, insulin resistance improvement and lipid profile optimization.[3] An altered dietary macronutrient composition, *i.e.*, a greater intake of monounsaturated fatty acids, would be recommended because saturated fatty acids have deleterious effects on liver function.[7,8]

Although the studies of the effects of different diets on NAFLD or NASH have been seldom performed in human, a Mediterranean diet has been proposed for the prevention of metabolic syndrome including hypertension and cardiovascular diseases. The main part of its beneficial effect is

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thought to be a high supply of energy coming from monounsaturated fatty acids, mainly from olive oil.[5,9] Olive products constitute many phenolic compounds such as oleuropein, hydroxytyrosol and tyrosol.[10] Most of these possess an antioxidant ability, which may have a beneficial effect on metabolic syndrome, and also, on NAFLD or NASH.[5,8] In this context, we recently reported that an olive leaf extract-rich diet had a preventive effect on the occurrence of NASH or NAFLD, and this effect was presumably due to the antioxidative activity in the spontaneous NASH model of spontaneously hypertensive/NIH-corpulent [SHR/NDmcr-cp(cp/cp)] rats.[11] This strain has a genetic background from SHR and also a genetic mutation in the leptin receptor gene.[12] However, such a genetic background is not common in humans with NAFLD. Moreover, whether the effect of olive leaf extract on NASH or NAFLD is preventive or therapeutic remains unclear. The aim of the present study was to evaluate the preventive or therapeutic effects of olive leaf extract on hepatic fat accumulation in Sprague-Dawley (SD) rats fed a high-fat diet, since high-fat diet can induce NAFLD or NASH in SD rats.[13]

Materials and Methods

Animals and experimental design

Three-week-old male SD rats (n=37) were purchased from Nippon SLC (Hamamatsu, Japan). The rats were housed in individual cages that were kept in a room maintained at 22-24℃ with 50-60% relative humidity and a 12-hour light/12-hour dark cycle. Rats were randomly divided into four groups: low fat diet-low fat diet (LDLD) group (n=10), fed a low-fat (standard) diet (LFD) containing 10% fat (5.5% of soybean oil and 4.4% of lard, kilocalories) (D12450B; Research Diets, New Brunswick, NJ, USA); high fat diet-high fat diet (HDHD) group (n=10), fed a high-fat diet (HFD) containing 45% fat (5.5% of soybean oil and 39.4% of lard, kilocalories) (D12451; Research Diets); high fat diet-olive containing high fat diet (HDOD) group (n=5), fed a HFD for 23 weeks followed by a HFD containing 0.1% olive leaf extract (OD) (D08080103; Research Diets) for 15 weeks; and olive containing high fat diet- olive containing high fat diet (ODOD) group (n=12), fed an OD. The olive leaf extract (containing 35% oleuropein, 30-40% carbohydrate, 0-5% protein, 0-10% other polyphenols, and approximately 10% unknown components) was obtained from Eisai Food & Chemical Co. (Tokyo, Japan) and the OD was manufactured by Research Diets. The compositions of LFD, HFD and OD are shown in Table 1. In the LDLD, HDHD and ODOD groups, 5 rats each were sacrificed at 26 weeks of age in order to investigate the histopathology of the liver, and the other rats (5 LDLD, 5 HDHD, 5 HDOD and 7 ODOD) were sacrificed at 41 weeks of age under anesthesia (pentobarbital sodium) after 20-hour fasting (Figure 1).

In general, the rats had free access to food and water in both 12-hour light and 12-hour dark periods. However, in

Table 1. Dietary compositions by grams and kilocalories

Component	LFD (D12450B)		HFD (D12451)		OD (D08080103)	
	gram	kcal	gram	kcal	gram	kcal
Casein	200	800	200	800	200	800
L-cystine	3	12	3	12	3	12
Cornstarch	315	1260	72.8	291	72.8	291
Maltodextrin	35	140	100	400	100	400
Sucrose	350	1400	172.8	691	172.8	691
Cellulose	50	0	50	0	50	0
Soybean oil	25	225	25	225	25	225
Lard	20	180	177.5	1598	177.5	1598
Mineral mix	10	0	10	0	10	0
Dicalcium phosphate	13	0	13	0	13	0
Calcium carbonate	5.5	0	5.5	0	5.5	0
Potassium citrate, $1H_2O$	16.5	0	16.5	0	16.5	0
Vitamin mix	10	40	10	40	10	40
Choline bitartrate	2	0	2	0	2	0
Olive leaf extract	0	0	0	0	0.86	0
Yellow, red, or blue dye	0.05	0	0.05	0	0.05	0
Total	1055.05	4057	858.15	4057	859.01	4057
Protein (%)	19	20	24	20	24	20
Carbohydrate (%)	67	70	41	35	41	35
Fat (%)	4	10	24	45	24	45
Total		100		100		100
Energy (kcal/gram)	3.8		4.7		4.7	

LFD, low-fat (standard) diet; HFD, high-fat diet; OD, HFD supplemented with 0.1% olive leaf extract.

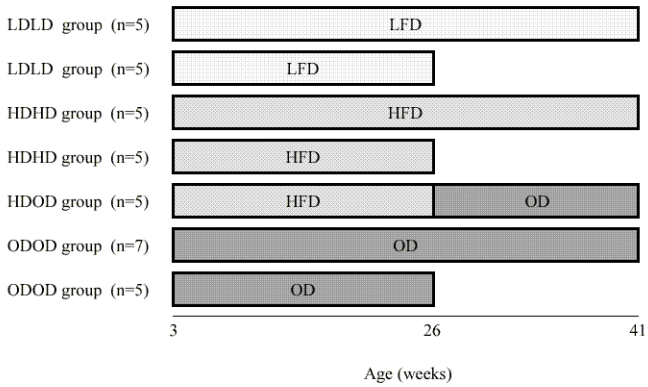

Figure 1. Experiment design. LFD, low-fat (standard) diet; HFD, high-fat diet; OD, HFD supplemented with 0.1% olive leaf extract.

order to avoid a significant difference in body weights among groups, daily food intake was maintained at 22 g of LFD from 10 to 41 weeks of age in the LDLD group, 22 g of HFD from 10 to 16 weeks of age and 20 g of HFD from 17 to 41 weeks of age in the HDHD and HDOD groups, 22 g of OD from 10 to 34 weeks of age, and 20 g of OD from 35 to 41 weeks of age in the HDOD and ODOD groups. Daily energy intake and body weights were monitored during the study. Systolic blood pressure was measured in conscious rats by the indirect tail-cuff method at 16 and 34 weeks of age.

All rats were sacrificed under anesthesia at 26 or 41 weeks of age, and blood samples were taken from the inferior vena cava or heart for measurement of serum glucose, insulin, total cholesterol, triglyceride, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels. Serum samples obtained were kept at -20℃ until analysis. The epididymal fat pads were removed and weighed. The livers were also removed, washed in a cold saline solution, and weighed. Liver portions (approximately 5 g) were fixed in 10% neutral buffered formalin for the histopathological examinations. Other liver portions (approximately 2 g) were diced and suspended in 0.25 M sucrose in 10 mM Tris-HCl (pH 7.4) and 1 mM ethylenediaminetetraacetic acid (EDTA) (HG buffer). The mixture was homogenized using a Potter homogenizer and centrifuged at 700 x g for 10 min at 4℃. The supernatants were again centrifuged at 10,000 x g for 10 min at 4℃ and the pellets were suspended in HG buffer (mitochondrial fraction). The supernatants were further centrifuged at 125,000 x g for 60 min at 4℃ and the pellets were again suspended in HG buffer (microsomal fraction). The supernatants were denoted as the cytosolic fraction. Each fraction was assessed for enzymatic activity. All procedures were performed in accordance with the Guidelines for Animal Experimentation and approved by the Animal Usage Committee of University of Nagasaki, Japan.

Serum biochemical analysis

Serum glucose levels were determined by the mutarotase and glucose oxidase method (Glucose C II test Wako, Wako Pure Chemical Industries, Osaka , Japan). Serum insulin levels were measured using a commercially available ELISA kit (Morinaga Institute of Biological Science, Kanagawa, Japan). Serum total cholesterol and triglyceride levels were measured by the cholesterol oxidase DAOS method (Cholesterol E test Wako, Wako Pure Chemical Industries) and the GPO DAOS glycerol method (Triglyceride E test Wako, Wako Pure Chemical Industries), respectively. Serum AST and ALT levels were estimated by an automatic analyzer (SRL, Tokyo, Japan).

Histopathological examinations

After fixation in neutral buffered formalin, the liver was sectioned and processed for routine hematoxylin-eosin (HE) staining for histopathological examination. All histopathological examinations were performed by a pathologist (K.T.) who was blinded to the experimental and serological data. Histopathological findings were scored using the NASH Clinical Research Network Scoring System based on four semi-quantitative factors: steatosis (0-3), lobular inflammation (0-3), hepatocyte ballooning (0-2), and fibrosis (0-4) as previously described.[14] The NAFLD activity score (NAS) was defined as the unweighted sum of the scores for steatosis, lobular inflammation, and hepatocyte ballooning; thus, scores ranged from 0 to 8. A NAS of 0 to 2 was considered not diagnostic of steatohepatitis, and scores of 5 or greater were diagnostic of steatohepatitis.[14]

For the immunohistochemical analysis of thioredoxin (Trx), which is a marker of oxidative stress,[15, 16] an enhanced immunostaining method using an enzyme-labeled polymer was performed on deparaffinized liver sections. Anti-mouse recombinant antibody against Trx-1 (Redox Bioscience, Kyoto, Japan; 1:100 dilution) was used as the primary antibody. Instead of primary antibody, normal rabbit serum was used as a negative control. For the following reactions, Envision-PO for mouse monoclonal antibodies (DAKO, Glostrup, Denmark) was used as the secondary antibody. The intensity of cytosolic or nuclear staining of Trx-1 in hepatocytes, hepatic sinusoidal endothelial cells and Kupffer cells in the liver section was classified into three grades as follows: grade 0, 0-10% of cells had positive staining; grade 1, 10-50% of cells had positive staining; grade 2, more than 50% of cells had positive staining.

Assays for enzyme activity

The activity of hepatic fatty acid synthase (FAS) in the cytosolic fraction was determined spectrophotometrically by the method of Kelley et al.[17] Malic enzyme activity in the cytosolic fraction was assayed by a previously reported method.[18] Glucose-6-phosphate dehydrogenase (G6PDH) activity in the cytosolic fraction was measured by the method of Kelley et al.[19] Carnitine palmitoyltransferase (CPT) activity in the mitochondrial fraction was confirmed spectrophotometrically by the release of CoA-SH from palmitoyl-CoA using the general thiol regent 5, 5'-dithiobis, as described previously.[20] Phosphatidic acid phosphohydrolase (PAP) activity in the cytosolic and microsomal fractions was measured by the method of Walton and Possmayer.[21] Protein concentrations

were determined by the method of Lowry et al.[22]

Statistical analysis

All values were expressed as mean ± standard error (SE). Differences between groups were tested for statistical significance using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test, Student's *t*-test, chi-square test, or Fisher's exact probability test. All analyses were performed using the SPSS 16.0J software program (SPSS, Inc., Chicago, IL, USA) on a Windows computer. $P < 0.05$ was considered significant.

Results

Food intake, body weight, systolic blood pressure, liver weight/rat weight ratio, and epididymal fat pad volume

In rats that were sacrificed at 26 weeks of age, the cumulative energy intake during the 23 weeks of the study was not significantly different among groups. In rats that were sacrificed at 41 weeks of age, the cumulative energy intake in the HDOD group during the 38 weeks was significantly larger than that in the LDLD group (p=0.014), de spite the limitation of the daily food intake. The body weight at 26 weeks of age was not significantly different among groups. On the other hand, the body weight at 41 weeks of age in the LDLD group was significantly lower than that in the other groups (p=0.004). There were no significant differences in systolic blood pressure among groups at both 16 and 34 weeks of age. The liver weight/body weight ratio was not significantly different among groups at both 26 and 41 weeks of age. The relative weight of the epididymal fat pad (g/100 g of body weight) was not significantly different among groups at both 26 and 41 weeks of age (Table 2).

Table 2. Food intake, body weight, systolic blood pressure, liver weight/rat weight ratio, and epididymal fat pad volume, and serum biochemical parameters

Parameter	LDLD group	HDHD group	HDOD group	ODOD group
Cumulative energy intake (kcal)				
26 weeks of age	11812 ± 238 (n=5)	13410 ± 535 (n=5)	-	13043 ± 414 (n=5)
41 weeks of age	21010 ± 345[a] (n=5)	22783 ± 492 (n=5)	24287 ± 567[a] (n=5)	23319 ± 768 (n=7)
Body weight (gram)				
26 weeks of age	562 ± 12 (n=5)	620 ± 34 (n=5)	-	606 ± 16 (n=5)
41 weeks of age	613 ± 6[b,c,d] (n=5)	684 ± 9[b] (n=5)	711 ± 21[c] (n=5)	680 ± 18[d] (n=7)
Systolic blood pressure (mmHg)				
16 weeks of age	148 ± 6 (n=5)	125 ± 8 (n=5)	-	130 ± 4 (n=5)
34 weeks of age	127 ± 6 (n=5)	134 ± 9 (n=5)	146 ± 9 (n=5)	144 ± 5 (n=7)
Liver weight/rat weight ratio (%)				
26 weeks of age	3.0 ± 0.2 (n=5)	2.5 ± 0.1 (n=5)	-	2.9 ± 0.2 (n=5)
41 weeks of age	2.8 ± 0.1 (n=5)	2.3 ± 0.1 (n=5)	2.5 ± 0.1 (n=5)	2.4 ± 0.1 (n=7)
Relative weight of epididymal fat pad (gram/100gram of body weight)				
26 weeks of age	2.7 ± 0.1 (n=5)	2.7 ± 0.3 (n=5)	-	2.7 ± 0.1 (n=5)
41 weeks of age	2.5 ± 0.1 (n=5)	2.9 ± 0.2 (n=5)	2.8 ± 0.2 (n=5)	2.7 ± 0.2 (n=7)
Serum glucose (mg/dL)				
26 weeks of age	205 ± 18 (n=5)	246 ± 29 (n=4)	-	290 ± 59 (n=5)
41 weeks of age	200 ± 42 (n=5)	158 ± 15 (n=5)	169 ± 10 (n=5)	188 ± 16 (n=7)
Serum insulin (ng/mL)				
26 weeks of age	5.0 ± 1.2 (n=5)	5.9 ± 0.9 (n=4)	-	7.2 ± 0.5 (n=5)
41 weeks of age	4.8 ± 0.4 (n=5)	5.4 ± 1.2 (n=5)	4.7 ± 0.6 (n=5)	5.3 ± 0.7 (n=7)
Serum total cholesterol (mg/dL)				
26 weeks of age	109 ± 13 (n=5)	91 ± 8 (n=4)	-	74 ± 4 (n=5)
41 weeks of age	123 ± 18 (n=5)	99 ± 5 (n=5)	93 ± 11 (n=5)	75 ± 14 (n=7)
Serum triglyceride (mg/dL)				
26 weeks of age	191 ± 33 (n=5)	89 ± 23(n=4)	-	139 ± 39 (n=5)
41 weeks of age	221 ± 43[e] (n=5)	134 ± 7 (n=5)	113 ± 27 (n=5)	84 ± 16[e] (n=7)
Serum AST (IU/L)				
26 weeks of age	218 ± 78 (n=4)	96 ± 1 (n=2)	-	84 ± 7 (n=4)
41 weeks of age	147 ± 46 (n=5)	94 ± 18 (n=5)	118 ± 29 (n=5)	87 ± 8 (n=7)
Serum ALT (IU/L)				
26 weeks of age	161 ± 78 (n=4)	45 ± 10 (n=2)	-	42 ± 5 (n=4)
41 weeks of age	109 ± 46 (n=5)	44 ± 7 (n=5)	72 ± 28 (n=5)	50 ± 9 (n=7)

Values are means ± SE.
a-e, Superscripts indicate significant differences as follows: [a]p=0.014, [b]p=0.049, [c]p=0.004, [d]p=0.043, [e]p=0.006.
AST, aspartate aminotransferase; ALT, alanine amintransferase.
The definitions of LDLD, HDHD, HDOD, and ODOD group: see text in Materials and Methods section.

Serum biochemical parameters

All serum biochemical data failed to be obtained in one rat in the HDHD group at 26 weeks of age, because insufficient serum samples were taken. Serum AST and ALT levels were not measured in one rat in the LDLD group, two rats in the HDHD group, and one rat in the ODOD group at 26 weeks of age, because of inadequate serum sample volumes.

There were no significant differences in serum levels of glucose, insulin, total cholesterol, AST, and ALT among the three (26 weeks of age) or four (41 weeks of age) groups. The serum triglyceride levels in the LDLD group were higher than those in the ODOD group at 41 weeks of age (Table 2).

Histopathological observation

Representative histopathological results in the livers of the LDLD, HDHD and ODOD groups at 26 weeks of age, and LDLD, HDHD, HDOD and ODOD groups at 41 weeks of age are shown in Figures 2 and 3, respectively. At 26 weeks of age, there were no significant differences in score/grade of hepatic steatosis, lobular inflammation, hepatocyte ballooning, NAS, and fibrosis among the three groups. The scores of hepatic steatosis, lobular inflammation, hepatocyte ballooning, and NAS tended to be higher in the HDHD group as compared to the LDLD group, although the differences were not statistically significant. Lobular inflammation and NAS of 5 points or greater were not observed in the ODOD group. At 41 weeks of age, the score of steatosis in the HDHD group was significantly higher than that in the LDLD group ($p=0.03$). The scores of hepatocyte ballooning and NAS also tended to be higher in the HDHD group as compared to the LDLD group, although the differences were not statistically significant. In the HDOD and ODOD groups, the scores of steatosis, hepatocyte ballooning, and NAS tended to be higher than those in the LDLD group, although the differences were not statistically significant (Table 3).

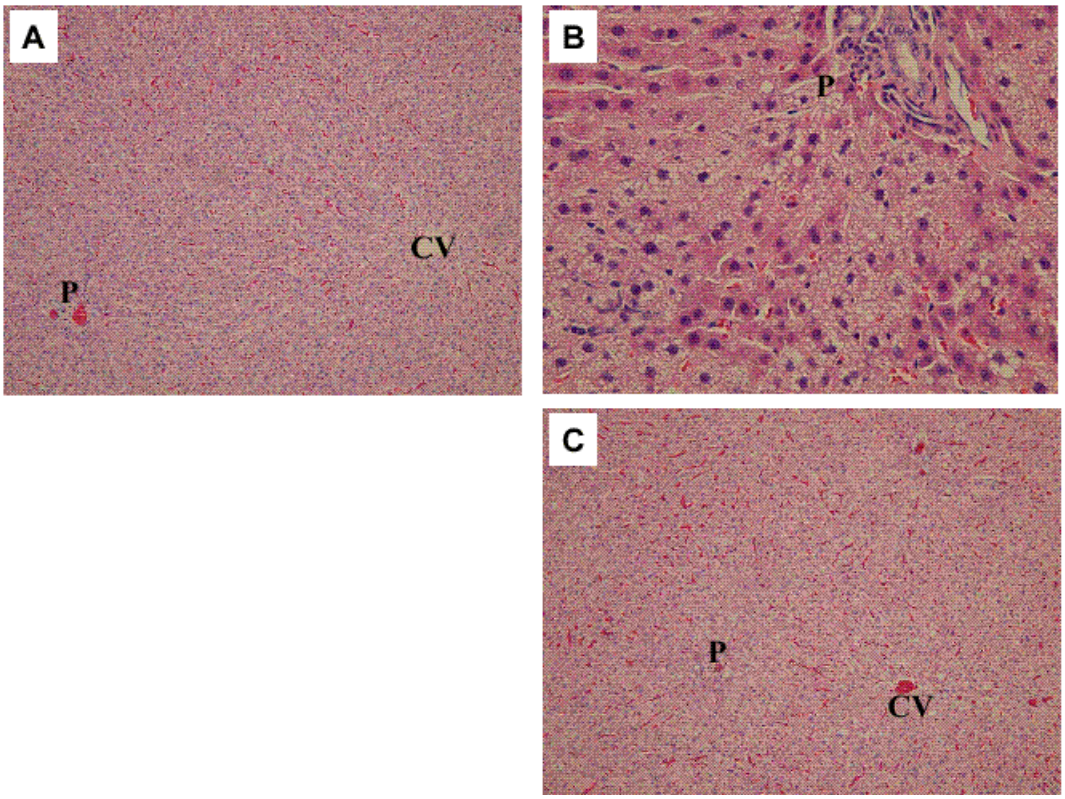

Figure 2. Representative histopathology (HE stain) of rat liver in LDLD, HDHD, and ODOD rats at 26 weeks of age. (A) Fatty change, lobular inflammation, hepatocyte ballooning, and fibrosis were seldom seen in the LDLD group (original magnification x100). (B) Moderate micro- or macrovesicular fatty change was seen in periportal area. Mild lobular inflammation and moderate hepatocyte ballooning without fibrosis were also seen in the HDHD group (original magnification x400). (C) Mild fatty change (perivenular) and hepatocyte ballooning without lobular inflammation and fibrosis were seen in the ODOD group (original magnification x100). P, portal tracts; CV, central vein.

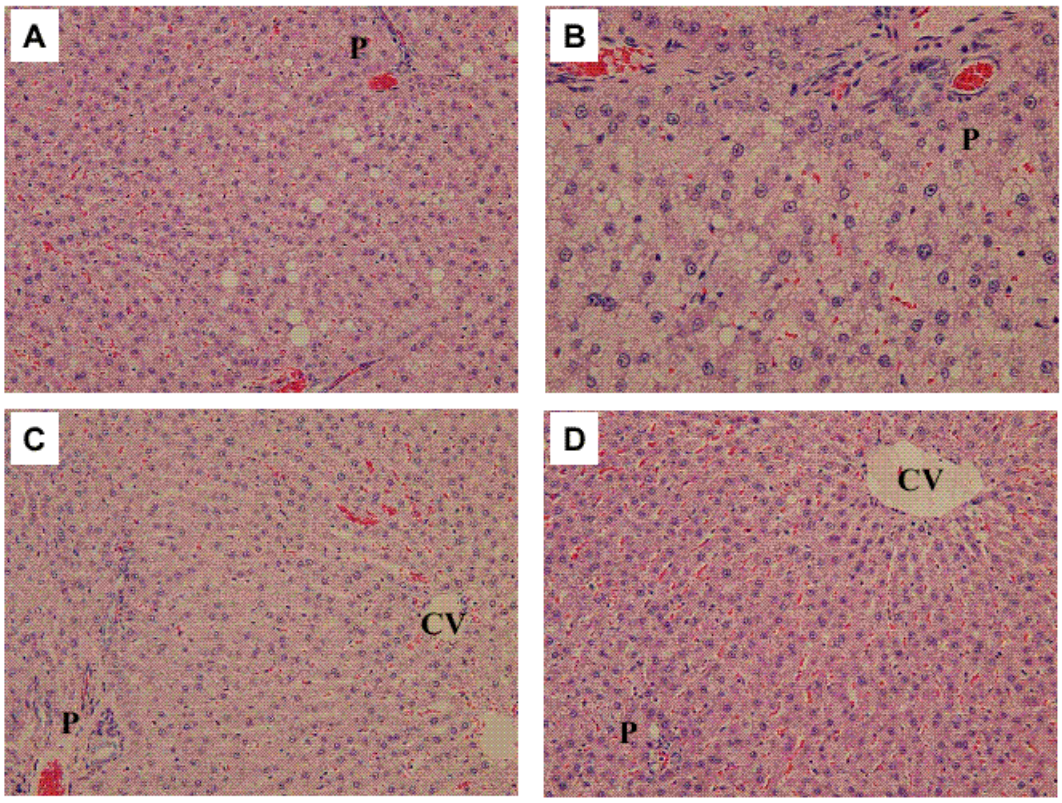

Figure 3. Representative histopathology (HE stain) of rat liver in LDLD, HDHD, HDOD, and ODOD rats at 41 weeks of age. (A) Mild fatty change without lobular inflammation, hepatocyte ballooning, and fibrosis was seen in the LDLD group (original magnification x100). (B) Moderate micro- or macrovesicular fatty change and mild hepatocyte ballooning without lobular inflammation and fibrosis were seen in the HDHD group (original magnification x400). (C) Fatty change, lobular inflammation, hepatocyte ballooning, and fibrosis were seldom seen in the HDOD group (original magnification x100). (D) Mild fatty change without lobular inflammation, hepatocyte ballooning, and fibrosis was seen in the ODOD group (original magnification x100). P, portal tracts; CV, central vein

Representative immunohistochemistry for Trx-1 in the liver of the LDLD, HDHD and ODOD groups at 26 weeks of age, and LDLD, HDHD, HDOD and ODOD groups at 41 weeks of age are shown in Figures 4 and 5, respectively. Although the differences were not statistically significant, the grades of Trx-1 staining in the HDHD and ODOD groups tended to be lower than those in the LDLD group at 26 weeks of age. In contrast, the grades of Trx-1 staining in the HDHD, HDOD, and ODOD groups tended to be higher than those in the LDLD group at 41 weeks of age. However, 4 out of 7 (57%) rats in the ODOD group were grade 2, whereas 4 out of 5 (80%) rats in the HDHD and HDOD groups exhibited grade 2 Trx-1 staining (Table 3).

Hepatic lipogenic enzyme activity

The activity of hepatic FAS, the enzyme required in fatty acid synthesis, was higher in the LDLD group than in the HDHD and ODOD groups at 26 weeks of age, although there were no significant differences among LDLD, HDHD, HDOD and ODOD groups at 41 weeks of age. The activity of malic enzyme, the enzyme involved in the synthesis of long-chain fatty acids and that supplies the NADPH required for palmitate synthesis, was also higher in the LDLD group than in the HDHD and ODOD groups at 26 weeks of age, although there were no significant differences among the four groups at 41 weeks of age. The activity of G6PDH, the rate-limiting enzyme of the pentose phosphate pathway in glucose metabolism that generates NADPH and regulates lipogenesis, was higher in the LDLD group than in the HDHD group at 26 weeks of age, and in the other three groups at 41 weeks of age. The activities of hepatic CPT (the rate-limiting step in fatty acid oxidation) and PAP (the enzyme that catalyzes the penultimate and rate-limiting step in triglyceride synthesis) were not significantly different between groups at both 26 and 41 weeks of age (Table 4).

Table 3. Histopathological assessment of steatosis, lobular inflammation, hepatocyte ballooning, NAFLD activity score (NAS), fibrosis, and thioredoxin (Trx)-1 staining

At 26 weeks of age Score/grade	LDLD group (n=5)	HDHD group (n=5)	ODOD group (n=5)	*p*
Steatosis				
0	2	1	0	
1	3	1	3	
2	0	3	2	n.s.
Lobular inflammation				
0	3	1	5	
1	2	4	0	n.s.
Hepatocyte ballooning				
0	4	0	1	
1	1	4	3	
2	0	1	1	n.s.
NAFLD activity score				
0-2	5	2	4	
3-4	0	2	1	
5-8	0	1	0	n.s.
Fibrosis				
0	5	5	5	n.s.
Trx-1 staining				
0	0	1	2	
1	3	4	3	
2	2	0	0	n.s.

At 41 weeks of age Score/grade	LDLD group (n=5)	HDHD group (n=5)	HDOD group (n=5)	ODOD group (n=7)	*p*
Steatosis					
0	1	0	0	0	
1	4	0	2	3	
2	0	5	3	4	0.030
Lobular inflammation					
0	2	3	2	3	
1	2	2	3	4	
2	1	0	0	0	n.s.
Hepatocyte ballooning					
0	5	2	3	4	
1	0	3	2	3	n.s.
NAFLD activity score					
0-2	5	1	2	3	
3-4	0	4	3	4	n.s.
Fibrosis					
0	5	4	3	6	
1A	0	1	2	1	n.s.
Trx-1 staining					
0	2	0	0	1	
1	3	1	1	2	
2	0	4	4	4	n.s.

* Scores are according to the NASH Clinical Research Network Scoring System.[14] n.s., not significant. The definitions of LDLD, HDHD, HDOD, and ODOD group: see text in Materials and Methods section.

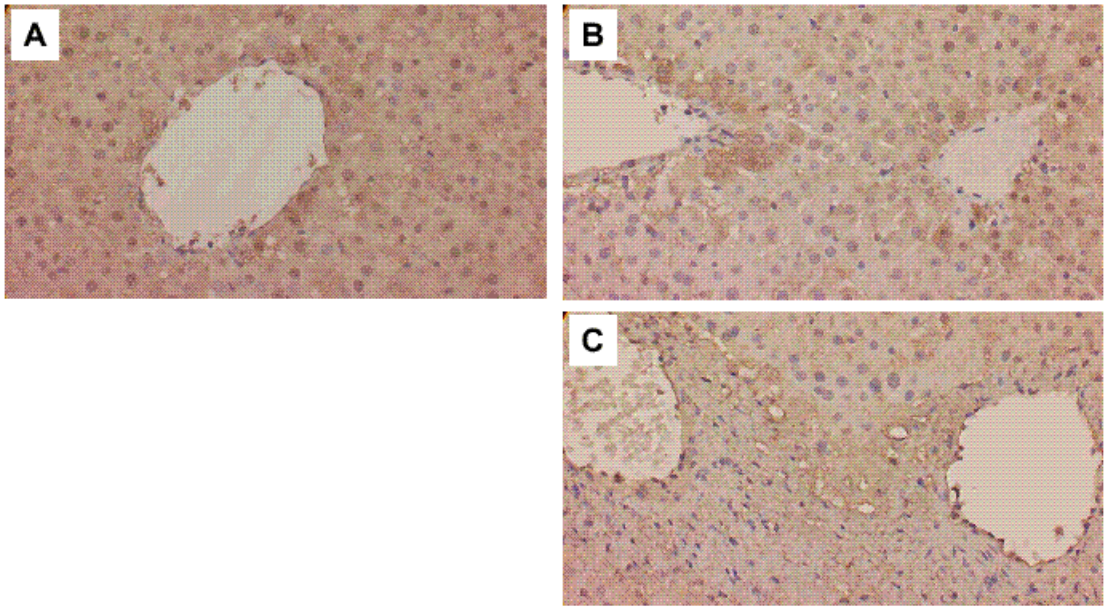

Figure 4. Representative immunohistochemistry for thioredoxin (Trx)-1 in livers from LDLD, HDHD, and ODOD rats at 26 weeks of age. (A) More than 50% of hepatocytes, hepatic sinusoidal endothelial cells and Kupffer cells had positive staining in the LDLD group. (B and C) Moderate (10-50%) cytosolic or nuclear staining of such cells was seen in the HDHD and ODOD groups (original magnification x400).

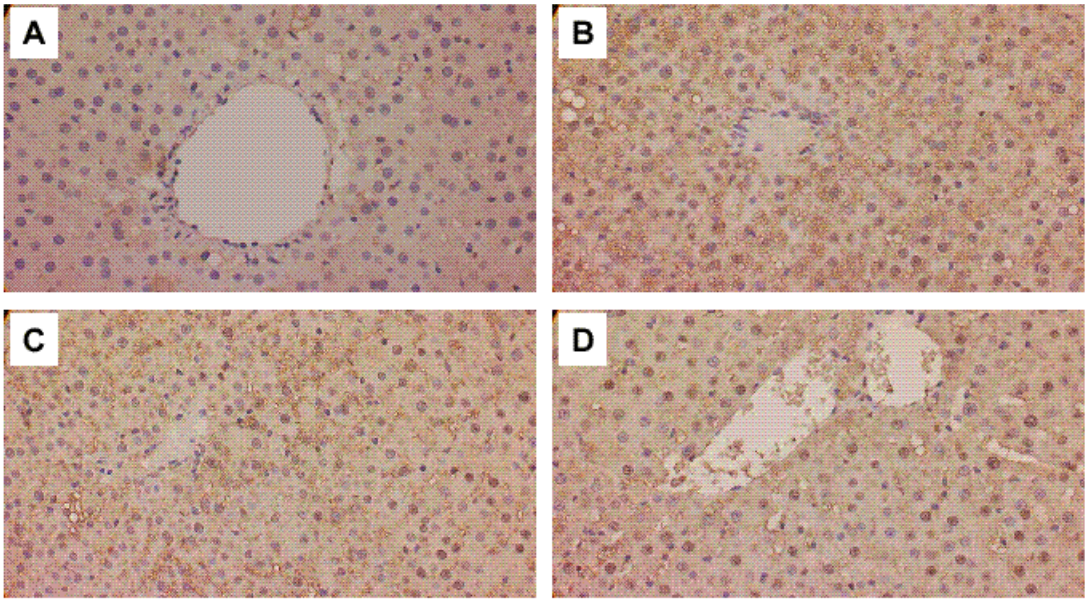

Figure 5. Representative immunohistochemistry for thioredoxin (Trx)-1 in livers from LDLD, HDHD, HDOD, and ODOD rats at 41 weeks of age. (A and D) Moderate (10-50%) cytosolic or nuclear staining of hepatocytes, hepatic sinusoidal endothelial cells and Kupffer cells was seen in the LDLD and ODOD groups. (B and C) More than 50% of such cells had positive staining in the HDHD and HDOD groups (original magnification x400).

Table 4. Hepatic lipogenic enzyme activities

Enzyme	LDLD group	HDHD group	HDOD group	ODOD group
FAS activity (nmol/min/mg protein)				
26 weeks of age	17.5 ± 2.2[a,b]	4.1 ± 1.3[a]	-	2.9 ± 0.7[b]
41 weeks of age	3.0 ± 0.8	3.1 ± 0.2	4.0 ± 1.4	1.9 ± 0.9
Malic enzyme activity (nmol/min/mg protein)				
26 weeks of age	54.4 ± 6.4[c,d]	22.3 ± 3.7[c]	-	29.6 ± 5.0[d]
41 weeks of age	37.3 ± 6.4	29.1 ± 4.8	24.9 ± 4.1	25.7 ± 3.3
G6PDH activity (nmol/min/mg protein)				
26 weeks of age	66.6 ± 11.6[e]	19.7 ± 3.2[e]	-	34.0 ± 9.1
41 weeks of age	68.5 ± 11.2[f,g,h]	35.6 ± 3.2[f]	37.6 ± 7.5[g]	25.3 ± 3.8[h]
CPT activity (nmol/min/mg protein)				
26 weeks of age	1.6 ± 0.3	2.9 ± 0.8	-	2.6 ± 1.3
41 weeks of age	1.3 ± 0.2	0.7 ± 0.1	1.6 ± 0.4	2.1 ± 0.8
PAP activity (nmol/min/mg protein)				
26 weeks of age	32.0 ± 12.8	18.8 ± 1.9	-	17.4 ± 1.8
41 weeks of age	6.9 ± 1.0	8.9 ± 1.2	8.7 ± 1.4	12.2 ± 2.1

Values are means ± SE.
a-h, Superscripts indicate significant differences as follows: [a]$p<0.001$, [b]$p<0.001$, [c]$p=0.003$, [d]$p=0.016$, [e]$p=0.008$, [f]$p=0.023$, [g]$p=0.036$, [h]$p=0.001$.
FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; CPT, carnitine palmitoyltransferase; PAP, phosphatidic acid phosphohydrolase.
The definitions of LDLD, HDHD, HDOD, and ODOD group: see text in Materials and Methods section.

Discussion

Epidemiological studies have shown that a Cretan Mediterranean diet, characterized by the high consumption of olive oil, fruits, vegetables, cereals, and legumes, lowers the risk of coronary heart disease, cancer, and mortality rates for all causes.[23, 24] Olive products constitute a rich source of polyphenols, such as oleuropein, that scavenge free radicals and inhibit the chemical oxidation of low-density lipoprotein.[25] According to the average consumption of olive drupes and olive oil in the Mediterranean basin, the estimated polyphenol (mainly originating from the hydrolysis of oleuropein) consumption is up to 100 mg/day.[22] Although oleuropein is present throughout the olive tree and in olive oil, the olive leaf is the richest source of this compound (60-90 mg/g dry weight).[26] Oleuropein, the principal active constituent of olive leaf and unprocessed olive drupes of *Olea europaea*, possesses a wide range of pharmacologic properties including anti-oxidant, anti-inflammatory, hypoglycemic, hypolipidimic, hypotensive, and anti-viral activities.[10, 25-33] The direct effect of olive oil or oleuropein on NAFLD is not fully understood,[8, 34] but we recently reported that diets rich in olive leaf extract (the richest source of oleuropein), containing more than 1,000 mg/kg, had a preventive effect on the occurrence of NASH in SHR/NDmcr-cp(cp/cp) rats.[11]

To determine whether olive leaf extract (containing more than 1,000 mg/kg diet) has a preventive or therapeutic effect on NASH or NAFLD in SD rats fed high-fat diets, we evaluated serum biochemical parameters and liver histology in rats treated with a variety of high-fat diet regimes (LDLD, HDHD, HDOD, and ODOD) in the present study. In the LDLD group, there were several unexpected results such as higher tendency in the serum levels of total cholesterol, triglyceride, AST, and ALT, despite the lower body weight than in other groups (Table 2). Overall, there were no significant differences in serum levels of biochemical parameters including glucose, insulin, total cholesterol, triglyceride, AST, and ALT among the three (26 weeks of age) or four (41 weeks of age) groups, with the exception of higher serum triglyceride levels in the LDLD group compared to those in the ODOD group at 41 weeks of age. However, the serum levels of total cholesterol, triglyceride and AST tended to be lower in the ODOD group as compared to the HDHD and HDOD groups, although there were no significant differences. These results suggest that olive leaf extract has a preventive effect on the development of a dyslipidemic state rather than a therapeutic effect. Histopathologically, definitive NASH was uncommon in the HDHD group at both 26 and 41 weeks of age. However, when comparing the HDHD and LDLD groups, the score of hepatic steatosis tended to be higher at 26

weeks of age, and was significantly higher in the HDHD group at 41 weeks of age. Conversely, the score of hepatic steatosis tended to be lower in the HDOD and ODOD groups as compared to the HDHD group at both 26 and 41 weeks of age. These results would indicate that olive leaf extract has a preventive or therapeutic effect on hepatic fat accumulation. It was noted that lobular inflammation was not observed in the ODOD group at 26 weeks of age, suggesting that olive leaf extract possesses the preventive effect on hepatic inflammation.

We also investigated the mechanism of olive leaf extract effects, including its anti-oxidative activity and its alteration of fatty acid metabolism in the liver. Oxidative stress may contribute to the pathogenesis of NASH.[1] Furthermore, an imbalance in the influx of free fatty acids to the liver, free fatty acid β-oxidation, hepatic secretion of triglyceride-rich lipoproteins, and lipid peroxidation can lead to the development of hepatic steatosis in NAFLD or NASH.[6] Sumida et al. reported that the level of serum Trx-1, a redox-active protein, was a marker of NASH.[35] Trx-1 plays a crucial role in reduction/oxidation regulation in signal transduction and is secreted from cells in response to oxidative stress. Up-regulation of Trx-1 in chronic liver diseases, as well as malignant diseases, human immunodeficiency virus infection and cardiovascular disorders is well described. However, whether Trx-1 contributes to or prevents the pathology of such conditions is not always clear.[15, 16] Moreover, hepatic Trx levels did not correlate with serum Trx levels because hepatic Trx consists of both reduced and oxidized forms whereas only the oxidized form is present in serum.[36] In our previous study, hepatic Trx-1 expression in SHR/NDmcr-cp rats was more evident in rats fed an olive leaf extract-rich diet than in rats fed an olive leaf extract-scarce diet; suggesting that a down-regulation of Trx-1 expression in response to oxidative stress is probably due to the exhaustion of tissue Trx secretion to the extracellular space by chronic exposure to oxidative stress.[11] In contrast, no differences were observed in hepatic Trx-1 staining between the HDHD group and ODOD or HDOD groups in the present study. The reason for these discrepancies is unclear, but we should consider that a rat strain-specific genetic mechanism may be involved in Trx regulation (SHR vs SD rats). Interestingly, hepatic Trx-1 staining in the HDHD group tended to be less evident at 26 weeks of age, and more evident at 41 weeks of age as compared to the ODOD and HDOD groups. The reason for these apparent discrepancies is also unclear, but it suggests that influence of aging (26 vs 41 weeks of age) should be investigated. In the present study, hepatic fatty acid metabolism analysis revealed that there were no significant differences in the activities of hepatic FAS, malic enzyme, G6PDH, CPT and PAP between HDHD group and ODOD/HDOD groups at both 26 and 41 weeks of age. These results suggest that olive leaf extract has little effect on hepatic fatty acid metabolism, as reported in our previous study,[11] although the activities of FAS (26 weeks of age), malic enzyme (26 weeks of age) and G6PDH (26 and 41 weeks of age) were up-regulated in the LDLD group.

In summary, the serum levels of total cholesterol, triglyceride and AST tended to be lower in the ODOD group as compared to the HDHD and HDOD groups, although there were no significant differences. Histopathologically, hepatic steatosis tended to be less evident in the HDOD and ODOD groups as compared to the HDHD group, and lobular inflammation that is an important finding suggestive NASH was not observed in the ODOD group at 26 weeks of age. Hepatic Trx-1 staining tended to be less evident in the ODOD group than in the HDHD and HDOD groups at 41 weeks of age. There were no significant differences in hepatic lipogenic enzyme activities between the ODOD group and HDHD/HDOD groups. These data suggest that olive leaf extract had a preventive, rather than therapeutic, effect on hepatic steatohepatitis in SD rats fed a high-fat diet. The mechanism of this effect is unclear, but anti-oxidative property of olive leaf extract may play some role in the preventive effect for the occurrence of NASH.[11] Further investigation will be needed to clarify the precise mechanism of this favorable effect of olive leaf extract on chronic high-fat diet-induced NAFLD or NASH.

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