Eur J Nutr (2012) 51:57–68
DOI 10.1007/s00394-011-0182-7

ORIGINAL CONTRIBUTION

Dietary *d*-limonene alleviates insulin resistance and oxidative stress–induced liver injury in high-fat diet and L-NAME-treated rats

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Received: 6 October 2010 / Accepted: 28 February 2011 / Published online: 29 March 2011

Abstract
Background Nonalcoholic fatty liver disease (NAFLD) is one of the most common etiologies of chronic liver disease worldwide. The pathogenesis of metabolic syndrome associated with NAFLD is still under debate.
Aim of the scope This study has investigated the hepatic biochemical and histological changes and also insulin resistance in metabolic syndrome associated with NAFLD.
Methods Young male Wistar rats fed a high-fat diet (*HFD* 42.2% beef tallow) together with N^{ω}-nitro-L-arginine methyl ester (L-NAME; 80 mg/L in drinking water) for 8 weeks and subsequently with 2% *d*-limonene for the final 4 weeks.
Results HFD-fed rats treated with L-NAME showed increased systolic blood pressure, heart rate, fasting blood glucose, plasma insulin, hepatic marker enzymes, hepatic lipids, circulatory lipid peroxidation by-products, and hepatic phase I enzyme activities with decreased circulatory nonenzymic antioxidant concentrations and hepatic phase II enzyme activities. Dietary supplementation with *d*-limonene reversed the HFD and L-NAME-induced changes and restored pathological alteration of liver and pancreas.
Conclusions These data provide new insights into the therapeutic approach of *d*-limonene against the development of the metabolic syndrome associated with NAFLD.

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Keywords Nonalcoholic fatty liver disease · Metabolic syndrome · High-fat diet · L-NAME · *d*-limonene

Background

Nonalcoholic fatty liver disease (NAFLD) is one of the most common etiologies of chronic liver disease. NAFLD is an increasingly recognized condition that may progress to end-stage liver disease. It refers to a wide spectrum of liver damage, ranging from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis. The pathological picture resembles that of alcohol-induced liver injury, but it occurs in patients who do not abuse alcohol [1, 2]. The pathogenesis of NAFLD is still under debate. Clinical, epidemiological, and biochemical data strongly support the concept that NAFLD is the hepatic manifestation of the metabolic syndrome, a cluster of closely related clinical features linked to visceral obesity, hyperlipidemia, insulin resistance, and type 2 diabetes [3–5].

NAFLD is rapidly becoming a worldwide public health problem. It is the most common liver disease in the United States and, indeed, throughout the world. Current estimates are that ~20% of the general US population suffers from NAFLD. The prevalence in the morbidly obese population has been estimated as 75–92% while that in the pediatric population, as 13–14%. At present, it is estimated that ~6 million individuals in the US general population have progressed to nonalcoholic steatohepatitis (NASH) and about 600,000 to NAFLD-related cirrhosis [6].

The changes in hepatic fat accumulation-related parameters (hepatic total cholesterol (TC), triglycerides (TG), free fatty acid (FFA), and phospholipids (PL)) [7–10] and liver function indicators (aspartate aminotransferase (AST),

alanine aminotransferase (ALT), and alkaline phosphatase (ALP)) [8–11] are in accordance with the degree of obesity, hypertension, and insulin resistance linked with the NAFLD. Members of the microsomal cytochrome P-450 participate in the generation of oxidative changes in the liver via increased production of the free oxygen radical H_2O_2. Hepatic CYP2E1 increases with fasting, diabetes, obesity, and insulin resistance and initiates oxidative stress in the fatty liver [12].

d-Limonene, a monocyclic monoterpene, also known as 1-methyl-4-(1-methyl ethenyl) cyclohexene. *d*-Limonene (orange oil/essence oil) is widely distributed as a natural nonnutritive constituent in a variety of foods particularly fruits (citrus fruits, especially lemon and orange) [13, 14], vegetables (carrots) [14], coffee, beverages, meat, and spices (nutmeg) [15]. *d*-Limonene is used primarily as a lemon fragrance in soaps, detergents, creams, lotions, and perfumes and as a flavoring agent in foods, beverages, and chewing gum [16]. It is found in nonalcoholic beverages (31 ppm), ice cream (68 ppm), candy (49 ppm), baked goods (120 ppm), gelatins and puddings (48–400 ppm), and chewing gum (2,300 ppm). It is found naturally in orange juice at an average concentration of 100 mg/L [16].

d-Limonene is known to inhibit lipid peroxidation, arrest the free radical-induced damage [17], and prevent physical stress, psychological stress [18], stress-induced hypertension [19], and stress responses in stroke-prone spontaneously hypertensive rats [20]. *d*-Limonene is also known to regulate the development of pulmonary hypertension [21], induce glutathione (phase II detoxification) [22], and inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity [23, 24]. In addition, *d*-limonene is reported to exert potent biological activities, such as antioxidant properties [22, 25], chemopreventive or chemotherapeutic properties against many types of cancers [26], antiinflammatory properties, hepatoprotective activities [27], and immunomodulatory effects [28].

The present study was aimed to evaluate the therapeutic efficacy of *d*-limonene against hepatic biochemical and histological alterations in high-fat diet (HFD) and N^{ω}-nitro-L-arginine methyl ester (L-NAME)-induced metabolic syndrome associated with NAFLD.

Materials and methods

Chemicals

d-Limonene and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were procured from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals and biochemicals used in the present study were of analytical grade.

Animal care and diet

All the experiments comply with the recommendations and guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and the experimental protocol was approved by the Animal Ethical Committee of Annamalai University (Reg. No. 160/1999/CPCSEA: 558/2008). Male Wistar rats (150–180 g, 4–6 weeks old) were supplied by the Central Animal House, Rajah Muthiah Medical College and Hospital (RMMCH), Annamalai University. They were housed individually in polypropylene cages with paddy husk for bedding at a room temperature of 25 ± 2 °C with relative humidity (45% ± 5%) under a 12-h light–dark cycle. Upon arrival, the rats were allowed 1 week to acclimatize to the new conditions. They were allowed free access to standard rat chow and water. The standard diet consisted of a balanced diet containing protein 21.1%, fat 5.1%, carbohydrate 60.0%, fiber 3.9%, minerals 7.9%, and vitamins 2.0%. High-fat diet (HFD) comprised of protein 12.2%, fat 2.8%, beef tallow 42.2%, carbohydrates 34.7%, fiber 2.3%, minerals 4.6%, and vitamins 1.2%. Two percent of *d*-limonene diet (w/w) was prepared by mixing with standard pellet diet everyday. All measures were taken to ensure uniform mixing of the additives of the diet before kneading using a little water.

Experimental procedure

After the acclimatization period, young male albino Wistar rats were divided into 6 groups ($n = 8$) randomly and treated for 8 weeks as follows: *group 1* (*CON*) Rats received standard pellet diet for the total experimental period of 8 weeks, *group 2* (*LIM*) Rats received 2% *d*-limonene diet for the last 4 weeks of the experiment, *group 3* (*HFD*) Rats received HFD (42.2% beef tallow) for the total experimental period of 8 weeks, *groups 4* (*HFD + L-NAME*) Rats received a HFD together with L-NAME (80 mg/L in drinking water) for 8 weeks, *groups 5* (*HFD + LIM*) Rats received HFD together with 2% *d*-limonene diet for the last 4 weeks of the experiment, *groups 6* (*HFD + L-NAME + LIM*) Rats received HFD and L-NAME together with 2% *d*-limonene diet for the last 4 weeks of the experiment.

Blood pressure measurement

Blood pressure was determined in conscious rats by means of the tail-cuff method. Each animal was introduced into a restrainer and kept in a quiet and warm environment for 10 min. After prewarming the rats at 37 °C, the blood pressure and heart rate were measured. This procedure was repeated for 2–3 days in order to familiarize the rats with

the restrainer. A rubber cuff (proximally) and a photoelectric sensor of pulsations (more distally) were placed around the tail. The sensor was connected to an amplifier and pulsations were recorded on a Power Lab recording unit (IITC blood pressure system, Model No: 32, Inc. /Life Science Instruments, USA). A large number of recordings were taken, but only 6–12 readings were selected, and the mean of measurements was obtained from each rat once a week until the end of the experiment.

Biochemical assays

On the day before euthanasia, the animals were deprived of food for overnight and anaesthetized, and then, rats were killed by cervical decapitation. Blood was collected in heparinized tubes, after 60-min rest in the supine position, and centrifuged at $160\times g$ for 10 min at 20 °C. The plasma separated was stored at −20 °C until assay. Liver was carefully cleaned of adherent fat, connective tissue, weighed accurately, and stored at −80 °C until use.

Plasma glucose was estimated according to the method of Trinder using a commercial kit (Sigma Diagnostics Pvt. Ltd., Baroda, India). Plasma insulin was assayed by an enzyme-linked immunosorbent assay (ELISA) method using a commercial kit (Catalog No. SP-401) from United Biotech Inc., Mountain View, CA, USA. Homeostatic model assessment for insulin resistance (HOMA-IR) index indirectly determines the insulin resistance as the multiplication of fasting insulinemia (μU/mL) and glycemia (mM) divided by 22.5 corrected factor. The activities of serum aspartate transaminase (AST, EC 2.6.1.1), alanine transaminase (ALT, EC 2.6.1.2), and alkaline phosphatase (ALP, EC 3.1.3.1) were assayed spectrophotometrically according to the standard procedures using commercially available diagnostic kits (Sigma Diagnostics (I) Pvt. Ltd., Baroda, India). Lipids were extracted from hepatic tissues by the method of Folch et al. [29]. Total cholesterol was estimated by the method of Zlatkis et al. [30], triglycerides by the method of Foster and Dunn. [31], and free fatty acids by the method of Falholt et al. [32]. Lipid peroxidation as indicated by thiobarbituric acid reactive substances (TBARS) was measured using the method of Ohkawa et al. [33]. The levels of conjugated dienes (CD) were determined by the method of Rao and Racknagel [34] and lipid hydroperoxides (LOOH) by the method of Jiang et al. [35]. Reduced glutathione (GSH) was determined by the method of Boyne and Ellman [36], based on the reaction with Ellman's reagent. Vitamin C (ascorbic acid) and vitamin E (α-tocopherol) concentrations were measured by the method of Roe and Kuether [37] and Baker and Frank [38], respectively.

Cytosolic and microsomal fractions were prepared from individual hepatic tissues. Tissues were homogenized in 0.25 M sucrose, centrifuged at $9{,}000\times g$ for 20 min, and the supernatant was collected. To this, 0.2 volumes of 0.1 M $CaCl_2$ in 0.25 M sucrose was added, the samples were kept on ice for 30 min and centrifuged at $27{,}000\times g$ for 20 min, and the clear cytosolic fractions obtained were promptly assayed for the activities of phase II enzymes. Microsomal pellets were washed twice by suspending in 7 ml of 10 mM Tris–HCl (pH 7.4) and 0.25 M sucrose. The tubes were centrifuged at $9{,}000\times g$ for 20 min to obtain microsomal fractions, which were promptly assayed for the activities of phase I enzymes.

Cytochrome P450 and cytochrome b5 were assayed by the method of Omura and Sato [39]. NADPH-cytochrome P450 reductase (EC 1.6.2.4) was assayed by the method of Omura and Takesue [40]. Cytochrome P4502E1 (CYP4502E1) activity was assayed by the method of Watt et al. [41]. NADH-cytochrome b5 reductase (EC 1.6.2.2) activity was assayed by Mihara and Sato [42]. DT-diaphorase (EC. 1.6.9.92) activity was assayed by the method of Ernster et al. [43]. Glutathione S-transferase (GST, EC. 2.5.1.18) was assayed by the method of Habig et al. [44]. Total protein concentrations were determined by the method of Lowry et al. [45].

Histological analysis

Hepatic tissue was quickly removed after euthanasia, fixed in 10% buffered formalin for 48 h, dehydrated by passing successively in different concentrations of ethanol–water, cleaned in xylene, embedded in paraffin, and sectioned (5–6 μm thickness) using a microtome. Sections were stained with hematoxylin and eosin (H and E) dye and Milligan's trichrome stain and then mounted in a neutral deparaffinated xylene (DPX) medium using standard protocols. For Oil *red* O staining, liver samples were removed and immediately frozen in liquid nitrogen. Frozen hepatic tissues were sliced and embedded using sucrose medium. Cryosectioning was performed at a temperature of –25 °C where the embedded tissues were sectioned into 8- to 10-μm slices and adhered onto glass slides. Sections were stained with Oil *Red* O and mounted in glycerine jelly. Pancreas was isolated from the duodenal portion of intestine and fixed with neutral-buffered formalin for 48 h. The samples were then dehydrated and embedded in paraffin wax. Thick sections (5–6 μm) were cut and stained for hematoxylin and eosin dye and aldehyde fuchsin stain for the islets of langerhans. They were assessed using light microscopy and photographed.

Statistics

The data are expressed as mean ± SE. Comparisons of the determined variables among all the grouped data for

biochemical and physiological parameters were analyzed statistically using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using the SPSS software package, version 11.01 for windows and statistical significance was defined as $P < 0.05$.

Results

Figure 1a illustrates the initial and final systolic blood pressure of control and experimental rats. After 8 weeks of treatment, HFD-fed rats showed increased systolic blood pressure as compared to control by 41% (152 ± 5.6 mm Hg vs. 108 ± 3.7 mm Hg), which was further increased by the addition of L-NAME to HFD-fed (group 4) rats by 81% (195 ± 6.7 mm Hg vs. 108 ± 3.7 mm Hg). Dietary *d*-limonene supplementation reduced the systolic blood pressure by 26% (112 ± 4.3 mm Hg vs. 152 ± 5.6 mm Hg) and by 37% (123 ± 4.3 mm Hg vs. 195 ± 5.6 mm Hg) in HFD + *d*-limonene- and HFD + L-NAME + *d*-limonene-administered rats, respectively. Figure 1b depicts the initial and final heart rate of control and experimental rats. Heart rate was elevated in the HFD-fed (group 3) rats, which was further increased by the addition of L-NAME to HFD-fed (group 4) rats as compared to the control (group 1). Supplementation with *d*-limonene reduced the heart rate in HFD-treated group and HFD + L-NAME-treated rats (group 5 and 6) as compared to the HFD alone– and HFD + L-NAME alone–treated rats (group 3 and 4) but not in *d*-limonene-supplemented control rats (group 2).

The activities of AST, ALT and ALP in the liver were significantly elevated in the HFD-fed and HFD + L-NAME (group 3 and 4) rats as compared to the control rats (group 1). Dietary *d*-limonene feeding decreased the activities of all the above enzymes in the liver of HFD and HFD + L-NAME (group 5 and 6) rats as compared to the HFD alone– and HFD + L-NAME alone (group 3 and 4)–treated rats. The concentrations of fasting blood glucose, plasma insulin, and HOMA-IR were elevated in the HFD-fed and HFD + L-NAME (group 3 and 4)-treated rats as compared to the control (group 1). Dietary *d*-limonene decreased the concentrations of fasting blood glucose plasma insulin and HOMA-IR of the HFD and HFD + L-NAME (group 5 and 6) rats as compared to the HFD alone– and HFD + L-NAME alone (group 3 and 4)–treated rats (Table 1).

The level of lipid peroxidation by-products in the circulation was elevated in the HFD-fed and HFD + L-NAME (group 3 and 4) rats as compared to the control rats (group 1). Two percent of *d*-limonene diet reduced the levels of lipid peroxidation by-products in the circulation of HFD and HFD + L-NAME (group 5 and 6) rats as compared to HFD alone– and HFD with L-NAME alone

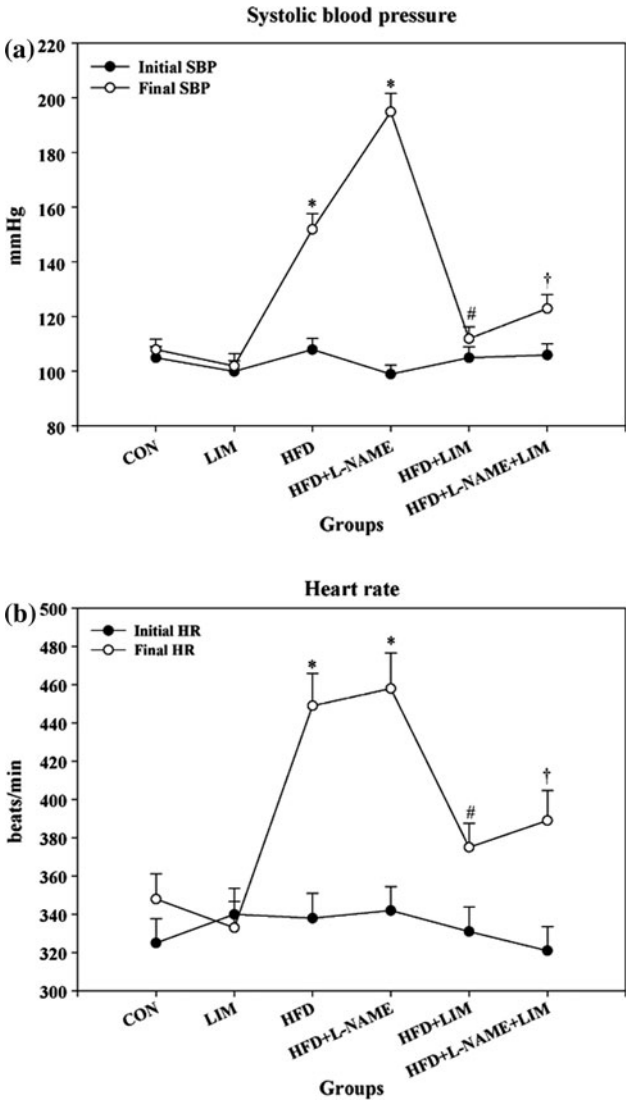

Fig. 1 **a** Changes in the initial and final systolic blood pressure of different experimental groups. **b** Changes in the initial and final heart rate of different experimental groups. *CON* Control group, *LIM* *d*-limonene (2%)-treated group, *HFD* High-fat diet fed group, *HFD + L-NAME* High-fat diet with L-NAME (80 mg/L in drinking water)-treated group, *HFD + LIM* High-fat diet with *d*-limonene (2%)-treated group, *HFD + L-NAME + LIM* High-fat diet with L-NAME (80 mg/L in drinking water)- and *d*-limonene (2%)-treated group. *Data* are expressed as mean ± SEM of 6 rats in each group. *Significantly different from control group, $P < 0.05$. #Significantly different from HFD group, $P < 0.05$. †Significantly different from HFD + L-NAME-treated group, $P < 0.05$

(group 3 and 4)–treated rats, respectively. The level of nonenzymic antioxidants such as vitamin C, vitamin E, and reduced glutathione (GSH) in the circulation was decreased in HFD- and HFD + L-NAME-treated rats (group 3 and 4) as compared to the control (group 1). Dietary *d*-limonene supplementation restored the nonenzymic circulatory antioxidant in HFD- and HFD + L-NAME alone (group 5 and 6)–treated rats as compared to the HFD-fed and HFD

Table 1 Effect of *d*-limonene on hepatic function indicators and insulin function indicators of control and experimental rats

Groups	CON	LIM	HFD	HFD + L-NAME	HFD + LIM	HFD + L-NAME + LIM
Hepatic functions indicators						
AST (IU/L)	56.18 ± 2.13	55.98 ± 2.09	76.90 ± 3.03*	77.12 ± 3.13*	62.89 ± 2.37$^{\#}$	63.11 ± 2.51†
ALT (IU/L)	26.89 ± 1.03	26.61 ± 1.02	36.39 ± 1.28*	37.13 ± 1.52*	29.82 ± 1.14$^{\#}$	30.122 ± 1.17†
ALP (IU/L)	88.93 ± 3.42	88.10 ± 3.33	142.35 ± 5.09*	143.10 ± 4.94*	94.66 ± 3.58$^{\#}$	95.02 ± 3.41†
LW (mg)	8.25 ± 0.33	8.15 ± 0.33	10.12 ± 0.42*	10.21 ± 0.42*	9.85 ± 0.40$^{\#}$	9.99 ± 0.40†
HI	0.034 ± 0.001	0.033 ± 0.001	0.027 ± 0.001*	0.026 ± 0.001*	0.032 ± 0.001$^{\#}$	0.032 ± 0.001†
Insulin resistance markers						
Blood glucose (mg/dL)	94.65 ± 3.56	87.90 ± 3.35	195.28 ± 16.86*	216.98 ± 8.28*	116.83 ± 4.22$^{\#}$	120.21 ± 4.63†
Plasma insulin (μU/mL)	16.80 ± 0.63	15.76 ± 0.60	30.57 ± 2.29*	33.90 ± 1.24*	22.40 ± 0.87$^{\#}$	23.18 ± 0.85†
HOMA-IR	3.95 ± 0.29	3.64 ± 0.29	17.07 ± 1.27*	18.27 ± 1.34*	6.50 ± 0.48$^{\#}$	6.92 ± 0.51†

All the values are expressed as mean ± SE of 6 rats in each group

AST Aspartate aminotransferase, *ALT* Alanine aminotransferase, *ALP* Alkaline phosphatase, *LW* Liver weight, *HI* Hepatic index (liver weight–body weight ratio), *HOMA-IR* Homeostatic model assessment for insulin resistance

* Significantly different from control group, $P < 0.05$

$^{\#}$ Significantly different from HFD group, $P < 0.05$

† Significantly different from HFD + L-NAME-treated group, $P < 0.05$

Table 2 Effect of *d*-limonene on circulatory lipid peroxidation, antioxidant status, and hepatic lipids of control and experimental rats

Groups	CON	LIM	HFD	HFD + L-NAME	HFD + LIM	HFD + L-NAME + LIM
Circulatory lipid peroxidation						
TBARS (nmoles/mL)	1.802 ± 0.072	1.785 ± 0.069	6.875 ± 0.255*	7.241 ± 0.288*	2.324 ± 0.092$^{\#}$	2.658 ± 0.103†
CD (mmoles/mL)	0.802 ± 0.033	0.786 ± 0.029	1.658 ± 0.066*	1.724 ± 0.069*	0.925 ± 0.036$^{\#}$	0.966 ± 0.037†
LOOH (μmoles/mL)	0.484 ± 0.016	0.442 ± 0.017	0.902 ± 0.034*	0.995 ± 0.037*	0.498 ± 0.017$^{\#}$	0.508 ± 0.020†
Circulatory nonenzymic antioxidants						
Vitamin C (mg/dl)	1.810 ± 0.071	1.832 ± 0.073	1.118 ± 0.047*	1.058 ± 0.040*	1.754 ± 0.070$^{\#}$	1.710 ± 0.068†
Vitamin E (mg/dL)	1.332 ± 0.045	1.385 ± 0.049	0.789 ± 0.031*	0.821 ± 0.033*	1.310 ± 0.044$^{\#}$	1.285 ± 0.049†
GSH (mg/dL)	18.862 ± 0.755	18.921 ± 0.649	13.845 ± 0.544*	13.424 ± 0.527*	19.250 ± 0.761$^{\#}$	19.985 ± 0.765†
Hepatic lipids						
Cholesterol (mg/gm tissue)	3.32 ± 0.13	3.28 ± 0.12	5.21 ± 0.20*	5.32 ± 0.20*	3.96 ± 0.13$^{\#}$	3.99 ± 0.15†
Triglycerides (mg/gm tissue)	3.11 ± 0.12	3.20 ± 0.13	6.22 ± 0.24*	6.54 ± 0.23*	4.13 ± 0.16$^{\#}$	4.24 ± 0.16†
Free fatty acids (mg/gm tissue)	6.98 ± 0.26	7.02 ± 0.28	10.44 ± 0.43*	10.54 ± 0.42*	8.25 ± 0.32$^{\#}$	8.54 ± 0.32†

All the values are expressed as mean ± SE of 6 rats in each group

TBARS Thiobarbituric acid reactive substances, *CD* Conjugated dienes, *LOOH* Lipid hydroperoxides, *GSH* Reduced glutathione

* Significantly different from control group, $P < 0.05$

$^{\#}$ Significantly different from HFD group, $P < 0.05$

† Significantly different from HFD + L-NAME-treated group, $P < 0.05$

with L-NAME (group 3 and 4) rats in which *d*-limonene supplementation did not have any marked effect on the control rats (group 2). The concentrations of hepatic lipids (total cholesterol, triglycerides, and free fatty acids) were elevated in the HFD and HFD + L-NAME (group 3 and 4) rats as compared to the control (group 1). Dietary supplementation with *d*-limonene reduced the concentration of hepatic lipids in the HFD and HFD + L-NAME (group 5 and 6) rats as compared to the HFD alone– and HFD + L-NAME alone (group 3 and 4)–treated rats, respectively (Table 2).

HFD- and HFD + L-NAME (group 3 and 4)-treated rats showed increased activities of hepatic microsomal phase I enzymes, such as cytochrome P450, cytochrome b5, cytochrome P4502E1, NADPH-cytochrome P450 reductase, and NADH-cytochrome b5 reductase, as compared to the control rats (group 1). *d*-Limonene supplementation to HFD- and HFD + L-NAME (group 5 and 6)-treated rats

Table 3 Effect of *d*-limonene on hepatic xenobiotic-metabolizing enzymes of control and experimental rats

Groups	CON	LIM	HFD	HFD + L-NAME	HFD + LIM	HFD + L-NAME + LIM
Hepatic phase I enzymes						
Cyt P450[Φ]	5.13 ± 0.21	5.02 ± 0.20	8.57 ± 0.32*	9.01 ± 0.35*	5.25 ± 0.20[#]	5.85 ± 0.22[†]
Cyt b5[Ω]	2.15 ± 0.08	2.08 ± 0.08	3.25 ± 0.12*	3.69 ± 0.11*	2.32 ± 0.08[#]	2.43 ± 0.08[†]
CYT2E1[$]	5.25 ± 0.20	5.06 ± 0.20	10.03 ± 0.35*	10.87 ± 0.35*	5.90 ± 0.20[#]	5.98 ± 0.19[†]
NADPH Cyt P450 reductase[¥]	60.60 ± 2.25	60.02 ± 2.06	71.99 ± 2.48*	72.87 ± 2.82*	61.26 ± 2.47[#]	61.81 ± 2.51[†]
NADH Cyt b5 reductase[£]	17.59 ± 0.61	17.04 ± 0.68	26.12 ± 0.83*	26.98 ± 0.84*	18.24 ± 0.64[#]	18.69 ± 0.68[†]
Hepatic phase II enzymes						
Glutathione S-transferase[¶]	2.12 ± 0.09	2.00 ± 0.08	1.22 ± 0.04*	1.09 ± 0.04*	1.98 ± 0.08[#]	1.90 ± 0.07[†]
DT-Diaphorase[‡]	1.83 ± 0.07	1.92 ± 0.08	0.96 ± 0.04*	0.90 ± 0.04*	1.77 ± 0.06[#]	1.72 ± 0.05[†]

All the values are expressed as mean ± SE of 6 rats in each group

Cyt P450 Cytochrome P450, [Φ] μ moles/mg protein, *Cyt b5* Cytochrome b_5 [Ω] μ moles/mg protein, *CYT2E1* CytochromeP4502E1, [$] m moles of p-nitrocatechol liberated/mg protein/min, *NADPH* Cyt P450 reductase NADPH-cytochrome P450 reductase, [¥] moles of NADPH oxidized/min/mg protein, *NADH* Cyt b5 reductase NADH-cytochrome b5 reductase, [£] moles of ferricyanide reduced/min/mg protein, *GST* Glutathione S-transferase, [¶] μ moles of CDNB-GSH conjugate formed/min/mg protein, *DT-Diaphorase* DT-diaphorase, [‡] μ moles of 2,6-dichlorophenolindophenol reduced/min/mg protein

* Significantly different from control group, $P < 0.05$

[#] Significantly different from HFD group, $P < 0.05$

[†] Significantly different from HFD + L-NAME-treated group, $P < 0.05$

reduced the activities of all the above phase I enzymes in the liver as compared to HFD alone– and HFD + L-NAME alone (group 3 and 4)–treated rats (Table 3). The activities of the liver cytosolic phase II enzymes such as glutathione S-transferase and DT-diaphorase showed decreased activities in the HFD- and HFD + L-NAME (group 3 and 4)-treated rats as compared to the control. *d*-Limonene supplementation to HFD and HFD + L-NAME rats (group 5 and 6) increased the activities of GST and DT-diaphorase as compared to the HFD alone– and HFD + L-NAME alone (group 3 and 4)–treated rats (Table 3).

Figure 2 represents the photomicrographs of the hematoxylin and eosin staining of the liver sections of the control and experimental rats. HFD and HFD + L-NAME (group 3 and 4) treatment caused changes in the hepatic tissue characterized by microvesicular steatosis, sinusoidal fibrosis, polymorphonuclear infiltrates, centrilobular inflammation, and marked inflammatory cell infiltration. Dietary *d*-limonene supplementation rats (group 5 and 6) showed reduced hepatosteatosis. Figure 3 represent the photomicrographs of the Oil red O staining of the liver sections of the control and experimental rats. HFD and HFD + L-NAME (group 3 and 4) administration caused changes in the hepatic tissue revealed marked lipid deposition (stained *red*). Dietary *d*-limonene supplementation to HFD and HFD + L-NAME (group 5 and 6) rats showed reduced in the lipid deposition in the hepatic tissue as compared to HFD alone– and HFD + L-NAME alone (group 3 and 4)–treated rats. Figure 4 illustrates photomicrographs of Masson's trichrome staining of the hepatic tissue of HFD- and HFD + L-NAME-administered rats. Both HFD- and HFD + L-NAME-treated (group 3 and 4) rats exhibited increased perisinusoidal, pericellular, and interstitial collagen accumulation (stained *blue*). Dietary supplementation with *d*-limonene to HFD and HFD + L-NAME (group 5 and 6) revealed reduced collagen accumulation (stained *blue*) as compared to the HFD alone– and HFD + L-NAME alone–treated (group 3 and 4) rats. Figure 5 summarizes the hematoxylin and eosin staining of pancreatic tissue of the control and experimental rats. HFD and HFD + L-NAME (group 3 and 4) administration caused changes in the pancreatic tissue which was characterized by increased pancreatic β-cell mass and hyperplasia. Dietary *d*-limonene supplementation to the HFD- and HFD + L-NAME-treated rats (group 5 and 6) showed reduced pancreatic β-cell mass and hyperplasia as compared to HFD alone– and HFD + L-NAME alone–treated rats (group 3 and 4). Figure 6 illustrates photomicrographs of aldehyde fuchsin staining of the pancreatic tissue of control and experimental rats. Aldehyde fuchsin staining of the pancreatic tissue of HFD- and HFD + L-NAME-treated rats (group 3 and 4) exhibited significant increase in the β-cell nucleus inside the islets. Dietary supplementation with *d*-limonene to HFD- and HFD + L-NAME-treated group (group 5 and 6) showed significant reduction in the β-cell nucleus inside the islets as compared to the HFD alone– and HFD with L-NAME alone–treated rats (group 3 and 4).

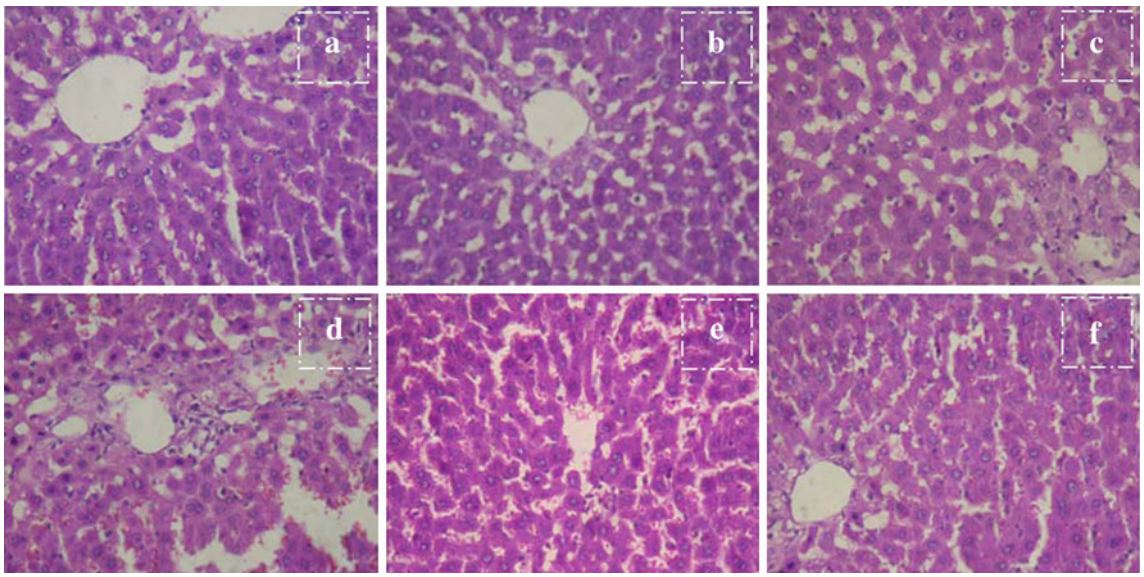

Fig. 2 Photomicrographs of hematoxylin- and eosin-stained sections of rat liver from different experimental groups under 20× standard light magnifications. **a** *CON* Control group, shows normal hepatocytes with central portal vein. **b** *LIM d*-limonene (2%)-treated group, shows normal hepatocytes with central portal vein with no pathological alterations. **c** *HFD* High-fat diet fed group, shows sinusoidal fibrosis, polymorphonuclear infiltrates, and marked inflammatory cell infiltration. **d** *HFD* + *L-NAME* High-fat diet with L-NAME (80 mg/L in drinking water)-treated group, shows microvesicular steatosis, centrilobular inflammation, and marked inflammatory cell infiltration. **e** *HFD* + *LIM* High-fat diet with *d*-limonene (2%)-treated group, shows reduced sinusoidal fibrosis, polymorphonuclear infiltrates, and marked inflammatory cell infiltration. **f** *HFD* + *L-NAME* + *LIM* High-fat diet with L-NAME (80 mg/L in drinking water)- and *d*-limonene (2%)-treated group shows reduced microvesicular steatosis, centrilobular inflammation, and marked inflammatory cell infiltration

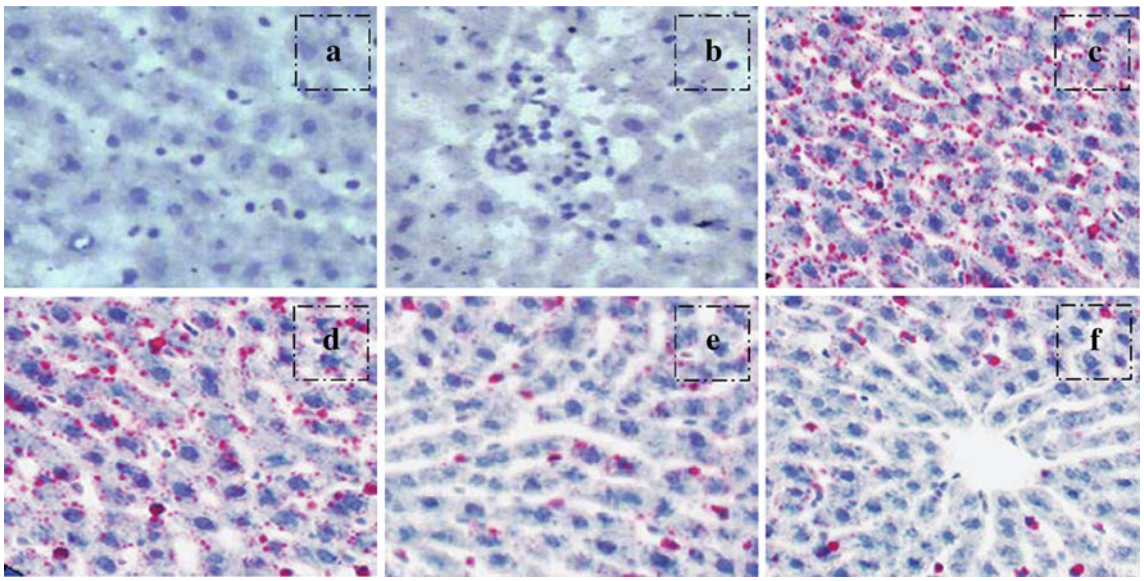

Fig. 3 Photomicrographs of Oil *red* O-stained sections of rat liver from different experimental groups under 40× standard light magnifications. **a** *CON* Control group, **b** *LIM d*-limonene (2%)-treated group, **c** *HFD* High-fat diet fed group, **d** *HFD* + *L-NAME* High-fat diet fed with L-NAME (80 mg/L in drinking water)-treated group, **e** *HFD* + *LIM* High-fat diet fed with *d*-limonene (2%)-treated group, **f** *HFD* + *L-NAME* + *LIM* High-fat diet fed with L-NAME (80 mg/L in drinking water)- and *d*-limonene (2%)-treated group. Fat accumulation appears *red* in color

Discussion

Nonalcoholic fatty liver disease (NAFLD) is usually caused by two 'hits': the 'first hit' is induced by peripheral insulin resistance, causing hepatic steatosis. The 'second hit' is thought to be caused by reactive oxygen species, inducing vicious cycles of oxidative injury leading to inflammation and fibrosis [46]. This study reports the

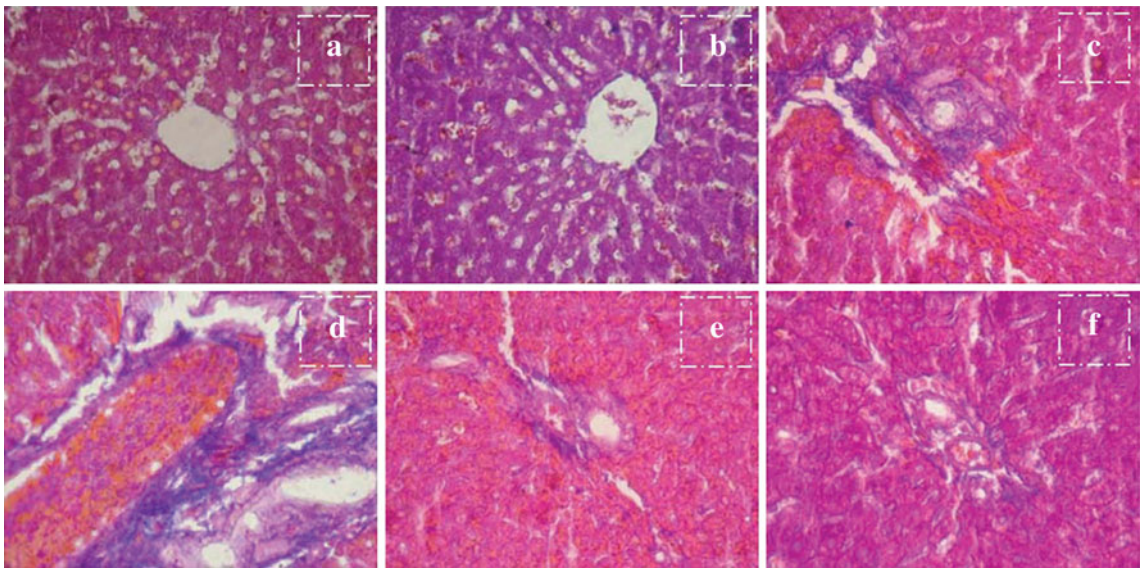

Fig. 4 Photomicrographs of Milligan's trichrome-stained sections of rat liver from different experimental groups under 20× standard light magnifications. **a** *CON* Control group, **b** *LIM* *d*-limonene-(2%) treated group, **c** *HFD* High-fat diet fed group, **d** *HFD* + *L-NAME* High-fat diet with L-NAME (80 mg/L in drinking water)-treated group, **e** *HFD* + *LIM* High-fat diet with *d*-limonene (2%)-treated group, **f** *HFD* + *L-NAME* + *LIM* High-fat diet with L-NAME (80 mg/L in drinking water)- and *d*-limonene (2%)-treated group. Perisinusoidal, pericellular deposition of collagen and interstitial collagen accumulation appears *blue* in color

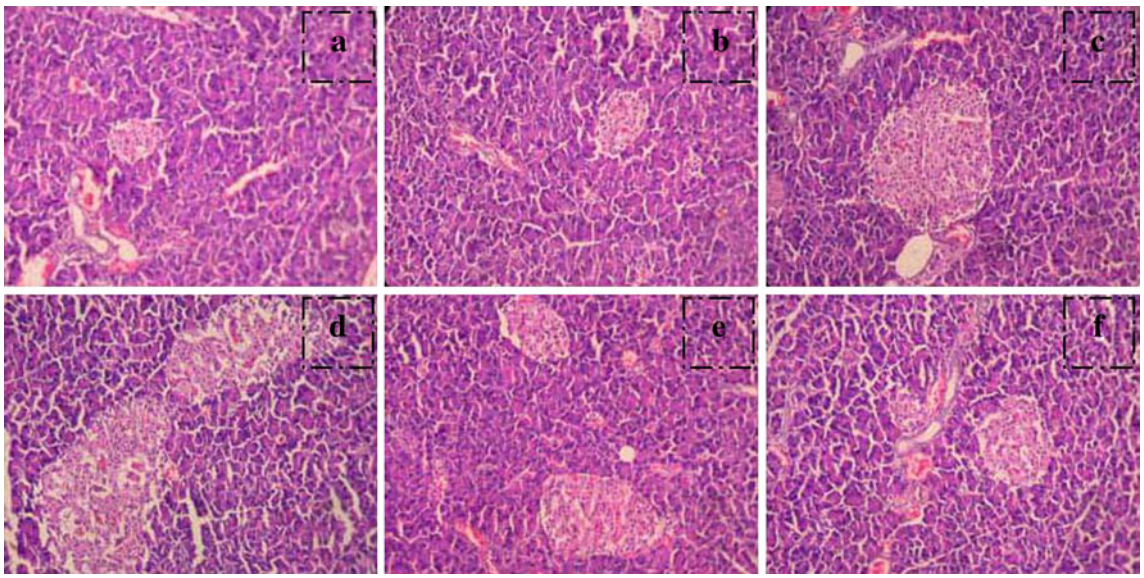

Fig. 5 Photomicrographs of hematoxylin- and eosin-stained sections of rat pancreas from different experimental groups under 10× standard light magnifications. **a** *CON* Control group, shows normal pancreatic β-cell mass. **b** *LIM* *d*-limonene (2%)-treated group, shows normal pancreatic β-cell mass with no pathological alterations. **c** *HFD* high-fat diet fed group, shows increased pancreatic β-cell mass and hyperplasia. **d** *HFD* + *L-NAME* High-fat diet with L-NAME (80 mg/L in drinking water)-treated group, shows increased pancreatic β-cell mass and hyperplasia. **e** *HFD* + *LIM* High-fat diet with *d*-limonene (2%)-treated group, shows reduced pancreatic β-cell mass and hyperplasia. **f** HFD + L-NAME + LIM High-fat diet with L-NAME (80 mg/L in drinking water)- and *d*-limonene (2%)-treated group shows reduced pancreatic β-cell mass and hyperplasia

synergistic interaction between HFD and L-NAME on NAFLD in male Wistar rats. We have characterized the changes in liver pathology, lipid composition, and xenobiotic-metabolizing enzymes activities; plasma insulin and glucose levels; and pancreatic β-cell mass in the rats treated with HFD and L-NAME. *d*-Limonene supplementation significantly reversed the changes induced by feeding HFD and HFD + L-NAME.

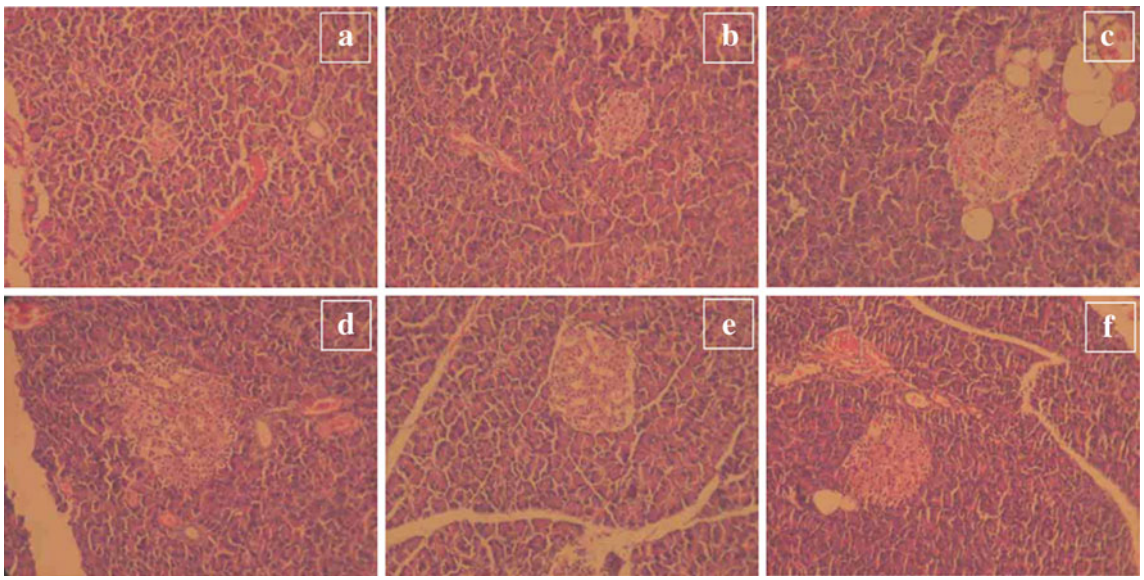

Fig. 6 Photomicrographs of aldehyde fuchsin-stained sections of rat pancreas from different experimental groups under 10× standard light magnifications. **a** *CON* Control group, shows normal *β*-cell nucleus. **b** *LIM d*-limonene (2%)-treated group, shows normal *β*-cell nucleus with no pathological alterations. **c** *HFD* High-fat diet fed group, shows increased the *β*-cell nucleus. **d** *HFD + L-NAME* High-fat diet with L-NAME (80 mg/L in drinking water)-treated group, shows increased the *β*-cell nucleus. **e** *HFD + LIM* High-fat diet with *d*-limonene (2%)-treated group, shows reduced the *β*-cell nucleus. **f** *HFD + L-NAME + LIM* High-fat diet with L-NAME (80 mg/L in drinking water)- and *d*-limonene (2%)-treated group shows reduced *β*-cell nucleus

AST, ALT, and ALP are the relatively liver-specific enzymes. Elevation of AST, ALT, and ALP activities in the plasma is the result of leakage from damaged cells and therefore reflects hepatocyte damage [47]. Mild to moderate elevation in the plasma activities of AST and ALT or both is the most common and often the only laboratory abnormality found in patients with NAFLD. The ratio of AST to ALT is usually <1, but this ratio increases as fibrosis advances, leading to a loss of its diagnostic accuracy in patients with cirrhotic NAFLD. Consistent with the previous findings by Fu et al. [9, 10] Loria et al. [47], and Byrne et al. [48] and Fallo et al. [49], in our study feeding HFD and HFD + L-NAME to rats showed elevated hepatic enzyme (ALT, AST, and ALP) activities which are strongly correlated NAFLD associated with metabolic syndrome. On supplementation with dietary *d*-limonene, the activities of the hepatic marker enzymes were significantly reduced as compared to untreated rats.

An imbalance between the uptake, synthesis, oxidation, and export of lipids results in excessive fat accumulation in the liver. High-fat or high-carbohydrate diet is inclined to result in fat accumulation in the liver [50], and similarly, L-NAME is associated with accelerated lipid deposition [51]. Elevated serum TC, TG, FFA and reduced serum HDL, the indicators of hyperlipidemia, are independent predictors of NAFLD. Increased retention of lipids in the hepatocytes, mostly in the form of TC, TG, and FFA, is known to be the common early trait of NAFLD [52]. Moreover, there is a strong link between insulin resistance and excessive deposition of lipids in hepatocytes, which is the hallmark for diagnosis of NAFLD [53]. In our study, accumulation of TC, TG, and FFA in the hepatocytes was observed in the HFD- and HFD + L-NAME-treated rats resulting in insulin resistance which is in accordance with the previous findings [50, 54] and also represents the "first hit" in the pathogenesis of NAFLD [54]. The excessive/ectopic fat depositions in the liver could be due to increased fatty acid delivery from adipose tissue, increased synthesis of fatty acid via the de novo pathway, increased dietary fat, decreased mitochondrial *β*-oxidation, decreased clearance of VLDL (very LDL) particles, or all of these factors in combination [55]. *d*-Limonene supplementation to HFD- and HFD + L-NAME-treated rats decreased the hepatic fat deposition. This may be due to the decreased activities of the key enzymes involved in the synthesis of FFA and TG.

HFD- and HFD + L-NAME-treated rats showed moderate increase in the plasma glucose levels after 8 weeks. Normally, to compensate the elevated levels of the glucose, the pancreas increases its mass as well as the number of beta cells to produce more insulin. Increased mass of islets is already well documented in experimental HFD animal model [56, 57]. Again, it is a matter of debate that increased insulin or glucose levels contribute to the hyperplasia of the islets. In agreement with the "first hit" hypothesis, insulin resistance was observed in the diet-induced NAFLD in our study. Dietary *d*-limonene supplementation reduced the levels of plasma glucose, insulin,

and the pancreatic β-cell mass in HFD- and HFD + L-NAME-treated rats, indicating that *d*-limonene alleviates insulin resistance.

The second hit leading to NAFLD/NASH involves oxidative stress, activation of cytochrome P450 2E1 (CYP 2E1), lipid peroxidation, increased inflammatory cytokines production, activation of hepatic stellate cells, and apoptosis [12]. Insulin resistance and FFA predispose to oxidative stress and reactive oxygen species (ROS) production by stimulating microsomal lipid peroxidases and by decreasing mitochondrial beta-oxidation [12, 58]. In our present study, increased levels of lipid peroxidation by-products and decreased levels of nonenzymic antioxidants were observed in the HFD- and HFD + L-NAME-treated rats. Lipid peroxidation by-products produced by the fatty liver causes cell damage by impairing the nucleotide, DNA, or protein synthesis and thereby the membrane structure and function [58, 59]. Administration of *d*-limonene diet to the HFD- and HFD + L-NAME-treated rats showed decreased levels of lipid peroxidation by-products and increased levels of nonenzymic antioxidants, which could explain the arrest of free radical-induced damage and antioxidant potential of *d*-limonene. These results are consistent with the studies of the previous researchers [17, 22].

Phase I detoxification enzymes, the cytochrome P450 s (CYP), comprise a multigene superfamily of microsomal heme-thiolate proteins that play critical roles in endogenous as well as xenobiotic metabolism and their detoxification [60, 61]. Elevated activities of cytochrome P450 enzymes both in the liver and extrahepatic tissues can result in extremely low bioavailabilities of a number of orally administered phytochemicals [62] and activation of the CYP 2E1 can lead to NAFLD [12]. In our present study, increased activities of the cytochrome P450 family enzymes was observed in the liver of HFD- and HFD + L-NAME-treated rats which was reversed on supplementation with dietary *d*-limonene. This strategic inhibition of P450 enzymes could be used to improve bioavailability of highly metabolized drugs. *d*-Limonene supplementation inhibited the activities of cytochrome b5 reductase and cytochrome b5 in HFD- and HFD + L-NAME-treated rats, which in turn can improve the bioavailability of the orally administered drugs. Phase II enzymes such as glutathione S-transferase and DT-diaphorase help in conjugating the xenobiotics to endogenous ligands like glutathione (GSH), glucuronic acid, acetic acid, or sulfuric acids, thus enhancing their solubility and excretion. Generally, inhibition of phase 1 enzymes concomitantly with induction of phase II enzymes is considered a logical strategy in chemoprevention [63]. HFD- and HFD + L-NAME-treated rats showed decreased activities of the phase II enzymes. Supplementation with *d*-limonene enhanced the phase II enzymes activities thereby helping the regulation of xenobiotic metabolism. These data are consistent with those of the previous studies by Kaji et al. [64]; Reicks and Crankshaw [22].

Histopathology of HFD- and HFD + L-NAME-treated rats liver showed features of NAFLD and/or nonalcoholic steatohepatitis (NASH) such as microvesicular steatosis, sinusoidal fibrosis, polymorphonuclear infiltrates, centrilobular inflammation, perisinusoidal, and pericellular deposition of collagen (fibrosis). In addition, the pancreas of HFD- and HFD + L-NAME-treated rats showed increased pancreatic β-cell mass and hyperplasia. These evidences demonstrate a systemic pathological change on long-term treatment with HFD and HFD + L-NAME, which are features of metabolic syndrome, associated with NAFLD. Dietary supplementation with *d*-limonene reversed the HFD- and HFD + L-NAME-induced changes.

Conclusion

In conclusion, the current study provides proof that HFD and HFD + L-NAME induce severe biochemical and histological changes and also aggravate the insulin resistance related hepatic injury. Furthermore, HFD and HFD + L-NAME can strengthen ‘‘the first hit’’ and promote ‘‘the second hit’’ hypothesis leading to NAFLD and/or NASH. Dietary *d*-limonene supplementation ameliorates the biochemical changes in the liver especially the hepatic lipid accumulation, liver function indicators, circulatory antioxidant, hepatic histology, and insulin resistance. These experimental evidences indicate that dietary *d*-limonene could be considered as a promising complementary treatment against development of the metabolic syndrome associated with NAFLD.

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