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ARTICLE

Flaxseed Oil Attenuates Nonalcoholic Fatty Liver of Hyperlipidemic Hamsters

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Hyperlipidemia of hamsters was induced by high-fat/cholesterol diets formulated by the addition of coconut oil (CO), butter (BU), and flaxseed oil (FX). Lower ($p < 0.05$) serum lipids, liver size, and hepatic cholesterol and triacylglycerol contents were observed in the FX group compared to both CO and BU groups. The liver damage indices [glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) values] in the FX group were lower ($p < 0.05$) than those in the CO and BU groups, which may result from higher ($p < 0.05$) glutathione (GSH) levels and a tendency toward lower malondialdehyde (MDA) levels in livers. Besides, lower ($p < 0.05$) gene expression and activity of hepatic matrix metalloproteinases-9 (MMP-9) in the FX group were lower ($p > 0.05$) compared to those in the CO and BU groups; however, no ($p > 0.05$) differences in gene expression activities of hepatic MMP-2 were observed among treatments. Those beneficial effects could explain the attenuation of FX on nonalcoholic fatty liver (NAFL) induced by a high-fat/cholesterol dietary habit.

KEYWORDS: Flaxseed oil; hepatic cholesterol/triacylglycerol contents; GSH; MDA; matrix metalloproteinases; NAFL

INTRODUCTION

The World Health Organization (*1*) projected that worldwide approximately 2.3 billion adults will be overweight and more than 700 million will be obese by 2015. The major causes of obesity are increased consumption of energy-dense, nutrient-poor foods, with high sugar and saturated fat contents, and reduced metabolic expenditure. Generally, nonalcoholic fatty liver (NAFL) is linked to obesity, diabetes, and hyperlipidemia (*2*). Consumption of high-fat diets results in high body weights, serum lipids, and hepatic lipids (*3*). As we know, the major component of lipid accumulation in livers is triacylglycerol. NAFL also causes a chronic inflammation and, thus, may be associated with progression to the end-stage of liver disease (*3*). Therefore, food scientists and nutritionists strive to reduce or attenuate the occurrence of NAFL by normalizing serum lipids and decreasing hepatic lipid accumulation through a dietary modification.

The cardioprotective and antiatherogenic natures of omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been reported for a number of years (*4*). However, due to increased demand for marine oils and dwindling natural resources, there is a significant interest in long-chain omega-3 PUFA (n-3 LC-PUFA) replacement at a cardioprotective and antiatherogenic point. It was reported that n-3 LC-PUFAs can be converted from their precursor, α-linolenic acid (ALA, C18:3n-3) in mammals (*5*). Unlike n-3 LC-PUFA from marine organisms, ALA is mainly derived from plants, in which there are plenty of omega-6 fatty acids (n-6 FA), such as linoleic acid (LA, C18:2n-6). However, due to competition for ALA for the 6-desaturase by LA, the conversion of ALA to eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acids (DHA, C22:6n-3) is slow (*6*). Therefore, the coexistence of ALA and LA in plant oils might impose a decreasing hypolipidemic effect of ALA. Zhao et al. (*7*) found that the level of C-reactive protein, a marker of inflammation strongly associated with heart disease, declined for both the LA and ALA diets, but much more significantly for the ALA diet, which could be attributed to its lipid-lowering effects. Although high fat consumption yielded higher serum alanine aminotransferase (ALT), free fatty acids, and tumor necrosis factor-α (TNF-α) levels, as well as hepatic cholesterol, triacylglycerol, malondialdehyde (MDA), and hydroperoxide levels in a rat model, ameliorations of fatty liver and degree of liver damage by supplementing n-3 PUFAs were attributed to restoration of hepatic adiponectin and PPAR-α expression and reduction of hepatic TNF-α level (*8*). Reduction of adipocyte size and hepatic triacylglycerol accumulation by n-3 LC-PUFAs, that is, EPA and DHA, were also reported in a high-fat-dietary diabetic mouse model (*9*).

Flaxseed (*Linum usitatissimum*) is the richest dietary source of ALA, phytoestrogen, lignans, and soluble fibers that are approved as having lipid-lowering characteristics (*10*). The National Research Council, Washington DC (*11*), reported that flaxseed oil (FX) contains 53.3% ALA and 12.7% LA, yielding

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the highest n-3/n-6 FA ratio among plant sources. The hypocholesterolemic effect of FX was also demonstrated previously (*12*). Liver fibrosis is caused by recurrent wound healing in response to various sources of chronic liver damage, for example, hepatic fat deposition and peroxidation, drug and chemical toxicity, alcoholism, and viral infection, which cause chronic inflammation and extracellular matrix (ECM) remodeling. Major components of the ECM in liver fibrosis are collagen types I, III, and IV. Matrix metalloproteinases (MMPs), that is, MMP-1, -2, and -9, play a central role in degradations of these collagens, resulting in ECM remolding (*13*). In response to chronic liver injury, hepatic stellate cells (HSCs) undergo transdifferentiation to myofibroblastic cells. The recurrence of collagen degradation and ECM remolding by MMPs may cause myofibroblastic cells to produce excessive interstitial ECM. Therefore, increased MMP-2 and -9 gene expressions or activities are regarded as major causes of liver fibrosis (*14*, *15*). No studies regarding the hepatoprotection of FX on NAFL by a high-fat/cholesterol-diet were found in the literature. Hence, the objectives of the present study were to investigate if the occurrence of nonalcoholic fatty liver attenuated by FX supplementation in hyperlipidemic hamsters is via (1) decreasing liver lipid accumulation, (2) lowering liver lipid peroxidation or increasing antioxidant capacity, or (3) reducing hepatic MMP-2 and -9 gene expressions and activities.

MATERIALS AND METHODS

Animals and Diets. Animal use and protocol were reviewed and approved by the Chung-Shan Medical University Animal Care Committee. Thirty-two 5-week-old male Golden Syrian hamsters were purchased from National Laboratory Animal Center (National Science Council, Taipei, Taiwan). Two hamsters were housed in each cage in an animal room at 22 ± 2 °C with a 12/12 h light/dark cycle. Chow diet containing 48.7% (w/w) carbohydrate, 23.9% (w/w) protein, 5.0% (w/w) fat, 5.1% (w/w) fiber, and 7.0% ash (Laboratory Rodent Diet 5001, PMI Nutrition International/Purina Mills LLC, St. Louis, MO) and water were provided for one week of acclimation. Following the acclimation period, 32 hamsters (2 hamsters per cage) were randomly assigned to one of the following diets: (1) control group, chow diet; (2) coconut oil (CO) group, 92.9% (w/w) chow diet supplemented with 7% (w/w) coconut oil/0.1% (w/w) cholesterol; (3) butter (BU) group, 92.9% (w/w) chow diet supplemented with 7% (w/w) butter/0.1% (w/w) cholesterol; (4) flaxseed oil (FX) group, 92.9% (w/w) chow diet supplemented with 7% (w/w) flaxseed oil/0.1% (w/w) cholesterol. The fat contents of the control, CO, BU, and FX diets were 5, 11.65, 11.65, and 11.65%, respectively. The control, CO, BU, and FX diets contained 335, 375, 375, and 375 kcal/100 g of diet, respectively. There were eight hamsters in each dietary group.

Coconut oil, butter, and cholesterol were purchased from ICN Biomedicals, Inc., Irvine, CA, and flaxseed oil was purchased from Master Asia Marketing Co., Ltd., Taipei, Taiwan. The fatty acid profiles of coconut oil, butter, and flaxseed oil can be found in **Table 1**. All hamsters were fed the assigned diets and water ad libitum. The experimental period lasted for 6 weeks.

Body Weight and Daily Food and Water Intakes. Hamsters were weighed individually at the beginning and at the end of the experiment. Feed (g) and water (mL) intakes were determined daily for each cage and divided by two to approximate intake on a per hamster basis.

Collection of Serum and Liver. At the end of the experiment (week 6), all feed was removed 16 h before killing. All hamsters were euthanized by CO_2. The liver from each hamster was removed and weighed. Livers were stored at −80 °C until further analyses. Blood samples were collected by intracardiac puncture. Serum was separated from blood samples by centrifugation at 3000*g* for 10 min and then stored at −80 °C until further analyses.

Determination of Serum Lipids and Liver Damage Indices. Serum total cholesterol (TC) and triacylglycerol (TAG) were measured by using commercial kits (Randox Laboratories Ltd., Antrim, U.K.) and are based on detection of colored end-products at 500 nm. The serum liver damage indices [glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) values] were determined by using commercial enzymatic kits (Ortho-Clinical Diagnostics, Inc., Rochester, NY) with an EKTACHEM DT 60 II/DTSC (Estman Kodak Co., Rochester, NY).

Table 1. Fatty Acid Profiles of Coconut Oil, Butter, and Flaxseed Oil Used in the Experimental Diets

	% of total fatty acids		
fatty acid	coconut oil	butter	flaxseed oil
4:0	nd[a]	4.3	nd
6:0	nd	2.2	nd
8:0	17.2	8.5	nd
10:0	8.8	10.5	nd
12:0	49.0	6.0	nd
14:0	14.0	11.8	nd
16:0	4.7	23.2	5.6
16:1	nd	5.5	nd
18:0	1.3	7.0	4.1
18:1	4.1	18.9	20.7
18:2(n-6)	0.9	2.1	14.8
18:3(n-3)	nd	nd	53.1
20:1	nd	nd	1.1
20:2(n-6)	nd	nd	0.7
total unsaturates	5.0	26.5	5.6
total saturates	95.0	73.5	94.4
total omega-3	nd	nd	53.1
total omega-6	0.9	2.1	15.5

[a] Not detectable.

Determination of Liver Cholesterol and Triacylglycerol. Hepatic lipid extractions were measured according to the procedures of Tzang et al. (*12*). Briefly, hepatic lipids were extracted by chloroform and methanol (2:1, v/v). The extract was dried under N_2 and resuspended in isopropanol and then efficiently dissolved by using an ultrasonic cleaner (model DC150H, Taiwan Delta New Instrument Co. Ltd.). Hepatic cholesterol and triacylglycerol levels were measured using commercial kits (Randox Laboratories Ltd., Antrim, U.K.).

Preparation of Liver Homogenate. A 0.5 g portion of liver was homogenized on ice in 4.5 mL of phosphate buffer saline (PBS, pH 7.0, containing 0.25 M sucrose) and centrifuged at 12000*g* for 30 min. The supernatant was collected for further analyses. The protein content in the supernatant was measured according to the procedures of a Bio-Rad protein assay kit (catalog no. 500-0006, Bio-Rad Laboratories, Inc., Hercules, CA) and using bovine serine albumin as a standard.

Determination of Hepatic Malondialdehyde (MDA) and Glutathione (GSH) Contents. The hepatic MDA content was measured according to a modification of the 2-thiobarbituric acid reactive substances (TBARS) assay described by Dhar et al. (*16*). A 0.5 mL volume of liver homogenate was mixed with 0.75 mL of TBA solution in a Teflon tube, and then 4.25 mL trichloroacetic acid−HCl (TCA-HCl) reagent was added. The tube was flushed with nitrogen and closed. A blank was prepared in the same manner, but PBS (pH 7.0) replaced liver homogenate. The tubes were boiled for 30 min and then cooled. The colored solution was centrifuged at 4000*g* for 15 min. A clear and colored supernatant was transferred to a cuvette, and the absorbance was measured at 535 nm using a UV−vis spectrophotometer (model U3000, Hitachi, Tokyo, Japan). The hepatic MDA content was calculated by taking the extinction coefficient of MDA to be 1.56×10^5 M^{-1} cm^{-1} at 535 nm. Due to the unique thiol compound in GSH, 2,2-dithiobisnitrobenzoic acid (DTNB) is commonly used for thiol assay. Therefore, the hepatic GSH content was measured according to a modification of the procedures of Hung et al. (*17*) with a modification. Equal volumes of liver homogenate and 10% TCA solution were mixed well, placed in an ice box for 5 min, and then centrifuged. The clear layer, Tris base (0.25 M)−ETDA (20 mM) buffer (pH 8.2), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (10 mM) solution were mixed well. After 5 min, the absorbance was measured at 412 nm using a UV−vis spectrophotometer (Hitachi model U3000). The hepatic GSH content was calculated based on the total contents of thiols by taking the extinction coefficient of TNB to be 1.36×10^4 M^{-1} cm^{-1}.

Table 2. Performances of the Experimental Hamsters[a]

group	control	CO	BU	FX
initial body weight (g)	83.6 ± 0.8a	80.6 ± 0.8a	80.3 ± 1.4a	82.0 ± 0.9a
final body weight (g)	103.3 ± 1.1b	107.6 ± 1.5ab	109.1 ± 2.0a	104.2 ± 1.5b
body weight gain (g)	19.7 ± 0.8b	27.1 ± 0.8a	28.8 ± 1.3a	22.2 ± 1.1b
food intake (g/hamster/day)	7.9 ± 0.1a	8.0 ± 0.1a	7.9 ± 0.1a	7.9 ± 0.0a
water intake (mL/hamster/day)	9.1 ± 0.3b	12.1 ± 0.3a	12.0 ± 0.2a	11.3 ± 0.1a

[a] Data are given as means ± SEM (initial and final body weight; weight gain, $n = 8$; food intake and water intake, $n = 4$). Mean values with different letters in each testing parameter were significantly different ($p < 0.05$). CO, coconut oil group; BU, butter group; FX, flaxseed oil group.

Table 3. Serum Lipids, Liver Size, Liver Lipids, and Liver Damage Indices of the Experimental Hamsters[a]

group	control	CO	BU	FX
serum lipid				
cholesterol (mg/dL)	110.2 ± 7.2c	220.9 ± 5.7a	230.1 ± 6.3a	177.6 ± 6.1b
triacylglycerol (mg/dL)	135.6 ± 6.5c	181.2 ± 3.6a	182.0 ± 3.8a	153.7 ± 1.5b
liver size (g/100 g of body weight)	3.3 ± 0.1c	4.0 ± 0.1a	3.8 ± 0.1ab	3.8 ± 0.1b
liver cholesterol (mg/g of tissue)	3.0 ± 0.3d	8.8 ± 0.49b	14.2 ± 0.4a	6.7 ± 0.7c
liver triacylglycerol (mg/g of tissue)	5.7 ± 0.2c	7.8 ± 0.6b	8.9 ± 0.3a	7.2 ± 0.2b
liver damage index				
GOT (U/L)	33.8 ± 4.7b	65.6 ± 4.6a	60.6 ± 5.2a	45.4 ± 3.2b
GPT (U/L)	77.0 ± 3.0d	152.6 ± 5.9b	360.0 ± 5.7a	115.4 ± 4.0c

[a] Data are given as means ± SEM ($n = 8$). Mean values with different letters in each testing parameter were significantly different ($p < 0.05$). CO, coconut oil group; BU, butter group; FX, flaxseed oil group.

Hepatic mRNA Expressions of MMP-2 and -9 and Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH). Total RNA was isolated from the stored frozen liver tissues by using the protocol described by Rneasy Mini Kits (Qiagen, Valencia, CA). Reverse transcription was carried out with 2 μg total RNA, 8 μL of reaction buffer, 2 μL of dNTPs, 4.8 μL of $MgCl_2$, 4 μL of oligo-dT (10 pmol/L), and 200 U of RTase (Promega, Madison, WI) with diethyl pyrocarbonate (DEPC) H_2O in a final volume of 40 μL at 42 °C at 1 h. After a heat inactivation, 1-μL of cDNA product was used for a PCR amplification. The appropriate primers of target genes (*18*) were designed for hamster's MMP-2, MMP-9, and GAPDH as follows: MMP-2 sense 5′-GCACTGGTGTTGGGGGAGATT-3′, antisense 5′-CAGGAAGGTGAAGGGGAAGAC-3′; MMP-9 sense 5′-ACCGCCCCGCCCACCGTCTGC-3′, antisense 5′-TCCATGTCATCAAAGGTA-3′; GAPDH sense 5′-GACCCCTTCATTGACCTCAAC-3′, antisense 5′-GGAGATGATGACCCTTTTGGC-3′. The size of reaction products is as follows: MMP-2, 476 bp; MMP-9, 705 bp; GAPDH, 264 bp. GAPDH was used as an internal control in all reactions. The PCR amplification was performed using a DNA thermal cycler (ASTEC PC-818, ASTEC Co., Ltd., Fukuka, Japan) under the following conditions: MMP-2, 28 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 2 min followed by 10 min at 72 °C; MMP-9, 28 cycles at 94 °C for 1 min, 61 °C for 1 min, and 72 °C for 2 min followed by 10 min at 72 °C; GAPDH, 25 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min followed by 10 min at 72 °C. The final products were subjected to electrophoresis on a 2% agarose gel and detected by ethidium bromide staining and visualization via UV light. The relative expression levels of the mRNAs of the target genes were normalized using the GAPDH internal standard.

Activities of MMP-2 and MMP-9 in Liver Tissues. The activities of MMP-2 and MMP-9 in liver tissues were measured by gelatin zymography protease assays as described by Yang et al. (*19*). Briefly, liver homogenates were prepared with SDS sample buffer without boiling or reduction and subjected to 0.1% gelatin−8% SDS-PAGE. After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in a reaction buffer (40 mM Tris-HCl, pH 8.0; 10 mM $CaCl_2$ and 0.01% NaN_3) at 37 °C for 12 h. Gels were stained with Coomassie brilliant blue R-250, and gelatinolytic activities were detected as clear bands against the blue background.

Statistical Analysis. The experiment was conducted using a completely random design (CRD). Data were analyzed using analysis of variance (ANOVA). A significant difference was used at 0.05 probability level, and differences between treatments were tested using the least significant difference (LSD) test. All statistical analyses of data were performed using SAS.

RESULTS

Body Weight, Weight Gain, and Daily Food and Water Intakes. Although the final body weights in high-fat/cholesterol dietary (HFCD) groups (CO, BU, and FX groups), except the BU group, were not ($p > 0.05$) different from that of the control dietary group (control group) after a 6 week feeding period, weight gains of CO and BU groups were higher ($p < 0.05$) than those of FX and control groups (**Table 2**). Food intakes of hamsters during the experimental period were not ($p > 0.05$) different among treatments, whereas water intakes in the three HFCD groups were higher ($p < 0.05$) than that in the control group.

Serum Lipids, Liver Size, and Determination of Hepatic Cholesterol and Triacylglycerol. After a 6 week feeding period, serum TC and TAG were increased ($p < 0.05$) in HFCD groups when compared to the control group, but the FX group apparently showed a lipid-lowering ($p < 0.05$) effect compared to the CO and BU groups (**Table 3**). After the hamsters had been sacrificed at the end of the experiment, livers were collected. Hamsters fed a control diet had the smallest ($p < 0.05$) liver size (g/100 g of body weight) when compared to those fed HFCD diets. In HFCD groups, the FX group had the smallest ($p < 0.05$) liver size, followed by BU and CO groups. Besides, hepatic cholesterol and triacylglycerol levels in HFCD groups were higher ($p < 0.05$) than those of control groups. However, among the HFCD groups, the hepatic cholesterol level of FX groups was dramatically lower ($p < 0.05$) than that of the other two HFCD groups, whereas the lowering ($p > 0.05$) effect of FX on hepatic triacylglycerol level was detected against only BU.

Hepatic Malondialdehyde (MDA) and Glutathione (GSH) Contents. Measurements of hepatic MDA and GSH contents were determined for liver oxidative damage induced by HFCD diets (**Figure 1**). Although there was no ($p > 0.05$) difference in MDA contents among all groups, there was a tendency toward higher MDA contents in the CO and BU groups compared to the FX and control groups. In the hepatic GSH evaluations, FX yielded

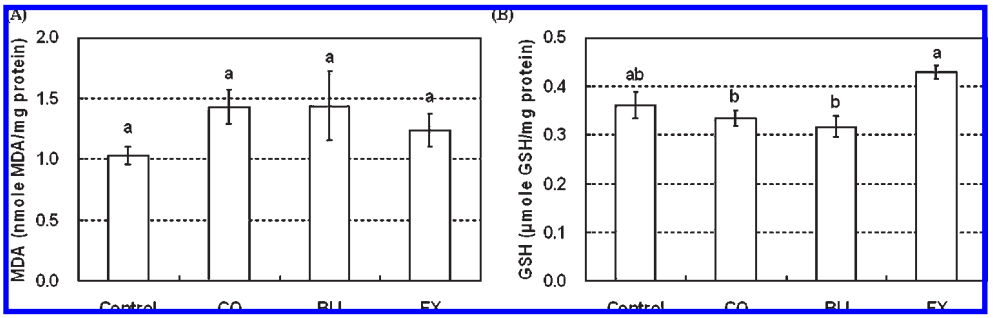

Figure 1. Hepatic MDA and GSH of the experimental hamsters. The data are given as means ± SEM ($n = 8$). Different letters on data bars indicate significant differences ($p < 0.05$). CO, coconut oil group; BU, butter group; FX, flaxseed oil group.

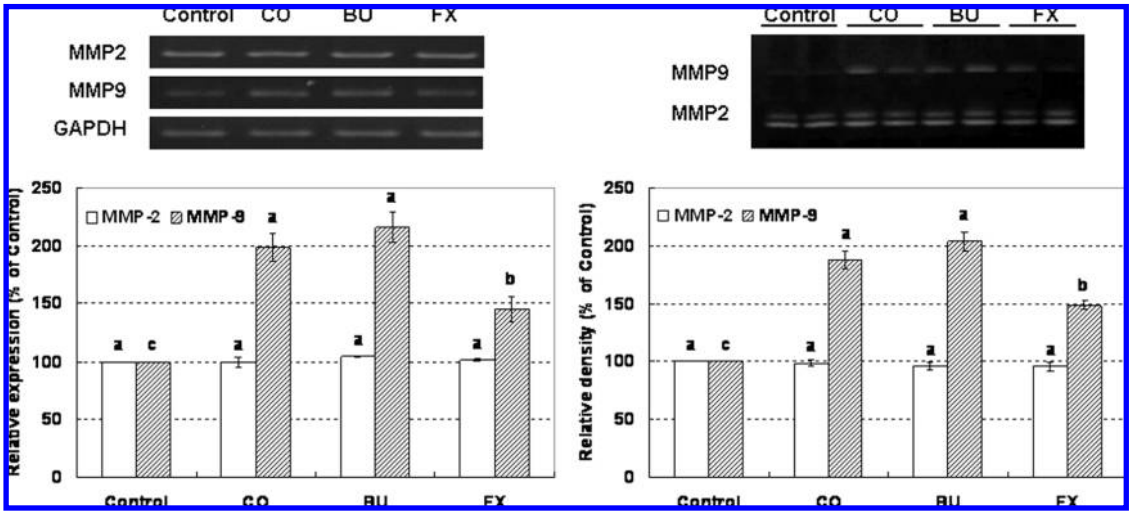

Figure 2. Hepatic MMP-2 and MMP-9 mRNA expressions (**A**, left) and activities (**B**, right) of the experimental hamsters. The data are given as mean ± SEM ($n = 8$). Different letters on data bars in each test parameter indicate significant differences ($p < 0.05$). CO, coconut oil group; BU, butter group; FX, flaxseed oil group. The values of MMP-2 and MMP-9 mRNA were normalized to the value of GAPDH, and values for the hamsters fed the CO, BU, and FX diets were expressed relative to the value for hamsters fed the control diet, which was set to 100%. Hepatic-cell homogenate was subjected to gelatin zymography to analyze the activities of MMP-2 and MMP-9. Activities of these proteins were determined by a densitometric analysis with that of control as 100%.

higher ($p < 0.05$) hepatic GSH contents compared to the other two HFCD groups (CO and BU groups).

Degree of Liver Damage. Clinically, the serum GOT and GPT values are means for determination of the degree of liver damage. The high-fat/cholesterol diets dramatically increased ($p < 0.05$) serum GOT and GPT values of hamsters (**Table 3**). It was also observed that high-fat/cholesterol diets formulated by addition of FX caused yielded lower ($p < 0.05$) GOT and GPT values than those by additions of CO and BU.

Hepatic Gene Expressions and Activities of MMP-2 and MMP-9. The relative hepatic gene expressions and activities of MMP-2 and MMP-9 were analyzed and are shown in **Figure 2**. The results demonstrate a similar pattern of both hepatic gene expressions and activities between MMP-2 and MMP-9. No ($p > 0.05$) differences in gene expressions and activities of hepatic MMP-2 were observed among different dietary groups. However, gene expressions and activities of hepatic MMP-9 were increased ($p < 0.05$) in HFCD groups compared to the control group. Interestingly, high-fat/cholesterol diets formulated by the addition of FX decreased ($p < 0.05$) gene expressions and activities of hepatic MMP-9.

DISCUSSION

Obesity is a prevalent chronic disease in Western countries. Excessive fat intake, leading to an energy imbalance, is considered to be one of the major causes of obesity. Generally, high-fat diets significantly increase body weight and hepatic lipid accumulation, leading to obesity, hyperlipidemia, and fatty liver. Yang et al. (*20*) observed no significant differences in the body weight gain among hamsters fed high-fat diets (12% fat), formulated by additions of conjugated linolenic acids with ALA, and normal diet (10% fat). The current data demonstrate that the body weight gain (**Table 2**) and liver size (**Table 3**) were decreased ($p < 0.05$) upon high-fat/cholesterol diets with FX. Kim et al. (*21*) demonstrated that ALA-rich perrilla oil could decrease the hepatic triacylglycerol content in male Sprague−Dawley rats by suppressing fatty acid synthesis. Because FX is also rich in ALA (53%) (*12*), most likely the decreases of body weight gain and liver size in the FX group may be due to a suppression of fatty acid synthesis.

Sinclair, Attar-Bashi, and Li (*5*) reported that n-3 LC-PUFA can be synthesized from their precursor, ALA, in mammals. It was concluded that ALA seems to lower CVD risk by inhibiting vascular inflammation beyond its lipid-lowering effects (*7*). Prasad (*22*) indicated that the lipid-lowering effect of FX in a high-cholesterol dietary group results from an increase in lipoprotein lipase activity. Flaxseed is the richest dietary source of several lipid-lowering compounds, specifically, ALA, phytoestrogens, lignans, and soluble fibers (*10*). Pellizzon et al. (*23*) reported that the lipid-lowering effect of flaxseed is not attributed to hepatic mediation and may be related to cholesterol absorption and/or bile acid absorption. In the present study, our data demonstrated that FX reduces serum TC and TAG (**Table 3**) compared to the other high-fat/cholesterol dietary diets (BU and

CO groups). However, the FX diet did not reduce serum TC or TAG to the levels of the control diet.

High cholesterol and triacylglycerol accumulations in the liver were demonstrated in a rodent model fed a high-fat/cholesterol diet (*8*). Decreases of adipocyte size and hepatic triacylglycerol accumulation by n-3 PUFAs were also reported in a high dietary fat-induced diabetic mouse model (*10*). Tzang et al. (*12*) concluded that FX can up-regulate cholesterol 7-α hydroxylase (CYP7A1) gene expression, causing increased cholesterol secretion into bile and leading to a depletion of the intrahepatic cholesterol pool. The decreased hepatic cholesterol concentration in the FX group was also observed in the present study (**Table 3**). A higher fecal lipid excretion related to lower serum lipid level was also reported (*12*). Therefore, it is assumed that the lowering effects of FX on serum and hepatic lipids are highly associated with higher fecal lipid outputs and hepatic lipid expenditure, as well as less hepatic lipid accumulation.

Higher fat consumption increases oxidative stress in the body, thus increasing the risk of inflammation and steatosis in the liver (*24*). High fat consumption resulted in higher serum GPT, free fatty acids, and TNF-α levels, as well as hepatic cholesterol, triacylglycerol, MDA, and hydroperoxide levels in a rat model; however, supplementation of n-3 PUFAs can restore hepatic adiponectin and peroxisome proliferator-activated receptor-α (PPAR-α) expression and reduce hepatic TNF-α level to ameliorate fatty liver and alleviate liver damage (*8*). Gonzalez et al. (*25*) reported that n-3 LC-PUFA in fish oil is noted to be particularly susceptible to lipid oxidation. However, hepatic MDA contents in laying hens were not ($p > 0.05$) increased when FX was added to a basal diet (40 g of FX/kg of diet) (*26*). The higher hepatic GSH contents were also observed in spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats fed diets formulated with n-3 LC-PUFA rich menhaden oil when compared to those formulated with butter and soybean oil (*27*). As we know, higher GSH contents in tissues results in lower oxidative stress, thus lowering MDA contents. In the present study, a tendency toward lower MDA contents in the FX and control groups compared to those in the CO and BU groups may be associated with lower hepatic cholesterol and triacylglycerol levels in the FX and control groups, which may decrease the depletion of hepatic GSH contents (**Figure 1** and **Table 3**). Higher hepatic MDA contents always couple with higher serum GOT and GPT values (*17*). Similar results were also observed in the present study (**Table 3**).

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent proteases that participate in the degradation of the extracellular matrix (ECM) (*28*). In addition, major components of ECM in liver fibrosis are collagen types I, III, and IV. MMP-1, -2, and -9 cause fibrolysis and tissue remolding, which will cause the progression of liver fibrosis (*13*). Hence, increased MMP-2 and -9 gene expression or activity is regarded as one of the major causes of liver fibrosis (*14*, *15*). Watanabe et al. (*29*) reported that MMP-9 may serve to recruit progenitor cells from the bone marrow to the injured liver. Ito et al. (*30*) indicated that increased gene expressions and activities of MMP-2 and -9 during acetaminophen intoxication cause severe hepatocellular damage, thus increasing alanine aminotransferase (ALT) levels in mice. Recently, it was reported that an inhibition of MMPs can minimize hepatic injuries (*30*, *31*). Moreover, a competition of n-3 PUFAs with n-6 PUFAs lowers the eicosanoid inflammatory mediators, such as prostaglandin E_2 (PGE_2) and leukotriene B4 (LTB_4), as well as cytokine inflammatory mediators, such as interleukin-1β (IL-1β) and TNF-α (*32*). Fernandes et al. (*33*) reported that MMPs are induced by TNF-α as a consequence of the inflammatory response. FX contains more ALA (53.3%) (*11*), which can be converted to n-3 LC-PUFAs, i.e., EPA and DHA (*6*). Hence, we speculate that consumption of FX is capable of increasing the concentration of n-3 PUFAs in the body, which could modulate inflammation by inactivation of cytokines, thus reducing gene expressions and activities of MMP-9. Meanwhile, FX also decreases the hepatic lipid accumulation and hepatic GSH depletion induced by a high-fat/cholesterol diet. Those benefits of FX further minimize liver damage in a high-fat/cholesterol diet.

In summary, our results demonstrate that FX markedly attenuates NAFL of hyperlipidemic hamsters. The attenuation of liver damage was associated with lower hepatic cholesterol and triacylglycerol levels, GSH depletion, and hepatic MMP-9 gene expressions and activities. On the basis of those beneficial effects, FX may attenuate the NAFL induced by the consumption of a high-fat/cholesterol habit.

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