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d-limonene attenuates blood pressure and improves the lipid and antioxidant status in high fat diet and L-NAME treated rats

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Abstract
Obesity and hypertension are important risk factors for cardiovascular diseases. The present study investigates the effect of *d*-limonene on blood pressure, plasma lipids, circulatory lipid peroxidation byproducts and antioxidants status in 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; 80mg/L in drinking water) for 8 weeks and subsequently with 2% *d*-limonene for the final 4 weeks. HFD fed rats showed increased systolic blood pressure, increased concentrations of circulatory lipids and lipid peroxidation byproducts, increased activities of circulatory enzymic antioxidant and decreased concentrations of nonenzymic antioxidant, L-NAME supplementation further increased systolic blood pressure, lipid concentrations, lipid peroxidation byproducts and enzymic antioxidant and decreased nonenzymic antioxidant. Supplementation of 2% *d*-limonene to HFD and L-NAME treated rats decreased the blood pressure and reverses the changes in lipids, lipid peroxidation byproducts and antioxidant. *d*-limonene should be considered as a promising lipid lowering agent and antioxidant activities with blood pressure-lowering properties.

Keywords: *limonene; L-NAME; high fat diet; lipids; blood pressure;*

Introduction

Hypertension is an important public health problem across the world because of its high prevalence and concomitant risks of cardiovascular and renal diseases [1]. The increasing prevalence of hypertension has occurred in conjunction with a dramatic increase in the prevalence of overweight and obesity [2]. The National Health and Nutrition Examination Survey have shown a strong linear relationship between systolic/diastolic blood pressures and body mass index (BMI) [3]. The distribution of body fat is considered important in the genesis of the obesity-hypertension syndrome, with a predominantly central distribution being particularly prognostic [4]. Increased dietary fats increased blood pressure and abdominal obesity in humans and in animal models [5-8]. Obesity is closely associated with hypertension as well as with insulin resistance, elevated plasma concentrations of triglyceride, low HDL, high LDL cholesterol and increased lipid peroxidation byproducts [4, 8, 9], leading to atherosclerosis. Many mechanisms are probably involved in obesity-related hypertension, including oxidative stress, inflammation, endothelial dysfunction, sympathetic activation, insulin resistance, activated renin-angiotensin system (RAS), hyperleptinemia with leptin resistance and sleep apnea [10].

Dietary strategies such as increased consumption of fruits, vegetables, whole grains and fish are important in the control of cardiovascular disease including hypertension [11, 12]. *d*-limonene (l-methyl-4-(1-methylethenyl)-cyclohexane) is a monocyclic monoterpene principally found in the oils of orange, grapefruit and lemon [13]. Studies with *d*-limonene have shown antioxidant properties [14], chemopreventive or chemotherapeutic activity against many types of cancers [15], relief of the symptoms of heartburn or gastroesophageal reflux disorder due to neutralization of gastric acid and support for normal peristalsis [16], immuno–modulating properties [17] and regulation of the development of pulmonary hypertension [18]. In this study, we have evaluated the responses to d-limonene treatment on blood pressure, circulatory and tissue lipids, circulatory and tissue lipid peroxidation byproducts and circulatory and tissue antioxidant status in an obese hypertensive rat model of human disease.

Materials and Methods

Chemicals and drugs

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

Rats and diet

All the protocols were approved and conducted in accordance with the 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). Four week-old male albino Wistar rats (150-180 g) were obtained from the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. The animals were weighed and randomly assigned into different groups (n=12). They were housed in our Animal Research Facility under controlled temperature (25±2°C), humidity (45±5 %), and lighting (12:12 h light-dark cycle). The animals were acclimatized to their environment for one week, were fed standard rat chow (Lipton Lever Mumbai, India) and water *ad libitum* before the dietary intervention was initiated. At the start of the experiment, control groups were fed standard rat chow and water *ad libitum* and the other groups were fed HFD.

Preparation of diet

The following three types of diets were prepared: The standard diet comprised of protein 21.1%, fat 5.1%, carbohydrate 60.0%, fiber 3.9%, minerals 7.9% and vitamins 2.0%. HFD comprised of 42.2% beef tallow and 57.8% standard pellet diet. *d*-limonene (2% w/w) was mixed with standard pellet diet everyday. All measures were taken to ensure uniform mixing of the additives and powder in dry ingredients of the diet before kneading using a little water.

Table 1: Composition of the diet

Ingredients	Standard pellet diet	High fat diet
Protein	21.1%	12.2%
Fat	5.1%	2.8%
Beef tallow	-	42.2%
Carbohydrate	60.0%	34.7%
Fiber	3.9%	2.3%
Minerals	7.9%	4.6%
Vitamins	2.0%	1.2%

Experimental timeline

Group 1 (CON): Rats were fed standard pellet diet throughout the experimental period of 8 weeks. Group 2 (LIM): Rats were fed standard pellet diet for the first 4 weeks. % *d*-limonene added to the standard diet was fed for the last 4 weeks. Group 3 (HFD): Rats were fed HFD for 8 weeks of the experimental period. Group 4 (HFD+L-NAME): Rats were fed HFD and supplemented with L-NAME (80mg/L in drinking water) for 8 weeks. Group 5 (HFD+LIM): Rats were fed HFD as in group 3 for 8 weeks, and then 2% *d*-limonene diet supplemented for the last 4 weeks. Group 6 (HFD+L-NAME+LIM): Rats were fed HFD, administered L-NAME as in group 4 for 8 weeks, and then 2% *d*-limonene diet supplemented for the last 4 weeks.

Measurement of food and water intake

Metabolic evaluations were performed individually for every animal one week before the experiments by placing the rats in metabolic recording cages for 24 h. The food and water consumption was measured daily. The L-NAME dose was based on daily measurement of water consumption by the rats. The body weight of each animal was determined once a week.

Blood pressure measurement

To assess the development of hypertension, systolic blood pressure (SBP) was measured with the tail cuff method in conscious restrained rats at ambient temperature routinely with an IITC Electro-Sphygmomanometer

Table 2: Circulatory lipid levels (mg/dL) of the control and experimental rats

Groups	Total Cholesterol	Triglycerides	Free fatty acids	Phospholipids	HDL
CON	81.56 ± 3.34	65.89 ± 2.55	29.40 ± 1.18	92.19 ± 3.69	46.28 ± 1.69
LIM	80.55 ± 3.29	64.19 ± 2.59	28.56 ± 1.13	91.28 ± 3.70	47.06 ± 1.90
HFD	220.12 ± 8.63	150.22 ± 6.17	45.13 ± 1.81*	192.45 ± 7.81	21.03 ± 0.83
HFD+L-NAME	235.92 ± 9.19	162.20 ± 6.56	50.20 ± 2.04*	210.12 ± 8.57	17.01 ± 0.69
HFD+LIM	111.20 ± 4.15	70.87 ± 2.78	30.20 ± 1.24$^{\#}$	99.56 ± 3.74	43.93 ± 1.76
HFD+L-NAME+LIM	117.73 ± 4.52	73.93 ± 2.94	31.09 ± 1.28†	103.92 ± 4.11	42.10 ± 1.74

(IITC blood pressure system, Model No: 32, Inc./Life Science Instruments, USA). Briefly, telemetry transmitters were implanted 1 week before the experiment. Blood pressure of each rat was monitored every 10 min. Peaks and troughs in the blood pressure curve were detected. Each value recorded was derived from eight to ten consecutive measurements (within approximately 10 min), which were then averaged to give one value representative of each experimental condition.

Preparation of lysate

At the end of the experimental period, after an overnight fast, the rats were anaesthetized and sacrificed by cervical decapitation. Blood was collected in heparinized tubes and centrifuged at 160×g for 10 min at 4°C. The plasma was separated and stored at -20ºC until assay. After the separation of plasma, the buffy coat was removed and packed cells (RBCs) were washed thrice with cold physiological saline. To determine the activity of RBC antioxidant enzymes, RBC lysate was prepared by lysing a known volume of RBCs with hypotonic phosphate buffer, pH 7.4. Centrifuging at 3000×g for 10 min at 4 °C separated the hemolysate.

Extraction and determination of lipids

Lipids were extracted from plasma by the method of Folch et al [19]. The cholesterol content was estimated by the method of Zlatkis et al [20]. Triglycerides were estimated by the method of Foster & Dunn [21]. Free fatty acids were estimated by the method of Falholt et al [22]. Phospholipid content was estimated by the method of Zilversmit & Davis [23]. HDL was analysed in the supernatant obtained after precipitation of plasma with phosphotungstic acid/Mg^{2+}.

Enzymic antioxidant activities

Superoxide dismutase (SOD. EC. 1.15.1.1) was assayed by the method of Kakkar et al. [24]. Catalase (CAT. EC. 1.11.16) was assayed by Sinha [25]. Glutathione reductase (GR. EC.1.6.4.2) was determined by the method of Carlberg and Mannerik [26]. Glutathione peroxidase (GPx. EC.1.11.1.9) activity was assayed by the method of Folhe and Gunzler [27]. A known amount of the enzyme preparation was incubated with H_2O_2 and was determined using Ellman's method [28].

Statistical analysis

The data are expressed as mean ± SE. Comparisons of the determined variables among all the grouped data were conducted by one-way analysis of variance, and differences at 5% significance level were further assessed by Duncan's multiple range test for multiple comparisons. Data were analyzed using SPSS software package, version 11.01 for windows and line graphs have drawn using the sigma software 10.0 versions.

All the values are expressed as mean ± SE 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, $p<0.05$.

Results

General characteristics of rats

Figure 1 shows the average body weight of the rats in the different groups during the 8-week experimental period. The HFD and HFD+L-NAME (group 3 and 4) rats showed a higher final body weight gain than the control rats (group 1). Supplementation with 2% *d*-limonene diet decreased the weight gain in the HFD+LIM and HFD+L-NAME+LIM rats (group 5 and 6) as compared to the HFD alone and HFD+L-NAME alone treated rats.

Blood pressure

Systolic blood pressure was elevated in the HFD fed rats (group 3), which was further increased by addition of L-NAME in HFD+L-NAME treated rats (group 4) as compared to control rats (group 1). Supplementation of 2% *d*-limonene diet reduced blood pressure in HFD+LIM and HFD+L-NAME+LIM treated rats (groups 5 and 6) as compared to the HFD alone and HFD+L-NAME treated rats (groups 3 and 4) but not in *d*-limonene control rats (group 2) (Figure 2).

Plasma lipid

Table 2 shows the effect of *d*-limonene on plasma lipid (total cholesterol (TC), triglycerides (TG), free fatty acids (FFA), phospholipids (PL) and high density lipoprotein (HDL)) of control and experimental rats. The concentrations of plasma TC, TG, FFA, PL and HDL were increased in HFD and HFD+L-NAME treated rats (group 3 and 4) as compared to the control rats (group 1). Supplementation of 2% *d*-limonene diet reduced plasma lipids in HFD+LIM and HFD+L-NAME+LIM treated rats (groups 5 and 6) as compared to the HFD and HFD+L-NAME (groups 3 and 4) rats but not in *d*-limonene alone treated control rats (group 2).

Circulatory antioxidant

The activities of enzymic circulatory antioxidant superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) were increased in HFD and HFD+L-NAME (group 3 and 4) rats as compared to the control rats (group 1). Supplementation of 2% *d*-limonene diet restores the enzymic circulatory antioxidant in HFD+LIM and HFD+L-NAME+LIM (groups 5 and 6) rats as compared to the HFD and HFD+L-NAME (groups 3 and 4) rats but not in *d*-limonene control rats (group 2).

Discussion

The relationship between obesity related hypertension and cardiovascular disease has been extensively studied [30-32], but less information is available on obesity-induced hypertension and metabolic disturbances in the circulation and tissues. Recent research in our laboratory measured the lipid-lowering effects of piperine and black pepper in HFD induced hyperlipidemic rats [33, 34]. Our present study with combined HFD and endothelial nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) produced marked lipid deposition. Thus our data provides clear evidence that HFD feeding as well as the administration of L-NAME induces different degrees of obesity-related hypertension, differently affecting the blood pressure, circulatory lipids and antioxidant status. Supplementation of 2% *d*-limonene diet improved the blood pressure by regulating the circulatory lipids concentrations and antioxidant status in HFD and L-NAME-induced treated rats.

Previous studies on obese and/or hypertensive animals and humans showed a rise in blood pressure with weight gain, even over only a small period of few weeks, reinforcing the importance of obesity in causing hypertension. Marked reduction in body weight lowered blood pressure [29]. Our observations also correlate with these findings, as dietary *d*-limonene replacement reduced body weight gain, which in turn may be the cause for decreased blood pressure.

Administration of 2% *d*-limonene diet showed drastic reduction in the concentrations of cholesterol in HFD and HFD+L-NAME treated rats, which may reflect the inhibitory effect of *d*-limonene and its major metabolite perillyl alcohol on 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase) activity, thus decreasing farnesyl pyrophosphate synthesis [34] and the concentrations of plasma cholesterol [35]. On supplementation with 2%

Figure 1 Changes in the body weight

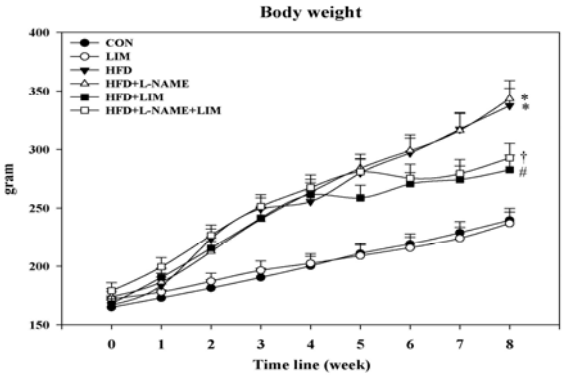


Figure 2- Changes in the systolic blood pressure

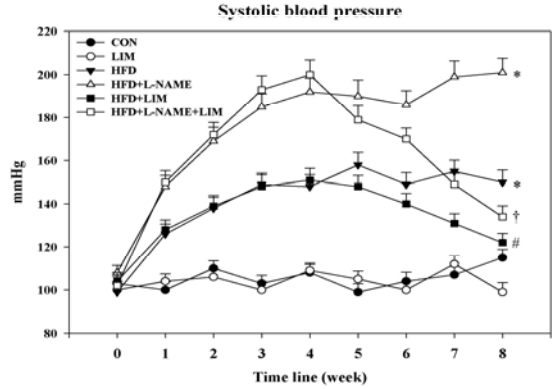
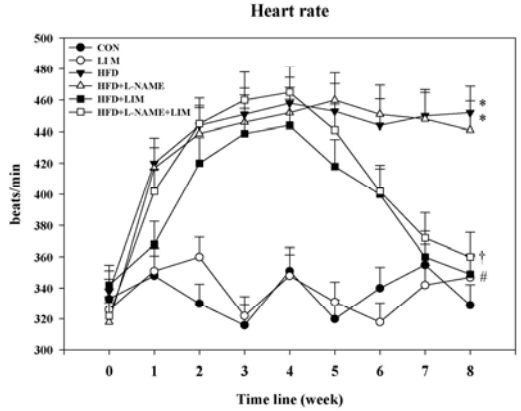


Figure 3- Changes in Heart Rate



Control (CON), *d*-limonene (LIM), high fat diet (HFD), high fat diet fed with L-NAME (HFD+L-NAME), high fat diet fed with *d*-limonene (HFD+LIM), high fat diet fed with L-NAME and *d*-limonene (HFD+L-NAME+LIM) groups over a period of 8 weeks.

Data are expressed as mean ± SEM. *Significantly different from control group at $p<0.05$. #Significantly different from HFD fed group at $p<0.05$. †Significantly different from HFD+L-NAME treated group at $p<0.05$.

Figure 6- Changes in the activity of erythrocyte lysate GPx

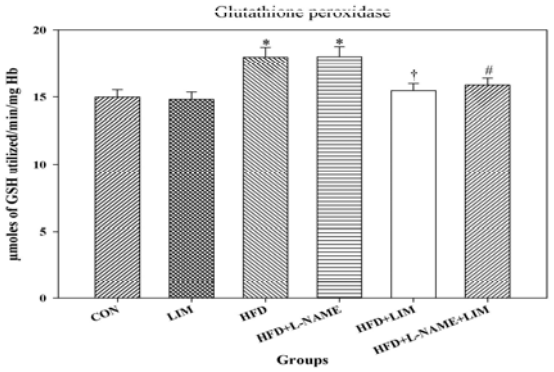


Figure 4- Changes in the activity of erythrocyte lysate SOD

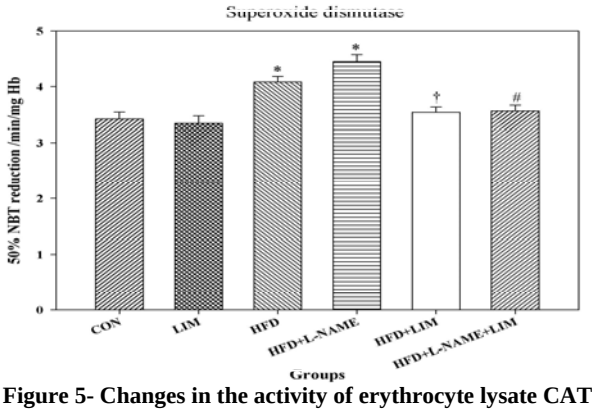


Figure 5- Changes in the activity of erythrocyte lysate CAT

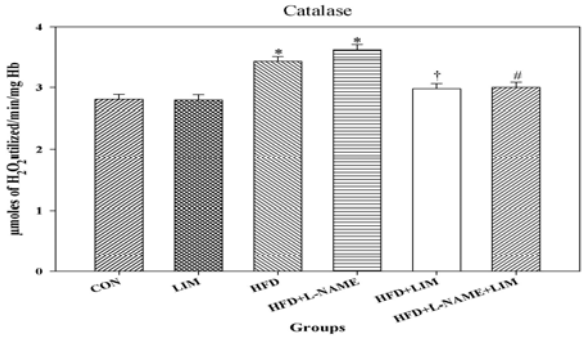
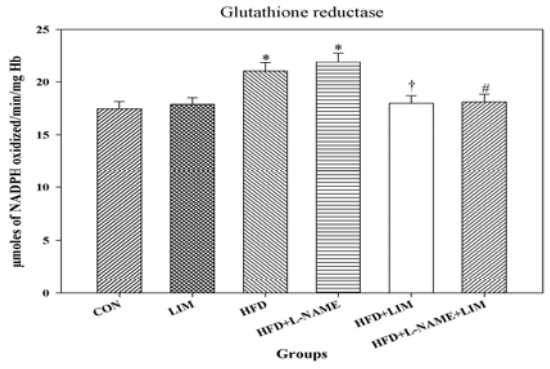


Figure 7- Changes in the activity of erythrocyte lysate GR



d-limonene diet to HFD and HFD+L-NAME treated rats showed reduced plasma triglyceride concentrations, possibly resulting from the stimulation of hepatic triglyceride lipase and lipoprotein lipase (LPL). Moreover, we observed on *d*-limonene supplementation to the HFD and HFD+L-NAME treated rats showed decreased increased HDL concentrations. These alterations in the lipoprotein concentrations could be due to the enhanced activities of LPL, and (lecithin:cholesterol acyl transferase) LCAT. In this context we have already reported increased plasma HDL concentrations following piperine supplementation to HFD-fed rats with concomitant increased activities of LPL and LCAT [33].

The cardiovascular system has a series of defense mechanism including antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and other endogenous free radical scavengers, vitamin E and C and reduced glutathione (GSH) to protect the cell against reactive oxygen species such as singlet-oxygen, superoxide anion, hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and lipid peroxidation byproducts (TBARS, CD and LOOH) [36].

HFD and HFD+L-NAME treated rats showed increased SOD activity which were restored back to near normal levels by supplementation with *d*-limonene diet in the circulation. The increased SOD activity in the erythrocyte lysate of HFD and HFD+L-NAME treated rats may be a compensatory response to nitric oxide deficiency [37]. CAT and glutathione dependent enzymes such as GPx and GR activities in the erythrocyte lysate of the HFD and HFD+L-NAME treated rats were slightly increased as compared to control. But, CAT and glutathione dependent enzymes activity was not more affected in the circulation of HFD and HFD+L-NAME treated rats. Although the reason for these increases is not clear, it may be related to the reduced $O_2^{\cdot -}$ inactivation caused by inhibition of nitric oxide synthesis, as indicated by the restoration of normal levels after supplementation with *d*-limonene diet. Our observations also correlate with the previous findings in hypertensive rats [37-39]

Administration of various lipid-lowering agents and antioxidants including vitamin E [40], tempol [37], melatonin [41], N–acetylcysteine (NAC) [9] attenuated the development of hypertension and protected the rats from the high blood pressure. Thus our present findings with *d*-limonene diet in HFD and HFD+L-NAME treated rat indicate that *d*-limonene attenuates blood pressure by the lipid lowering activity and antioxidant mimetic mechanisms. Moreover, previous findings with *d*-limonene showed reduced sympathetic responsiveness [42] regulating the development of pulmonary hypertension [42] which are in agreement with blood pressure related to cardiovascular benefits.

In conclusion, these extensive changes in both blood pressure and metabolic disturbances in the circulation suggest that an integrated therapeutic approach with *d*-limonene. This compound would be predicted to produce substantially change the circulatory lipids and antioxidant as well as decreased blood pressure. Thus, *d*-limonene by virtue of its lipid-lowering and antioxidant mimetic activities could reduce the pathological changes and restore the physiological functions and reduce the blood pressure.

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