

Comparative study of hydrochlorothiazide and indapamide on the anti-atherogenic potential of losartan in cholesterol fed rat

Md. Zakirul Islam and Md. Sayedur Rahman

*Department of Pharmacology, Bangabandhu Sheikh Mujib Medical University,
Shahbag, Dhaka 1000, Bangladesh. e-mail: srkhasru@bdcom.com*

Abstract

The study was conducted to evaluate the anti-atherogenic potential of losartan and to assess the effects of hydrochlorothiazide and indapamide on losartan activity in rat. Cholesterol diet (0.5%) for 12 weeks led to significant hyperlipidemia, increased body weight and oxidative stress in erythrocyte. While, losartan, hydrochlorothiazide and indapamide treatment continued for next 12 weeks, losartan showed anti-atherogenic activity reflected by hypolipidemic effect and antioxidant effect in erythrocyte. This activity was abolished by addition of hydrochlorothiazide with losartan but remained unaltered by addition of indapamide with losartan. Atherosclerotic change and oxidative stress were not found in rat aorta, which may be due to short duration and low dose of cholesterol feeding. Hydrochlorothiazide treatment was associated with hypokalemia, which was not present in losartan or indapamide treatment. This study suggests that indapamide might be co-administered with losartan conserving the essential anti-atherogenic potential of losartan.

Introduction

Hyperlipidemia is a well-established major risk factor for the development and progression of atherosclerosis and its associated conditions as ischemic heart disease & cerebrovascular disease¹. Atherogenesis is initiated as an inflammatory response to injury, usually with cholesterol². The renin-angiotensin system is known to be involved in atherogenesis through promotion of endothelial dysfunction and oxidative modification of LDL cholesterol by angiotensin II³. Angiotensin II Type 1 receptor blockers (ARBs) like losartan may have an important role contributing to attenuation of atherogenic process independent of lowering blood pressure⁴. Furthermore, losartan was well tolerated at high doses required for its additional pharmacological benefits⁵.

The addition of either a thiazide diuretic as hydrochlorothiazide or the thiazide-like indoline indapamide to an ARB results in further reduction in blood pressure⁶. Hydrochlorothiazide is known to raise the blood concentration of total cholesterol, low-density lipoproteins and triglycerides both in high and low doses⁷. A study describes that combination therapy of hydrochlorothiazide with ACE inhibitor can abolish the beneficial effect of ACE inhibitor with regard to endothelial function and atherosclerosis⁸.

It is claimed that standard doses of indapamide are metabolically neutral, specifically with regard to lipid profile⁹. Indapamide reduces the progression and development of atherosclerotic lesions⁷. Therefore, the present study aims to find out a thiazide diuretic that can be added to ARBs in hypertension treatment conserving the beneficial anti-atherogenic effect of ARBs.

Materials and Methods

Chemicals and reagents: Cholesterol powder $\geq 99\%$ was procured from E. Merck, Germany. Cholesterol, triglycerides and HDL cholesterol assay kit were procured from Randox Laboratories, UK. Trichloroacetic acid, disodium hydrogen phosphate, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH) and thiobarbituric acid were procured from Sigma Aldrich, Germany. All other chemicals used were of analytical grade. Losartan potassium, hydrochlorothiazide and indapamide were obtained from Square Pharmaceuticals Limited (Bangladesh).

Animals: The study was carried out on Long Evans Norwegian strains of adult healthy rats of both sexes. The rats were 2-3 months aged and weighing between 150-160 g. Rats were housed in standard stainless cages. They were allowed living at room temperature in a clean, well ventilated rodent room

where a 12/12 hours light/dark cycle was maintained. They were fed on standard pellets of rat food and allowed to drink *ad libitum*.

Experimental design: The study was conducted in two parts. In part I, 90 rats were divided into 3 groups and fed standard rat diet in group A consisted of 10 rats, soybean oil in Group B consisted of 10 rats and 0.5% cholesterol-enriched diet in Group C consisted of 70 rats, for 12 weeks. 10 rats of Group A, Group B and Group C were sacrificed under chloroform anesthesia at the end of 12 weeks and the remaining 60 rats of Group C were continued to part II of the study. In part II, 60 rats were divided into 6 groups each consisted of 10 rats and all groups were fed 0.1% cholesterol-enriched diet with losartan (25 mg/kg/day) in Group II, hydrochlorothiazide (12.5 mg/kg/day) in Group III, indapamide (1.25 mg/kg/day) in Group IV, losartan (25 mg/kg/day) plus hydrochlorothiazide (12.5 mg/kg/day) in Group V and losartan (25 mg/kg/day) plus indapamide (1.25 mg/kg/day) in Group VI, for next 12 weeks and at the end they were sacrificed under chloroform anesthesia. Rats were kept fasting overnight the day before sacrifice.

Body weight measurement: Properly calibrated analogue weight measurement machine was used to measure body weight of rats. In part I of the study body weight of 30 rats was measured at day 0 and after 12 weeks. In part II of the study body weight of 60 rats was measured at day 0, after 12 weeks and after 24 weeks.

Sample collection: Blood samples were collected by cutting throat with sharp blade and were treated with and without heparin. The aorta was dissected out and divided into two parts. The upper part of the aorta was packed in separate polyethylene packets with accurately labeled and preserved at -20°C until the period of analysis. The lower part of the aorta was preserved in separate accurately labeled container with 10% formal saline for histopathological study.

Preparation of erythrocytes: Heparinized blood samples were centrifuged at 4,000 rpm for 5 min and the plasma and buffy coat was discarded by removing from the top. The packed RBCs were washed three times with five volumes buffered saline (0.9% saline in 10 mM phosphate buffer, pH 7.4) by centrifugation at 4,000 rpm for 5 min. The packed cells were then suspended in an equal volume of the distilled deionized water to lyse RBCs.

Preparation of serum: Blood samples without heparin were centrifuged at 4,000 rpm for 5 min and the serum was collected by pasture pipette into labeled test tube.

Preparation of aortic tissue homogenate: Aortic tissue was cleaned properly in a petri dish containing Tyrode's solution at 0-4°C (resting in ice bath) and carefully weighed of 100 mg. Clean tissue was then chopped into small pieces and homogenated properly by a hand tissue homogenizer with 2 mL Tyrode's solution and keeping the homogenizer in the ice bath.

Estimation of serum cholesterol: Serum cholesterol was assayed by enzymatic (CHOD-PAP) method¹⁰. One mL of the cholesterol reagent was added to each of the test tubes containing 10 µL of standard solution, 10 µL of distilled water and 10 µL of serum and the mixtures were mixed properly and incubated at 37°C for 5 min. The absorbance was measured at 500 nm against the reagent blank using UV-Vis spectrophotometer.

Estimation of serum triglyceride: Serum triglyceride was assayed by enzymatic (GPO-PAP) method¹¹. One mL of the triglyceride reagent was added to each of the test tubes containing 10 µL of standard solution, 10 µL of distilled water and 10 µL of serum and the mixtures were mixed properly and incubated at 37°C for 5 min. The absorbance was measured at 500 nm against the reagent blank using spectrophotometer.

Estimation of serum HDL cholesterol: Serum HDL cholesterol was assayed by enzymatic (CHOD-PAP) method after precipitation of other lipid component of the sample¹². One mL of the precipitant reagent was added to 500 µL of the sample. Then the mixture was mixed properly, allowed to stand for 10 min at room temperature and centrifuged at 4,000 rpm for 10 min. After that HDL cholesterol was assayed from the supernatant by enzymatic (CHOD-PAP) method.

Estimation of serum LDL cholesterol: Serum LDL cholesterol was calculated by Friedewald equation¹³. $LDL\ cholesterol = Total\ cholesterol - HDL\ cholesterol - Triglycerides/5$.

Estimation of GSH: GSH level was assayed by Ellman's method¹⁴. In brief, 1 mL of aortic tissues homogenate and erythrocytes was added to 1 mL of 5% trichloroacetic acid and the mixture was vortexed and centrifuged at 4,000 rpm for 5 min. Then 250 µL of supernatant was added to 2 mL Na₂HPO₄ (4.25%) and 250 µL of DTNB (0.04%). The mixture was allowed to stand for approximately 10 min, and forming a yellow substance. The absorbance was measured at 412 nm using spectrophotometer.

Estimation of MDA: MDA (malondialdehyde) level was assayed by thiobarbituric acid reaction method¹⁵. In brief, 1 mL of aortic tissues

homogenate and erythrocytes was added to 4.5 mL of 5.5% trichloroacetic acid and the mixture was vortexed and centrifuged at 4,000 rpm for 5 min. Then 1 mL of supernatant was added to 1 mL of 0.67% thiobarbituric acid. The mixture was kept in a water bath at 100°C for 10 min and forming a pink substance. The absorbance was measured at 532 nm using spectrophotometer. MDA content was calculated in mol/L using the molar extinction coefficient for MDA that is 1.56×10^5 . Then it is multiplied by 10^9 for conversion to nmol/L.

Estimation of serum electrolytes: Serum sodium, potassium and chlorine level were assayed by ion selective electrode method using Nova Electrolyte 4 Analyzer (Nova biomedical corporation, USA).

Histopathological procedure: The aorta was fixed in 10% formalin, dehydrated in graded alcohol and embedded in paraffin wax, sectioned at 5 μ m thickness and stained with hematoxylin and Eosin (H&E) for microscopic examination. Microscopically the lesion of the aorta was graded according to stary's classification¹⁶. Photomicrographs were taken from the representative histopathological sections of each group using a digital camera fitted with microscope. Intima-media ratio was measured using Image pro plus software (Media Cybernetics, USA).

Statistical analysis: Statistical analysis was done by Statistical Package for Social Science (SPSS), version 16 for windows. The quantitative variables were expressed as mean \pm SEM. ANOVA (multiple comparisons) was done for statistical analysis. Post hoc analyses of differences were done by Bonferroni 't' test.

Results

Losartan decreased serum cholesterol, triglyceride, LDL-C & increased HDL-C level, but hydrochlorothiazide increased serum cholesterol, triglyceride, LDL-C & decreased HDL-C level. Indapamide showed no effects on serum lipid profile in comparison to control group (Table I). Co-administration of hydrochlorothiazide with losartan diminished the hypolipidaemic effect of losartan, whereas conadiminisrration of indapamide did not interfere the effect of losartan.

The body weights of the rats were decreased with losartan, increased with hydrochlorothiazide and not changed with indapamide (Table II). In comparison to losartan treated rats, body weight increased when hydrochlorothiazide and losartan were co-administered and not changed when indapamide and losartan were co-administered.

Table I: Effect of drug treatment on serum lipid level of 0.5% cholesterol-fed rats

Group	Serum cholesterol (mg/dL)	Serum triglycerides (mg/dL)	Serum HDL cholesterol (mg/dL)	Serum LDL cholesterol (mg/dL)
Control	108.94 \pm 1.78	81.59 \pm 1.41	40.50 \pm 0.87	52.12 \pm 1.97
Losartan	84.24 \pm 1.21*	73.44 \pm 1.17*	45.97 \pm 0.68*	23.58 \pm 0.76*
Hydrochlorothiazide	125.82 \pm 1.23*	88.14 \pm 1.30*	32.16 \pm 1.38*	76.03 \pm 2.00*
Indapamide	104.63 \pm 1.19	77.06 \pm 1.11	37.94 \pm 1.01	51.28 \pm 1.08
Losartan + Hydrochlorothiazide	121.80 \pm 1.31 ^a	83.77 \pm 1.23 ^a	31.69 \pm 0.92 ^a	73.36 \pm 1.87 ^a
Losartan + Indapamide	82.80 \pm 1.20	71.67 \pm 1.22	43.58 \pm 1.40	24.89 \pm 1.15

Values are mean \pm SEM; n = 10; *p<0.01 compared to control group as analyzed by ANOVA (multiple comparisons by Bonferroni 't' test); ^a p<0.001 compared to losartan treated group as analyzed by ANOVA (multiple comparisons by Bonferroni 't' test)

Table II: Effect of drug treatment on body weight of 0.5%cholesterol-fed rats

Group	Body weight (g)		
	Day 0	12 weeks	24 weeks
Control	155.50 \pm 0.93	258.30 \pm 0.76	290.00 \pm 1.32
Losartan	154.40 \pm 0.99	256.80 \pm 1.00	281.80 \pm 1.23*
Hydrochlorothiazide	155.10 \pm 0.60	257.20 \pm 0.96	299.10 \pm 2.16 ^a
Indapamide	155.40 \pm 0.76	256.40 \pm 0.87	288.60 \pm 0.96
Losartan + Hydrochlorothiazide	154.80 \pm 1.01	255.50 \pm 1.02	291.20 \pm 1.41 ^a
Losartan + Indapamide	155.80 \pm 0.83	256.30 \pm 0.82	280.50 \pm 0.93

Values are mean \pm SEM; n = 10; *p<0.01 compared to control group as analyzed by ANOVA (multiple comparisons by Bonferroni 't' test); ^a p<0.001 compared to losartan treated group as analyzed by ANOVA (multiple comparisons by Bonferroni 't' test)

Table III: Effect of drug treatment on GSH and MDA levels in erythrocyte & aortic tissue of 0.5% cholesterol-fed rats

Group	GSH level		MDA level	
	Erythrocyte (mg/g Hb)	Aortic tissue (mg/g tissue)	Erythrocyte (nmol/g Hb)	Aortic tissue (nmol/g tissue)
Control	8.86 ± 0.29	4.04 ± 0.14	10.08 ± 0.24	1.61 ± 0.07
Losartan	10.11 ± 0.36*	4.11 ± 0.17	8.30 ± 0.11*	1.48 ± 0.07
Hydrochlorothiazide	7.44 ± 0.20*	3.92 ± 0.11	11.02 ± 0.19*	1.75 ± 0.06
Indapamide	8.97 ± 0.20	4.02 ± 0.20	9.89 ± 0.17	1.59 ± 0.08
Losartan + Hydrochlorothiazide	7.52 ± 0.20 ^a	3.88 ± 0.10	10.84 ± 0.17 ^a	1.55 ± 0.09
Losartan + Indapamide	10.23 ± 0.30	4.13 ± 0.17	8.18 ± 0.13	1.50 ± 0.11

Values are mean ± SEM; n=10; *p<0.01 compared to control group as analyzed by ANOVA (multiple comparisons by Bonferroni 't' test); ^a p<0.001 compared to losartan treated group as analyzed by ANOVA (multiple comparisons by Bonferroni 't' test)

Losartan have antioxidant effect observed by increased GSH and decreased MDA levels in erythrocyte. Hydrochlorothiazide decreased GSH and increased MDA levels in erythrocyte. In indapamide treated rats, there was tendency of increase in GSH and decrease in MDA levels in erythrocyte but the changes were not significant (Table III). The antioxidant effect of losartan was abolished with addition of hydrochlorothiazide, which effect was conserved while indapamide was added with losartan.

Morphological study of aortic tissue of rats revealed that there was no atherosclerotic change in the aorta. There were no significant changes observed in intima-media ratio of the aorta. There were no significant changes in serum electrolyte level in losartan and indapamide treated rats and in rats where losartan and indapamide were co-administered. Hypokalemia was observed in hydrochlorothiazide treated rats and also in rats where hydrochlorothiazide was added with losartan (Table IV).

Table IV: Effect of losartan, hydrochlorothiazide, indapamide, losartan plus hydrochlorothiazide and losartan plus indapamide on morphology of aortic tissue and serum electrolytes level of 0.5% cholesterol-fed rats

Group	Intima-media ratio of aorta	Serum sodium (mmol/L)	Serum potassium (mmol/L)	Serum chloride (mmol/L)
Control	0.54 ± 0.01	147.30 ± 1.14	7.09 ± 0.07	86.70 ± 0.52
Losartan	0.55 ± 0.02	146.40 ± 0.43	6.51 ± 0.16	87.40 ± 0.31
Hydrochlorothiazide	0.57 ± 0.01	146.40 ± 0.43	5.51 ± 0.12	87.50 ± 0.27
Indapamide	0.53 ± 0.01	147.00 ± 0.67	6.56 ± 0.13	87.40 ± 0.40
Losartan + Hydrochlorothiazide	0.56 ± 0.02	147.00 ± 0.79	5.62 ± 0.21	86.80 ± 0.68
Losartan + Indapamide	0.55 ± 0.01	146.70 ± 0.62	6.60 ± 0.17	86.90 ± 0.75

Values are mean ± SEM; n = 10; *p<0.01 compared to control group as analyzed by ANOVA (multiple comparisons by Bonferroni 't' test); ^a p<0.001 compared to losartan treated group as analyzed by ANOVA (multiple comparisons by Bonferroni 't' test)

Discussion

In the present study, serum cholesterol, triglyceride & LDL-C level was decreased and HDL-C level was increased by losartan, which resembles to the finding of a previous study¹⁷. Hypolipidemic benefit of losartan in this study may be due to its decreasing effect of lipoprotein(a) [Lp(a)] and fibrinogen level¹⁸. Serum cholesterol, triglyceride & LDL-C level was increased and HDL-C level was decreased by hydrochlorothiazide but indapamide had no effects on serum lipid profile. Both of these results support the finding of another earlier study⁷. Changes in lipid profile level by hydrochlorothiazide could be attributable to its effect on cholesterol metabolism probably via

alteration in receptors for cholesterol uptake, increase in the level of Lp(a) and interference on the enzyme of cholesterol metabolism¹⁸. Indapamide had no effect on lipid profile because it has no deleterious effect on cholesterol metabolism⁹.

The hypolipidaemic effect of losartan was diminished when hydrochlorothiazide was coadministered with losartan, whereas the effect was conserved when indapamide was coadministered with losartan. The observation might be explained by the fact that the above mentioned hyperlipidaemic mechanisms of hydrochlorothiazide perhaps interferes the beneficial effect of losartan on lipid profile;

whereas this hypolipidemic effect of losartan was conserved with indapamide, which have neutral or no effect on cholesterol metabolism⁹.

Body weights of the rats were decreased with losartan, increased with hydrochlorothiazide and not changed with indapamide. In comparison to losartan treated rats, body weight increased when hydrochlorothiazide and losartan were co-administered and not changed when indapamide and losartan were co-administered. These effects on body weight may correlates with the changes in serum lipid profile produced by losartan, hydrochlorothiazide and indapamide¹⁹.

The present study revealed that losartan exerted antioxidant effect observed by increased GSH and decreased MDA levels in erythrocyte, which might be due to its effect of blocking the angiotensin II mediated oxidative stress, which is similar to the findings of other study²⁰. Hydrochlorothiazide treated rats demonstrate decreased GSH and increased MDA levels in erythrocyte. The result elucidate that hydrochlorothiazide has no antioxidant effect, which corresponds to the finding of another study²¹. Although the changes were not significant, indapamide treated rats showed tendency of increase in GSH and decrease in MDA levels in erythrocyte, which is similar in another study²². However, antioxidant effect of losartan was abolished with co-administration of hydrochlorothiazide. This might be due to attenuation of nitric oxide (NO) mediated endothelial antioxidant mechanism of losartan by hydrochlorothiazide²³. While the antioxidant effect of losartan was conserved in rats, in which indapamide was added with losartan. This might be explained by the fact that indapamide have antioxidant property of its own and exerts its effect without interfering the antioxidant mechanism of losartan²⁴.

Morphological study of aortic tissue of rats in the present study revealed that there was no atherosclerotic change in the aorta as well as there were no significant changes observed in intima-media ratio of the aorta among different groups of rats. This might be explained by the fact that the duration of cholesterol enriched diet feeding was short or the amount of cholesterol in the rat diet was just only adequate to produce hyperlipidemia but not to develop atherosclerosis. However, these findings differ from the results of previous studies conducted in other species may be due to different and weaker anti-inflammatory and antioxidant mechanism in vascular endothelium than rat²⁵.

Atherosclerotic change in the aortic tissue was not observed in rats after 24 weeks, irrespective of drug

received. This might be due to the fact that oxidative stress usually not evidenced in aortic tissue of rats irrespective of the treatment received and is assumed to be related to presence of additional antioxidant mechanism in aortic tissue of rat²⁶.

In this study, there was no significant changes in serum electrolyte level in losartan treated rats. Though, it has been reported earlier that losartan sometimes causes hyperkalemia²⁷. Indapamide treated rats showed no alteration in serum electrolytes level. However, hypokalemia and hyponatremia have been reported to be associated with indapamide treatment²⁸. There was hypokalemia in hydrochlorothiazide treated rats and also in rats where hydrochlorothiazide was added with losartan. This finding conforms to the previous reports of hypokalemia associated with hydrochlorothiazide. However in those studies, there were occasional reports of hyponatremia and hypochloremia²⁹, which was not observed in the present study. Another study suggested a relationship between hypokalemia and atherosclerosis. So, hydrochlorothiazide-induced hypokalemia may be responsible for its atherogenic effect³⁰.

In conclusion, the present study reaffirms the anti-atherogenic benefit of losartan reflected by its' hypolipidemic effects. The study reveals that co-administration of hydrochlorothiazide abolishes that anti-atherogenic benefit of losartan and co-administration of indapamide does not hinder the anti-atherogenic benefit of losartan.

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