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Research Article

RESTORATION OF HEART RATE, ANTIOXIDANT STATUS AND LIPID LEVEL IN L-NAME INDUCED HYPERTENSIVE RATS BY TROXERUTIN

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Abstract

Hypertension is associated with dyslipidemia, which is a significant risk factor for cardiovascular complications. This study was undertaken to investigate the effects of troxerutin (TX) on heart rate, antioxidants and tissue lipid profile in N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME)-induced hypertensive rats. Hypertension was induced in adult male albino rats of Wistar strain, weighing 180–220 g, by oral administration of L-NAME (40 mg/kg) in drinking water for 4 weeks. Rats were treated with different doses of TX (25, 50 and 100 mg/kg) for 4 weeks. L-NAME treated rats showed significant increase in heart rate and water intake. The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were decreased in erythrocytes of L-NAME rats. L-NAME rats showed significant increase in the levels of lipids such as total cholesterol (TC), triglycerides (TG), free fatty acids (FFA) and significant decrease in the level of phospholipids (PL) in heart and aorta. Above pathological changes were considerably restored with the treatment of TX. Among the three doses (25, 50, and 100 mg/kg) of TX, 100 mg/kg dosage exerts optimum protection. These results suggest that TX acts as an antihypertensive and antihyperlipidemic agent against L-NAME induced hypertension.

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1. Introduction

Cardiovascular disease (CVD) has been recognized as the most common leading cause of mortality in developed countries. High blood pressure or hypertension is the leading preventable risk factor for cardiovascular diseases and is estimated to account for about 54% of deaths from stroke and 47% of deaths from coronary heart disease in adults worldwide (Lawes *et al*., 2008). Hypertension, frequently occurs in conjunction with metabolic disturbances and in particular with dyslipidemia (Chapman and Sposito, 2008). Dyslipidemia is a broad term which implies imbalance between elevated circulating levels of cholesterol and it occurs concomitantly in over one-third of patients with hypertension (Wong *et al*., 2006).

Chronic inhibition of nitric oxide (NO) produces volume-dependent elevation of blood pressure and its physiological and pathological characteristics resemble essential hypertension (Attia *et al*., 2001). The blockade of nitric oxide

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synthase (NOS) by L-NAME seems to be involved in lipid metabolism alterations: increases serum cholesterol levels in rats (Khedara *et al*., 1996) and impairs endothelial function in hypercholesterolemic rabbits (Cayatte *et al*., 1994), in which it also causes atherosclerosis (Naruse *et al*., 1994).

Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense systems so that the latter become overwhelmed (Becker, 2004; Juranek and Bezek, 2005). The generated ROS induce lipid peroxidation, a type of oxidative deterioration in polyunsaturated fatty acids (PUFAs), which has been linked with altered membrane structure and enzyme inactivation (Kumar *et al*., 2011). Bioavailability of NO can be maintained by inhibition of oxidative stress, and therefore the agents with antioxidant properties inactivating free radicals increase NO bioavailability and can improve regulation of vascular tone (Kumar *et al*., 2010).

Lifestyle and dietary habits may affect blood pressure and cardiovascular risk factors (Zhou *et al*., 2006). Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them (Argolo *et al*., 2004). In recent years, much attention has been focused on the protective properties of exogenous antioxidants in biological systems, and on the mechanisms of their action. The protective effects of plant ingestion can be due to the presence of phenolic compounds and flavonoids. Flavonoids are a group of plant polyphenols that are generally found in vegetables, fruits, herbs, tea, and wine as secondary metabolites and have received much attention due to their anti-inflammatory and antioxidant activities (Beecher, 2003). Troxerutin, a trihydroxyethylated derivative of the natural bioflavonoid rutin is present in tea, coffee, cereal grains and a variety of fruits and vegetables. Troxerutin possesses a variety of biological activities, such as vasoprotective, anti-oxidative, anti-inflammatory property (Fan *et al*., 2009). Therefore, the aim of the present study was to investigate the effect of TX on heart rate, enzymatic antioxidants and lipid level in L-NAME induced hypertensive rats.

2. Materials and Methods

Animals and chemicals

Healthy male albino Wistar rats (180 - 220 g), were obtained from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, India. They were housed (3 rats/cage) in polypropylene cages (47 × 34 × 20 cm) lined with husk, renewed every 24 h and maintained in an air-conditioned room (25 ± 3 °C) with a12 h light/12 h dark cycle. Feed and water were provided *ad libitum* to all the animals. The whole experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India and approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg No. 160/1999/CPCSEA, Proposal number: 925), Annamalai University, Annamalai Nagar.

L-NAME and troxerutin were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals used in this study were of analytical grade obtained from Merck and Hi-media, India.

L-NAME induced hypertensive animal model and troxerutin treatment

Animals were given L-NAME in drinking water at a dosage of 40 mg/kg bw for 4 weeks. Troxerutin was dissolved in water (vehicle) and administered to rats orally everyday using an intragastric tube for a period of 4 weeks.

Experimental protocol

Different doses of troxerutin (25, 50 and 100 mg/kg/body weight [bw]) were assessed to find out the antihypertensive effect in L-NAME-induced hypertension.

Group I : Control + vehicle
Group II : Control + troxerutin (100 mg/kg bw)
Group III : L-NAME control (40 mg/kg bw)
Group IV : L-NAME + troxerutin (25 mg/kg bw)
Group V : L-NAME + troxerutin (50 mg/kg bw)
Group VI : L-NAME + troxerutin (100 mg/kg bw)

After the completion of experimental period, the rats were anaesthetized and sacrificed by cervical dislocation. Blood samples were collected into heparinized tubes. After the separation of plasma, the buffy coat, enriched in white cells, was removed and the remaining erythrocyte was washed three times with physiological saline. A known volume of erythrocyte was lysed with hypotonic phosphate buffer at pH 7.4. The hemolysate was separated by centrifugation at 350 × g for 10 min and the supernatant was used for the estimation of enzymatic antioxidants.

Blood pressure measurement

Before commencement of the experiment, animals were trained with the instrument for measuring blood pressure. In all groups of animals, heart rate was measured every week during the entire period of the study noninvasively using a tail cuff method (IITC, model 31, USA) according to standard procedures. Values reported are the average of lowest three readings. All the recordings and data analyses were done using a computerized data acquisition system and software.

Determination of enzymatic antioxidants

Superoxide dismutase (SOD) activity was assayed in the erythrocyte by the method of Kakkar *et al*. (1984). Erythrocytes (0.5 ml) were diluted to 1.0 ml with distilled water followed by addition of 2.5 ml of ethanol and 1.5 ml of chloroform (all reagents chilled) was added. This mixture was shaken and then centrifuged. The enzyme activity in the supernatant was determined. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate, and 0.3 ml of nitroblue tetrazolium and appropriately diluted enzyme preparation in a total volume of 3 ml. The reaction was started by the addition of 0.2 ml of nicotinamide adenine dinucleotide (NADH). After incubation at 30 °C for 90 s, the reaction was arrested by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The mixture was allowed to stand for 10 min; centrifuged and n-butanol layer was separated. The color density of the chromogen in n-butanol was measured at 510 nm against butanol blank.

The activity of catalase (CAT) in the erythrocyte was assayed by the method of Sinha (1972). To 0.9 ml of phosphate buffer, 0.1 ml of erythrocyte and 0.4 ml of H_2O_2 were added. The reaction was arrested after 60 s by adding 2 ml of dichromate–acetic acid mixture. The tubes were kept in a boiling water bath for 10 min and the color developed was read at 620 nm.

The activity of glutathione peroxidase (GPx) in the erythrocyte was measured by the method of Rotruck *et al*. (1973). To 0.2 ml of Tris buffer, 0.2 ml of ethylene diamine tetraacetic acid (EDTA), 0.1 ml of sodium azide, and 0.5 ml of erythrocyte were added. To the mixture, 0.2 ml of glutathione followed by 0.1 ml of H_2O_2 was added. The contents were mixed well and incubated at 37 °C for 10 min along with a tube containing all reagents except the sample. After 10 min, the reaction was arrested by the addition of 0.5 ml of 10% TCA. The tubes were centrifuged and the supernatant was used for the estimation of glutathione.

Extraction of lipids

Total lipids were extracted from tissue according to the method of Folch et al. (1957) using chloroform: methanol mixture (2:1, v/v). Plasma was mixed with cold chloroform–methanol (2:1, v/v) and the contents were extracted after 24 hours. The extraction was repeated four times. The combined filtrate was washed with 0.7% of potassium chloride (0.1 N)

and the aqueous layer was discarded. The organic layer was made up to a known volume with chloroform and used for the analysis of lipids.

Estimation of total cholesterol

The levels of total cholesterol (TC) were estimated by the method of Zlatkis *et al*. (1953). Lipid extract of 0.5 ml was evaporated to dryness. To this, 5.0 ml of ferric chloride-acetic acid reagent was added. The tubes were mixed well and 3.0 ml of concentrated sulphuric acid (H_2SO_4) was added. A series of standards containing cholesterol in the range 3–15µg were made up to 5.0 ml with the reagent and a blank containing 5.0 ml of the reagent were prepared. The absorbance was read after 20 minutes at 560 nm.

Estimation of triglycerides

The content of triglycerides (TG) was estimated by the method of Fossati and Prencipe (1982). Lipid extract of 0.5 ml was evaporated to dryness. To this, 0.1 ml of methanol was added followed by 4.0 ml of isopropanol. About 0.4 g of alumina was added to all the tubes and shaken well for 15 minutes. It was centrifuged and then accurately 2.0 ml of the supernatant was transferred to appropriately labeled tubes. The tubes were placed in a water bath at 65°C for 60 minutes for saponification after adding 0.6 ml of the saponification reagent followed by 0.1 ml of sodium metaperiodate and 0.5 ml of acetyl acetone reagent. After mixing, the tubes were kept in a water bath at 65°C for an hour. A series of standards of concentration 8–40µg triolein were treated similarly along with a blank containing only the reagents. All the tubes were cooled and read at 405 nm.

Estimation of free fatty acids

Free fatty acid (FFA) level was estimated by the method of Falholt *et al*. (1973). An aliquot (0.5 ml) of the lipid extract was evaporated to dryness. To this, 1.0 ml of phosphate buffer, 6.0 ml of extraction solvent, and 2.5 ml of copper (Cu-TEA) reagent were added. All the tubes were shaken vigorously for 90 seconds and were kept aside for 15 minutes. Then the tubes were centrifuged and 3.0 ml of the upper layer was transferred to another tube containing 0.5 ml of diphenylcarbazide solution and mixed carefully. The absorbance was read at 550 nm after 15 minutes. A reagent blank containing (1.0 ml) of phosphate buffer was processed as blank.

Estimation of phospholipids

Phospholipid (PL) levels were estimated by the method of Zilversmit and Davis (1950). An aliquot of 0.5 ml of the lipid extract was pipetted out into a Kjeldahl flask and evaporated to dryness. To the extract/0.2 ml of plasma, 1 ml of 5 NH_2SO_4 was added and digested in a digestion rack till the appearance of light brown color. Two to three drops of concentrated nitric acid was added and the digestion continued till it became colorless. The Kjeldahl flask was cooled and 1.0 ml of distilled water was added and heated in a boiling water bath for about 5 minutes. Then, 1.0 ml of 2.5% ammonium molybdate and 0.1 ml of 1-amino-2-napthol-4-sulfonic acid were added. The volume was then made upto 5.0 ml with distilled water and the absorbance was measured at 660 nm within 10 minutes.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using statistical package for the social science (SPSS) software version 20.0. Values were expressed as mean ± S.D. for six rats in each group. Values were considered significant when $P<0.05$.

3. Results

Heart rate

Fig. 1 shows the effect of TX at three different doses (25, 50 and 100 mg/kg) on heart rate in L-NAME induced hypertensive rats. The L-NAME rats showed significantly increased heart rate while treatment with TX significantly reduced the heart rate.

Table 1. Effect of TX on SOD, CAT and GPx in erythrocytes of control and L-NAME induced hypertensive rats.

Parameter		Control	Control+TX	L-NAME	L-NAME+TX
SOD	Erythrocytes (U*/mg Hb)	6.54 ± 0.43^{a}	6.78 ± 0.36^{a}	3.11 ± 0.22^{b}	5.27 ± 0.34^{c}
CAT	Erythrocytes ($U^{\#}$/mg Hb)	170.21 ± 8.15^{a}	171.45 ± 7.23^{a}	108.2 ± 5.34^{b}	159.62 ± 6.31^{c}
GPx	Erythrocytes (U^{Δ}/mg Hb)	13.23 ± 0.71^{a}	13.74 ± 0.64^{a}	8.43 ± 0.52^{b}	11.24 ± 0.63^{c}

U^{*}, enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard condition. $U^{\#}$, µmol of H_2O_2 consumed/minute. U^{Δ}, µg of GSH utilized/minute. Values are mean ± S.D. for six rats in each group. Values not sharing common superscript are significant with each other at $P<0.05$ (Duncan's multiple range test).

Table 2. Effect of TX on lipid in heart of control and L-NAME induced hypertensive rats.

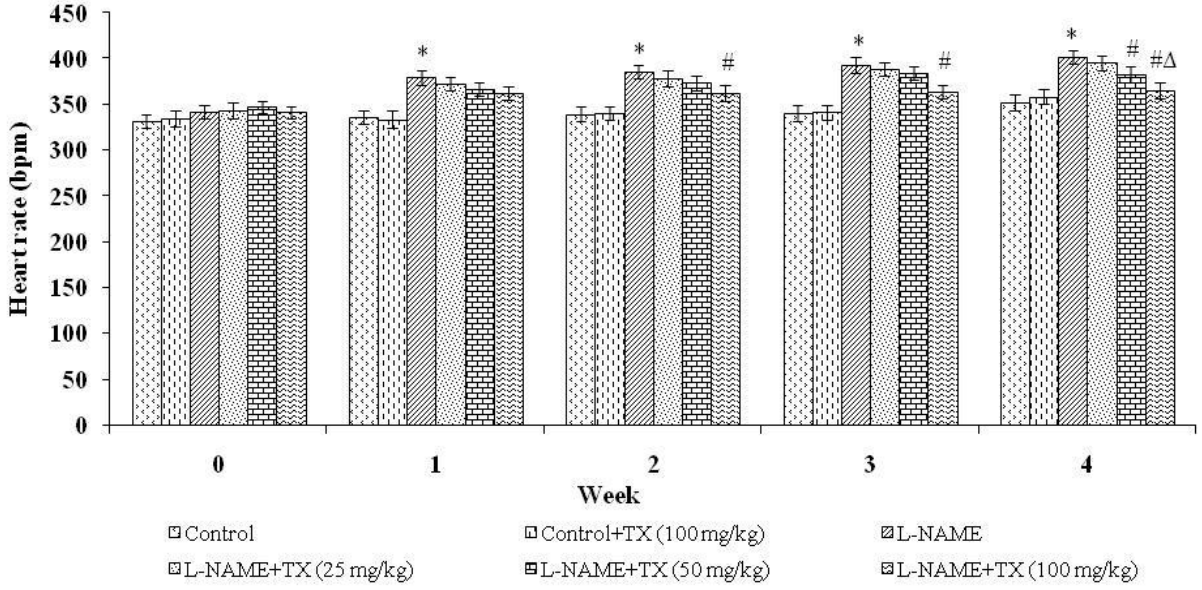
Groups	Heart (mg/g wet tissue)			
	TC	TG	FFA	PL
Control	2.56 ± 0.17^{a}	3.87 ± 0.22^{a}	4.39 ± 0.24^{a}	13.35 ± 0.73^{a}
Control + TX (100 mg/kg)	2.41 ± 0.18^{a}	3.61 ± 0.19^{a}	4.21 ± 0.19^{a}	13.23 ± 0.58^{a}
L-NAME	4.23 ± 0.26^{b}	5.37 ± 0.36^{b}	5.86 ± 0.36^{b}	9.45 ± 0.52^{b}
L-NAME + TX (100 mg/kg)	3.22 ± 0.23^{c}	4.35 ± 0.23^{c}	4.91 ± 0.24^{c}	11.61 ± 0.73^{c}

Values are mean ± S.D. for six rats in each group. Values not sharing common superscript are significant with each other at $P<0.05$ (Duncan's multiple range test).

Table 3. Effect of TX on lipid in aorta of control and L-NAME induced hypertensive rats.

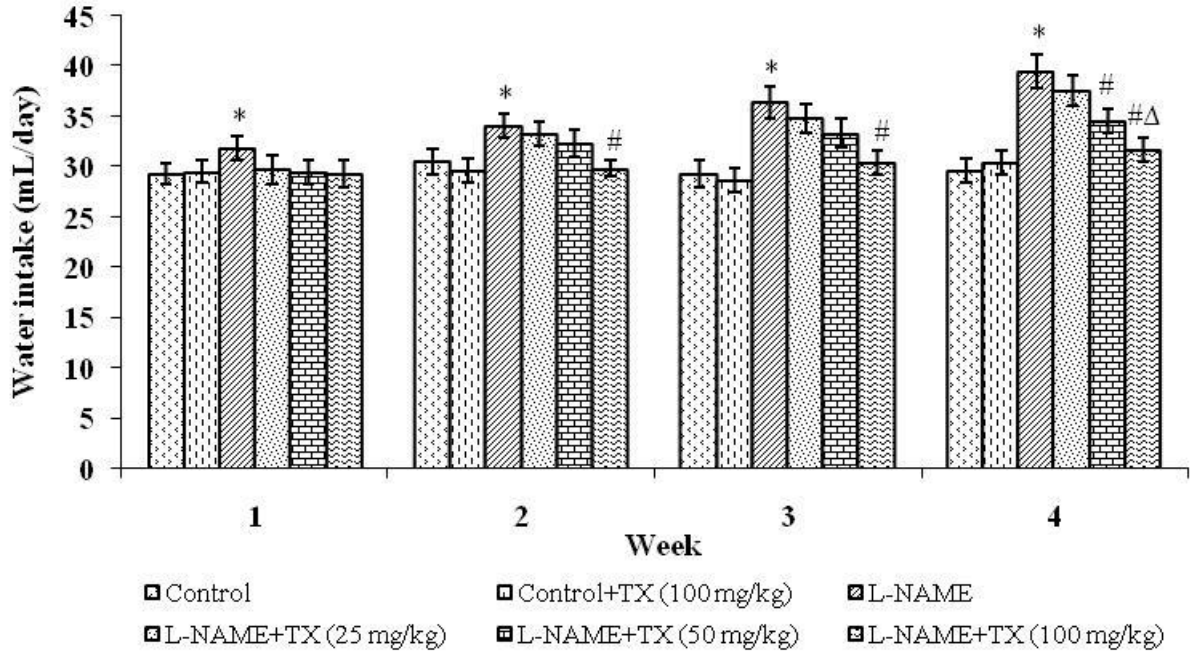
Groups	Aorta (mg/g wet tissue)			
	TC	TG	FFA	PL
Control	3.29 ± 0.22^{a}	3.1 ± 0.18^{a}	3.49 ± 0.22^{a}	5.13 ± 0.32^{a}
Control + TX (100 mg/kg)	3.17 ± 0.19^{a}	3.03 ± 0.23^{a}	3.36 ± 0.18^{a}	5.21 ± 0.27^{a}
L-NAME	5.67 ± 0.31^{b}	5.35 ± 0.31^{b}	5.27 ± 0.31^{b}	3.72 ± 0.21^{b}
L-NAME + TX (100 mg/kg)	4.34 ± 0.21^{c}	3.91 ± 0.19^{c}	4.12 ± 0.24^{c}	4.08 ± 0.28^{c}

Values are mean ± S.D. for six rats in each group. Values not sharing common superscript are significant with each other at $P<0.05$ (Duncan's multiple range test).



Values are mean ± S.D. for six rats in each group. Data were analyzed by one-way ANOVA followed by Duncan's multiple range test (DMRT). * P<0.05 compared with the control. # P<0.05 compared with the L-NAME. Δ P<0.05 compared with the TX (50 mg/kg) group.

Fig. 1. Effect of TX on heart rate in control and L-NAME induced hypertensive rats.



Values are mean ± S.D. for six rats in each group. Data were analyzed by one-way ANOVA followed by Duncan's multiple range test (DMRT). * P<0.05 compared with the control. # P<0.05 compared with the L-NAME. Δ P<0.05 compared with the TX (50 mg/kg) group.

Fig. 2. Effect of TX on water intake in control and L-NAME induced hypertensive rats.

Water intake

Fig. 2 shows the effect of TX at three different doses (25, 50 and 100 mg/kg) on water intake in L-NAME induced hypertensive rats. The L-NAME rats showed significantly increased water intake while treatment with TX significantly decreased the water intake. The 100 mg/kg dose showed better effect in reducing heart rate and water intake than other two doses (25 and 50 mg/kg), so we have chosen 100 mg/kg dosage for further evaluation.

Enzymatic antioxidants

Table 1 shows the effect of TX on the activities of SOD, CAT and GPx in the erythrocytes of L-NAME induced hypertensive rats. The activities of SOD, CAT and GPx decreased significantly in L-NAME rats and the administration of TX significantly increased these enzymatic antioxidants.

Tissue lipid level

Tables 2 and 3 portray the levels of lipids (TC, TG, FFA, and PL) in heart and aorta of control and L-NAME hypertensive rats. The increase in the levels of TC, TG and FFA and decrease in the level of PL were observed in L-NAME rats as compared to control rats. Treatment of TX significantly decreased the levels of TC, TG and FFA and increased the level of PL in L-NAME hypertensive rats.

4. Discussion

NO, essential for the proper functioning of the cardiovascular system, is derived from L-arginine by NO synthase in endothelial cells (de Champlain *et al*., 2004). NO plays a role in attenuating cardiac remodeling and apoptosis via suppression of oxidative stress-mediated signaling pathways (Smith *et al*., 2005). NO synthase inhibition produces hypertension, endothelial damage, cardiac hypertrophy, inflammation, atherosclerosis, ventricular contractile dysfunction, and fibrosis (Moncada *et al*., 1991). NO synthesis by the chronic administration of L-NAME, an inhibitor of NO synthesis, produces systemic arterial hypertension, vascular structural change and renal dysfunction (Siragy *et al*., 1992). In this study, L-NAME-treated rats showed significantly increased heart rate and water intake. Previous studies reported that the phenolic compounds reduce blood pressure and prevent target organ damage in hypertensive rats (Jalili *et al*., 2006). Treatment with TX significantly reduced the heart rate and water intake due to its antioxidant property (Fan *et al*., 2009).

Oxidative stress, characterized by increased bioavailability of ROS such as superoxide anion, hydrogen peroxide and lipid peroxides, plays an important role in the development and progression of cardiovascular dysfunction associated with hypertensive disease (Touyz, 2003). Oxidative stress occurs when there is an imbalance between the generation of ROS and the antioxidant defense systems so that the latter become overwhelmed (Becker, 2004; Juranek and Bezek, 2005). The loss of the balance between oxidation and antioxidation may lead to promote the generation of $OH^{\bullet}$ which is a powerful oxidant for many compounds. Crucial components of the antioxidant defense system in the body are cellular antioxidant enzymes, which play a primary role in the maintenance of a balanced redox status.

Intracellular defense against active oxygen species is performed by enzymatic antioxidants (SOD, CAT, and GPx) and non-enzymatic antioxidant reduced glutathione (Romero and Roche, 1996). SOD, CAT, and GPx balance together to eliminate ROS, and small deviations in physiological concentrations may have a dramatic effect on the resistance of cellular lipids, proteins, and DNA to oxidative damage (Mates and Sanchez-Jimenez, 1999).

SOD plays an important role in scavenging superoxide anion, which is the initial free radical, among the oxygen radicals. Catalase prevents oxidative hazard by catalyzing the formation of water and oxygen from hydrogen peroxide (Rajeshkumar and Kuttan, 2003). GPx offers protection to the cellular and subcellular

membranes from the peroxidative damage by eliminating hydrogen peroxide by utilizing reduced glutathione and H_2O_2 as substrates to yield H_2O and oxidized glutathione and its declined activity may be due to the reduced availability of GSH (Li *et al*., 2012). L-NAME induced hypertensive rats showed significantly depleted levels of antioxidant enzymes SOD, CAT, and GPx in erythrocytes. Administration of TX, improved the activities of SOD, CAT, and GPx in L-NAME-induced hypertensive rats. The increased activities of these enzymic antioxidants in TX treated rats might be due to the antioxidant ability of TX (Fan *et al*., 2009), which could exert a beneficial action against pathophysiological alterations caused by superoxide anion and hydroxyl radicals

Abnormalities in plasma lipids and lipoprotein metabolism play a central role in the pathogenesis of hypertension. The presence of high blood pressure and hyperlipidemia is so common in hypertension that many have argued that the high blood pressure itself may play a role in altering lipid metabolism, resulting in abnormalities (Friedwald *et al*., 1972). Large epidemiologic studies have demonstrated that subjects with hypertension have a marked increase in the prevalence of hypercholesterolemia (Yang *et al*., 2011). The blockade of NO synthase by L-NAME seems to be involved in lipid metabolism alterations: increases serum/plasma cholesterol levels in rats (Khedara *et al*., 1996). In our study, we observed increased levels of TC in tissues such as heart and aorta of hypertensive rats. High levels of circulating cholesterol and its accumulation in tissues are well associated with cardiovascular damage (Salter and White, 1996). TX supplementation decreased the levels of TC in hypertensive rats, which may be due to the suppression of endogenous cholesterol biosynthesis by inhibiting the activity of HMG-CoA reductase.

Hypertriglyceridemia is independent risk factor that can accelerate the development of coronary artery disease and progression of atherosclerotic lesions (McKenney, 2001). In this study, we observed a higher concentration of TG in L-NAME hypertensive rats. This may be due to increased secretion of VLDL by the liver, disturbed catabolism of VLDL and decreased removal of triglyceride due to diminished lipoprotein lipase activity. TX supplementation lowered the levels of TG in tissues of hypertensive rats. This beneficial action might be due to the antioxidant property of TX.

PLs are vital components of biomembrane. These PLs and FFA are important for the maintenance of cellular integrity, microviscosity, and survival (Iacono *et al*., 1975). The major targets of damaging free radicals are the cellular and membrane phospholipids. The oxidative tissue damage can release the membrane lipids such as FFA and PL into blood (Ohara *et al*., 1993). Elevated levels of FFA and PL in heart and aorta of L-NAME hypertensive rats may be due to membrane damage caused by increased lipid peroxidation. TX treatment protects the tissues from lipid peroxidation by scavenging free radicals and decreased the levels of FFA and PL in hypertensive rats.

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