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ORIGINAL ARTICLE

Changes in the Urinary Metabolites in Losartan-Treated Spontaneously Hypertensive Rats

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ABSTRACT

Introduction: This study examined the association of losartan induced changes in urinary metabolomic profile with the changes in blood pressure (BP) and renin-angiotensin-aldosterone system (RAAS) in spontaneously hypertensive rats (SHR). **Methods:** Male SHR were administered with either 0.5 mL of distilled water (control group, n=6) or 10 $mg.kg^{-1}$ of losartan (group 2, n=6) daily by oral gavage for 4 weeks. Body weight, BP, food and water intake were measured weekly. At week 4, urine was collected for urinary electrolyte analysis and metabolite profiling, after which the animals were euthanised by decapitation and blood was collected for analysis of components of RAAS and electrolyte concentrations. Urine metabolite profile of SHR was determined using proton nuclear magnetic resonance (^{1}H-NMR) spectrometry combined with multivariate data analysis. **Results:** At week 4, losartan-treated SHR had significantly lower BP than non-treated SHR. There were no differences in water and food intake, body weight, serum and urinary electrolyte concentrations or in their urinary excretions between the two groups. No differences were evident in the components of RAAS except that the angiotensinogen level was significantly higher in losartan-treated SHR compared to non-treated SHR. Orthogonal partial least squares discriminant analysis (OPLS-DA) showed clear separation of urinary metabolites between control and losartan-treated SHR. Losartan-treated SHR group was separated from the control group by changes in the intermediates involved in glycine, serine and threonine metabolism. **Conclusion:** Antihypertensive effect of losartan in SHR seems to be associated with changes in urinary metabolite profile, particularly involving the metabolism of glycine, serine and threonine.

KEYWORDS: ^{1}H-NMR, metabolomics, hypertension, losartan, spontaneously hypertensive rats, RAAS

INTRODUCTION

Hypertension is a significant risk factor for stroke, myocardial infarction, kidney failure and premature death if early treatment is not instituted [1]. Essential or primary hypertension is a complex, polygenic, heterogeneous, and multifactorial disease comprising of more than 90% of the cases of hypertension. Its precise mechanism remains unknown. Many pathophysiological mechanisms have been implicated in the pathogenesis of essential hypertension and some of these include activation of the renin–angiotensin–aldosterone system (RAAS) and the sympathetic nervous system. Although endothelial dysfunction, increased vascular reactivity, and vascular remodelling are often considered as consequences of hypertension, however, they may by themselves also be factors contributing to the pathogenesis of essential hypertension [2, 3].

The RAAS is a pivotal hormonal cascade that plays an essential role in the homeostatic control of arterial pressure, extracellular fluid volume and tissue perfusion [4]. Any disturbance to the components of RAAS contributes to the pathogenesis of hypertension [5]. Hence, the antihypertensive agents targeting RAAS system such as angiotensin-converting enzyme (ACE) inhibitors, AT_1-receptor blockers (ARBs) and aldosterone-receptor antagonists (ARAs) are highly effective in lowering blood pressure [6].

Considering the gaps in the understanding of the various aspects of the disease process, and the

therapeutic approaches used in the management of hypertension, it is important to adapt a comprehensive approach, to help understand the disease and also the mechanisms of action of the numerous antihypertensive agents currently used in the management of hypertension. It is possible that some of these agents may have more than one antihypertensive mechanism, affecting numerous other systems. In this regard, metabolomics has been shown to be a valuable tool in disease pathogenesis, diagnosis, identification of new therapeutic targets and the basis of potential treatment procedures [7]. Metabolomics provides an extensive understanding into the basis of metabolite changes emerging from interplays between genetic and environmental factors such as drugs and diet [8]. This can be achieved by focusing on non-biased global detection, identification and quantification of molecules or metabolites with low molecular weight in cells, tissues and all bio-fluids [9].

One of the widely used angiotensin receptor blockers (ARBs), losartan, have been shown to reduce blood pressure by effectively blocking the angiotensin II type 1 (AT_1) receptors in the RAAS. However, the changes in metabolites in relation to its systemic effect in hypertensive individuals have not been clearly elucidated. There is very little information in the literature on the effect of losartan on urinary metabolite profile. Evaluation of the urinary metabolite profile following losartan treatment could provide greater insights into the mechanism of actions of losartan. This study, therefore, examined the changes in the urine metabolomic profile as well as in the component of RAAS following treatment with losartan in spontaneously hypertensive rats.

MATERIALS AND METHODS

a) Chemical and reagents

Of the chemicals and reagents used in this study, sodium phosphate dibasic (Na_2HPO_4), deuterium oxide (D_2O), sodium phosphate monobasic (NaH_2PO_4) and trimethylsilyl propionic acid-d4 sodium salt (TSP) were purchased from Sigma Aldrich, St Louis, MO, USA. Methanol and ethanol were purchased from Merck Milipore, Darmstadt, Germany. Losartan was purchased from MSD, UK.

b) Animals and study design

The study protocol was approved by the institutional animal ethics committee of Universiti Teknologi MARA (UiTM), (UiTM Committee on Animal Research and Ethics, UiTM CARE No: 113/2015). Male, spontaneously hypertensive rats (SHR), aged 12-14 weeks and weighing 250 – 300 grams were obtained from Laboratory Animal Care Unit, Faculty of Medicine, UiTM Sungai Buloh Campus, Selangor, Malaysia. The animals were individually housed in metabolic cages and kept in the laboratory with a room temperature of 23 ± 2º C and a 12-hour light: 12-hour dark period with food (Rodent Diet Specialty Feeds, Australia) and water *ad libitum*. Animals were acclimatized to the metabolic cages and blood pressure measurement for one week before the commencement of the experiment. The cylindrical restrainer was placed together with the rat inside the cage. Only animals with a systolic blood pressure above 150 mmHg and diastolic blood pressure above 90 mmHg, measured after rest and acclimatization, were included in the study. The selected SHR were divided into two groups consisting of a control group (n = 6) and a group that received 10 mg kg^{-1} day^{-1} of losartan (n = 6). Control SHR were given 0.5 mL of distilled water. All treatments were given daily via oral gavage in a total volume of 0.5 mL, once daily for a total of 4 weeks. During the study period, blood pressure was measured once a week using tail-cuff plethysmography. Additionally, animals were observed daily for changes in body weight, water intake, food intake and urine output. Twenty-four-hour urine samples were collected over the last twenty-four hours of treatment using metabolic cages. The collection was done in bottles containing 0.1 % sodium azide and the urine was immediately stored at −80°C after collection until analysis. Animals were euthanized after 4 weeks of treatment. For euthanization, the animals were decapitated using a small animal guillotine under light anaesthesia with diethyl ether. Blood was collected and left at room temperature for 15 minutes to clot and then centrifuged for 10 min at 3000 rpm for the collection of serum. Sera were then stored at −80°C until further analysis.

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c) Blood pressure measurement using tail cuff plethysmography

The blood pressure measurement was done at 11 am daily using tail-cuff plethysmography, which is based on recordings of systolic blood pressure according to changes in tail volume, (Kent Scientific, Torrington, CT). The method has been validated and recommended by the American Heart Association particularly for high throughput screening in rodents [10]. All animals were acclimatized to the procedure for 15 minutes prior to the measurement of blood pressure. During acclimatization, each rat was placed in an appropriately-sized cylindrical animal restrainer with the tail exposed at one end. The restrainer together with the rat was placed on an infrared warming platform, measuring 6" x 8", where the temperature was maintained at 31-32° C to ensure good tail blood circulation. A blood pressure measuring cuff was then placed around the tail. Twenty recordings were obtained and recorded on the physiograph, with a time interval of two minutes between each reading. Three lowest readings were taken and the mean of these was taken as the blood pressure.

d) Serum and urinary biochemistry and electrolytes measurement

Calcium, total protein, potassium, albumin and sodium concentrations were measured in urine and serum spectrophotometrically (Hitachi, Japan). The measurement was done by Clinical Training Centre, Faculty of Medicine, UiTM Sungai Buloh Campus, Selangor, Malaysia.

e) Measurement of serum components of RAAS

i) *Measurement of serum Angiotensin I (Ang I), Angiotensin II (Ang II) and aldosterone concentration*

Commercially-available ELISA kits were used to measure the concentration of Ang I, Ang II and aldosterone (Elabscience, China). These kits use the competitive-ELISA principle. Briefly, 50 microlitre (µl) of standards and samples were pipetted into the designated wells. Immediately after that, 50 µl of Biotinylated Detection Antibody (Ab) was added to each well and incubated for 45 min at 37º C. After this the samples were aspirated, and the wells were washed 3 times with 350 µL of wash buffer before adding 100 µL HRP Conjugate to each well followed by incubation for 30 min at 37ºC. After incubation, the samples were aspirated, and the wells were washed 5 times with 350 µL of wash buffer. Ninety µL of substrate reagent was added into each well and then incubated for 15 min at 37º C. Lastly, 50 µL of stop solution was added and the plate was read for the optical density absorbance at 450 nm immediately.

ii) *Measurement of ACE, ACE2, angiotensinogen, and renin concentration*

Concentrations of renin, angiotensinogen, ACE and ACE2 were determined using ELISA kits (Elabscience, China). These kits use the sandwich-ELISA principle. Briefly, 100 µl of standard or samples were pipetted into the designated wells of a 96-well tray. The samples were incubated for 90 minutes at 37ºC after which 100 µl of Biotinylated Detection Antibody (Ab) was added to each well. The samples were then incubated for 1 hour and washed. One hundred µl of horseradish peroxidase (HRP) was added and the sample was incubated for 30 minutes and then washed once again. This was followed by the addition of 90 µl of substrate reagent to every well, after which the sample was incubated for 15 minutes. Lastly, fifty µl of stop solution was added and the absorbance was read at 450 nm immediately.

iii) *Measurement of renin activity*

Renin activity was quantified using commercially available Renin Assay Kit (Sigma-Aldrich, St. Louis, MO). This kit uses fluorescence resonance energy transfer (FRET) technique, which contains florescence quencher. Briefly, 50 µl of either standard or samples were added into the designated wells. Fifty µl of an assay mixture containing renin substrate and assay buffer was added for assay reaction. The wells were incubated for 20 minutes at 37ºC. During incubation, absorbance was read every 5 minutes at excitation and emission wavelengths of 540/590 nm using a

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microplate reader (Victor X5), Perkin Elmer. Renin activity was expressed as RFU/min.

f) Preparation of urine sample and ^{1}H-NMR data acquisition

Each urine sample was first thawed and then 400 µL of the sample was mixed with 200 µL of 0.2 M phosphate buffer (pH 7.40), containing 0.1% 3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionic acid (TSP). The mixture was vortexed and then centrifuged at 12000 rpm for 5 min. An aliquot of 550 µL from the sample was then transferred into an NMR tube [11]. Urine samples were subjected to NMR spectroscopy at 600 MHz (Bruker 600 AscendTM NMR). The ^{1}H-NMR spectra were recorded at 26^{o} C with a total acquisition time of 2.49 min for 32 scans. The settings for the ^{1}H-NMR spectra were with water suppression pulse sequence 1 dimensional (1D) nuclear Overhauser effect spectroscopy (NOESY)-presat. The pre-saturation was done to suppress the residual water signal. This involves the increment of a NOESY pulse sequence with water irradiation during the relaxation delay and mixing time of 10 ms. The sweep width, time domain size and relaxation delay (RD) were set at 20 ppm, 65 and 2.0 s, respectively. The Chenomx NMR Suite library (v. 6.2, Alberta, Canada) and the free online Human Metabolome Database (HMDB) as well as previously published data were used for metabolite identification.

g) Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). For comparison between 2 groups, Student's T-test was used. A $P<0.05$ was considered significant. For metabolomics studies, to reduce the effect of variation in ionic concentration, the bucketing of NMR dataset was accordingly done with a bucket size of 0.04 ppm prior to alignment to TSP peak. The removed regions were for urea (δ 5.60–5.96) and water (δ 4.70–5.10). The data was converted to ASCII file of the NMR spectra and used in multivariate data analysis using Simca-P software 14 (Umeå, Sweden) and scaled to Pareto scaling method for all the metabolite signals to have the same importance and for the effects of noise to be reduced.

RESULTS

a) Effect of losartan on systolic blood pressure in SHR

Mean systolic blood pressure was comparable between control and losartan-treated groups at the beginning of the study (day 0) (Figure 1). However, after 4 weeks of treatment, systolic blood pressure in the losartan-treated group was significantly lower ($P<0.001$) when compared to that in the control group (Figure 1).

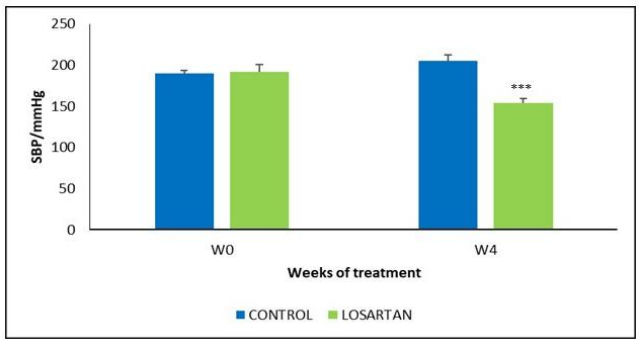

Figure 1 Effect of losartan on systolic blood pressure in losartan-treated and control SHR. (n=6 per group), ***p<0.001 versus control at respective week.

b) Effect of losartan on body weight, food intake, urine output and creatinine clearance in SHR

No difference was evident in body weight, food intake, urine output and creatinine clearance between the losartan-treated and control SHR before the start and after the completion of the 4-week treatment (Table 1).

c) Effect of losartan on the urinary electrolytes and protein excretion in SHR

No difference was evident in the urinary excretion of sodium, potassium, calcium and total protein between the losartan-treated and control SHR at W4 (Table 2).

d) Effect of losartan on serum electrolytes, albumin and total protein concentration

No difference was observed in the serum concentrations of sodium, potassium, calcium, albumin and total protein between control and losartan-treated SHR (Table 3).

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Table 1 Body weight, food intake, urine output and creatinine clearance in losartan treated and control SHR

Groups	**Control**		**Losartan**	
Parameters	Week 0	Week 4	Week 0	Week 4
Body weight (g)	239.5 ± 8.66	263.3 ± 5.02	239.8 ± 8.05	259.8 ± 7.37
Food intake (g)	13.2 ± 1.57	16.2 ± 2.10	17.8 ± 2.82	20.2 ± 1.71
Urine output (mL day^{-1})	6.7 ± 2.13	5.8 ± 0.56	5.4 ± 0.94	7.7 ± 0.95
Creatinine clearance (mL min^{-1})	-	1.14 ± 0.17	-	1.13 ± 0.19

n= 6. Data are expressed as mean ± S.E.M

Table 2 Urinary electrolyte and protein excretion rates in losartan treated and control SHR

Parameters	**Groups**	**Mean ± SEM**
		Week 4
Sodium (µmol day^{-1})	Control (n=6)	635.7 ± 128.93
	Losartan (n=6)	607.4 ± 138.74
Potassium (µmol day^{-1})	Control (n=6)	1484.3 ± 238.45
	Losartan (n=6)	1445.7 ± 286.68
Calcium (µmol day^{-1})	Control (n=6)	5.8 ± 1.39
	Losartan (n=6)	7.7 ± 2.21
Albumin (µg day^{-1})	Control (n=6)	11.15 ± 1.28
	Losartan (n=6)	9.19 ± 1.10
Total protein (mg day^{-1})	Control (n=6)	12.0 ± 2.43
	Losartan (n=6)	12.0 ± 2.50

n= 6. Data are expressed as mean ± S.E.M

Table 3 Serum electrolytes, albumin and protein concentrations in losartan treated and control SHR

Parameters	**Groups**	**Mean ± SEM**
Sodium (mmol. L^{-1})	Control (n=6)	140.33 ± 2.59
	Losartan (n=6)	145.40 ± 2.15
Potassium (mmol. L^{-1})	Control (n=6)	6.27 ± 0.23
	Losartan (n=6)	5.73 ± 0.27
Calcium (mmol. L^{-1})	Control (n=6)	2.51 ± 0.06
	Losartan (n=6)	2.52 ± 0.08
Albumin (g. L-^{1})	Control (n=6)	38.62 ± 1.08
	Losartan (n=6)	40.48 ± 1.22
Total protein (g.L^{-1})	Control (n=6)	64.83 ± 0.99
	Losartan (n=6)	67.44 ± 0.94

n= 6. Data are expressed as mean ± S.E.M

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Table 4 Serum concentrations of angiotensinogen, renin, Ang I, Ang II, ACE, ACE2, aldosterone and renin activity in losartan treated and control SHR

Parameters	Groups	Mean ± SEM
Agt (ng/ml)	Control (n=6)	2.72 ± 0.75
	Losartan (n=6)	11.28 ± 3.80**
Renin (pg/ml)	Control (n=6)	424.64 ± 54.47
	Losartan (n=6)	612.34 ± 11.72
Renin activity (rfu/min)	Control (n=6)	10.77 ± 1.42
	Losartan (n=6)	9.35 ± 1.67
Ang I (pg/ml)	Control (n=6)	388.47 ± 32.68
	Losartan (n=6)	314.12 ± 96.95
ACE (ng/ml)	Control (n=6)	5.28 ± 0.35
	Losartan (n=6)	7.42 ± 0.57
Ang II (pg/ml)	Control (n=6)	304.20 ± 22.73
	Losartan (n=6)	305.20 ± 50.69
ACE2 (ng/ml)	Control (n=6)	0.116 ± 0.0048
	Losartan (n=6)	0.112 ± 0.0023
Aldosterone (pg/ml)	Control (n=6)	791.38 ± 69.15
	Losartan (n=6)	681.42 ± 88.66

n= 6. Data are expressed as mean ± S.E.M, **p<0.01 versus control

e) Renin-angiotensin-aldosterone system

Serum angiotensinogen concentration was significantly higher in losartan-treated SHR when compared to that in the SHR controls (Table 4). No difference was observed in other parameters in the RAAS between the control and losartan-treated SHR (Table 4).

f) Effect of losartan on the urinary metabolite excretions in SHR- ^{1}H-NMR spectroscopy analysis

^{1}H-NMR spectral analysis of urine samples from non-treated controls and losartan-treated SHR is shown in Figure 2. A total of 48 metabolites (Table 5) were successfully identified in both groups (excluding water and urea). Some of the metabolites identified included acetate, glycine, betaine, succinate, methylamine, hippurate, trimethylamine-N-oxide (TMAO) and creatine. Visibly, the overlay ^{1}H-NMR spectra of urine samples of both groups shared high similarity in terms of the type of metabolites identified, and the changes were mainly in their intensities. As the variation between the control and losartan-treated group was difficult to observe visually, we applied multivariate data analysis; OPLS-DA to determine the differences between the 2 groups.

The OPLS-DA score plot obtained for both control and losartan-treated SHR is shown in Figure 3. Application of the OPLS-DA was done to identify metabolites which might be associated with hypertension or blood pressure regulation in both the control and losartan-treated SHR. The control and losartan groups were separated by the PC1 (Figure 3A). Based on the score plot of the OPLS-DA module, both groups are separated distinctly (Figure 3A), which suggests that they are represented by different sets of metabolites. In other words, these 2 groups might have differences in the blood pressure regulation. In the S-plot, metabolites which are clustered away from the centre of the plot are the ones which are highly significant in that particular group and play a crucial role in separating the two groups. As seen in Figure 3B, Dimethylamine (DMA) is situated away from the centre of the plot and is higher in the control SHR (Figure 4) when compared to that in the losartan-treated SHR. DMA could be one of the biomarkers that differentiates the control and losartan group. Based on the metabolites identified from the ^{1}H-NMR spectral analysis, significant amounts of glycine, serine and threonine metabolites were detected. Hence, it is likely that the metabolic pathways of glycine, serine and threonine might be involved in the hypotensive effect of losartan (Figure 5).

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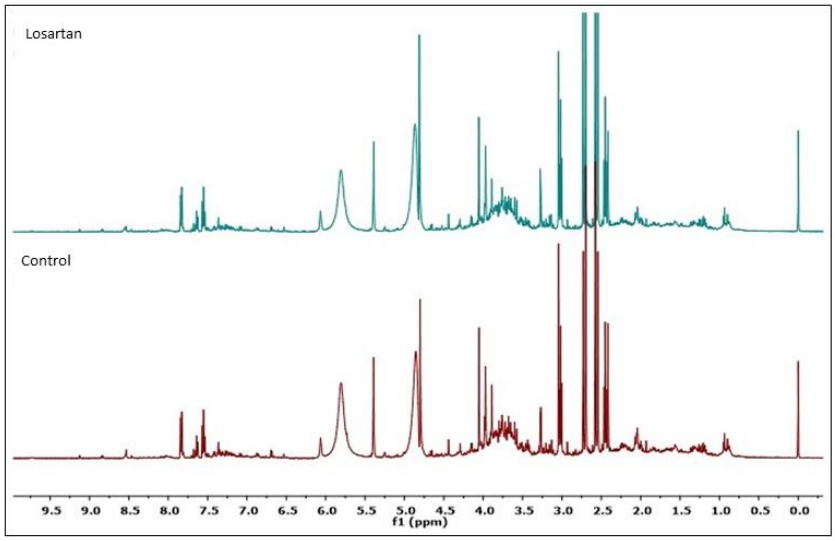

Figure 2 Representative 600 MHz 1H-NMR spectra of urine samples from control and losartan-treated SHR

Table 5 Summary of major metabolites detected in the urine of non-treated and losartan treated SHR as observed using OPLS-DA of ^{1}H-NMR spectral data.

Number	Metabolites	^{1}H-NMR Characteristics Signals
1	Hippurate	3.96 (d), 7.62 (t), 7.82 (d)
2	N-Phenylacetylglycine	3.76 (d), 7.34 (t), 7.4 (t)
3	Succinate	2.40 (s)
4	Citrate	2.54 (d), 2.70 (d)
5	Guanidinoacetatae	3.80 (s)
6	Glycine	3.56 (s)
7	Taurine	3.42 (t)
8	Acetate	1.92 (s)
9	2-Hydroxyphenylacetate	7.18 (m)
10	Dimethylamine	2.70 (s)
11	Creatine	3.02 (s)
12	Creatinine	4.04 (s)
13	Trimethylamine N-Oxide (TMAO)	3.26 (s)
14	α-Ketoglutarate	2.44 (t)
15	3-Indoxysulfate	7.22 (t), 7.30 (t), 7.68 (d)
16	Choline	3.20 (s)
17	Betaine	3.88 (s)
18	Alanine	1.48 (d)
19	Allantoin	5.38 (s)
20	Acetoacetate	2.26 (s), 3.44 (s)
21	Lysine	3.00 (t)
22	Glucose	5.22 (d)
23	3-Hydroxybutyrate	1.18 (d)
24	Formate	8.46 (s)
25	N,N-Dimethylglycine	2.92 (s)
26	Kynurenine	3.7 (d)
27	Trigonelline	4.42 (s), 8.06 (t)
28	Lactate	1.32 (d)

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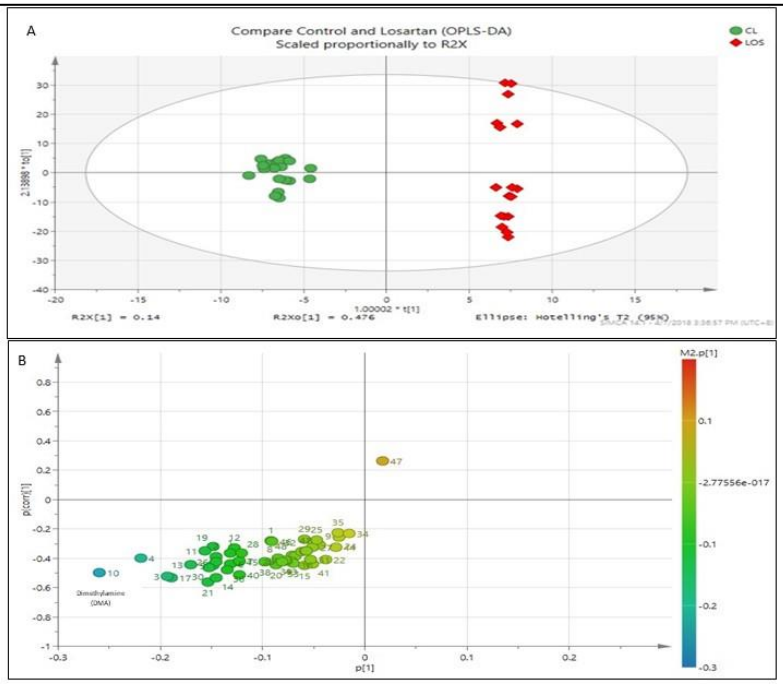
29	Isoleucine	0.92 (t)
30	Valine	0.98 (d), 3.6 (d)
31	Trimethylamine	2.94 (s)
32	Malonate	3.10 (s)
33	Sarcosine	2.74 (s)
34	Fumarate	6.50 (s)
35	Glutamine	6.86 (s)
36	N-Acetylglutamate	2.02 (s)
37	Methionine	2.12 (s)
38	Acetone	2.22 (s)
39	Pyruvate	2.36 (s)
40	Methylamine	2.60 (s)
41	Histidine	7.06 (s)
42	Methylguanidine	2.82 (s)
43	Ethanolamine	3.14 (t)
44	1-Methylhistidine	6.98 (s)
45	Homocysteine	3.90 (t)
46	Benzoate	7.54 (t)
47	Citrulline	6.40 (s)
48	Glucarate	4.06 (t)



The assignments: Hippurate ;1, N-Phenylacetylglycine; 2, Succinate; 3, Citrate; 4, Guanidinoacetate; 5, Glycine; 6, Taurine; 7, Acetate; 8, 2-Hydroxyphenylacetate; 9, DMA; 10, Creatine; 11, Creatinine; 12, TMAO; 13, a-ketoglutarate; 14, 3-indoxylsulfate; 15, Choline; 16, Betaine; 17, Alanine; 18, Allantoin; 19, Acetoacetate; 20, Lysine; 21, Glucose; 22, 3-hydroxybutryrate; 23, Formate ;24, N,N-dimethylamine; 25, Kynurenine; 26, Trigonelline ; 27, Lactate; 28, Isoleucine; 29, Valine; 30, TMA: 31, Malonate; 32, Sarcosine; 33, Fumarate; 34, Glutamine; 35, N-acetylglutamate; 36, Methionine; 37, Acetone; 38, Pyruvate; 39, Methylamine; 40, Histidine; 41, Methylguanidine; 42, Ethanolamine; 43, 1-methylhistidine; 44, Homocysteine; 45, Benzoate; 46, Citrulline ;47, Glucarate; 48.

Figure 3 Figure 3. (A) The OPLS-DA score, (B) S-plot of the effects of losartan after 4 weeks of treatment in SHR. CL; control, LOS; losartan.

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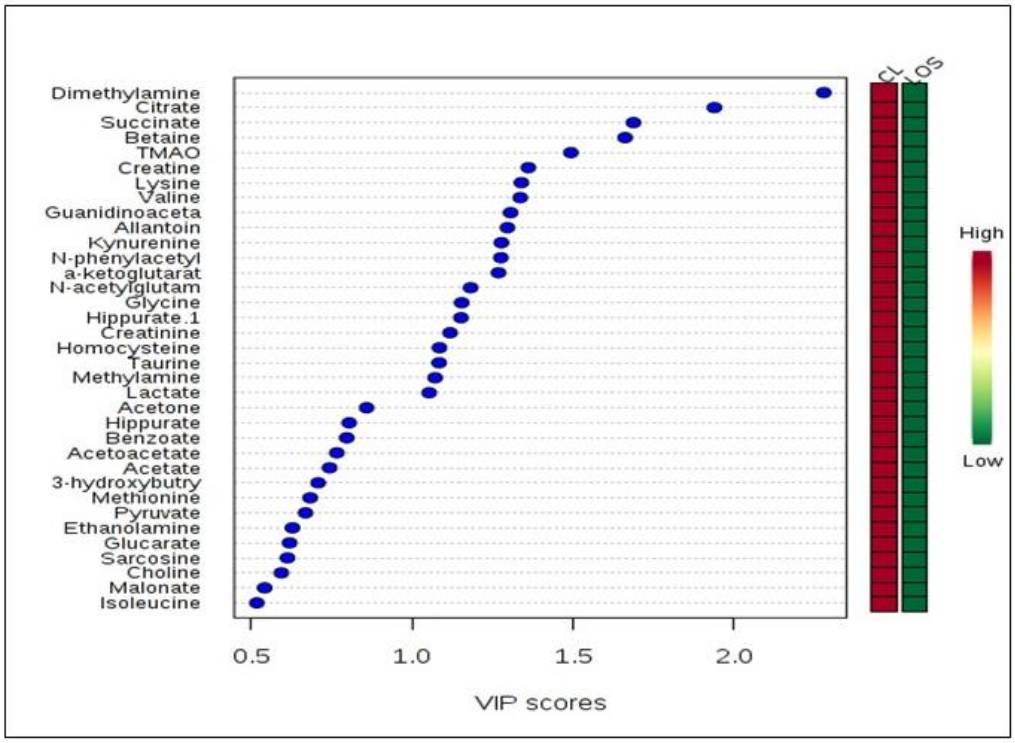

Figure 4 The VIP values derived from OPLS-DA. CL: control (hypertensive). LOS: 10 mg kg-1 day-1 of losartan

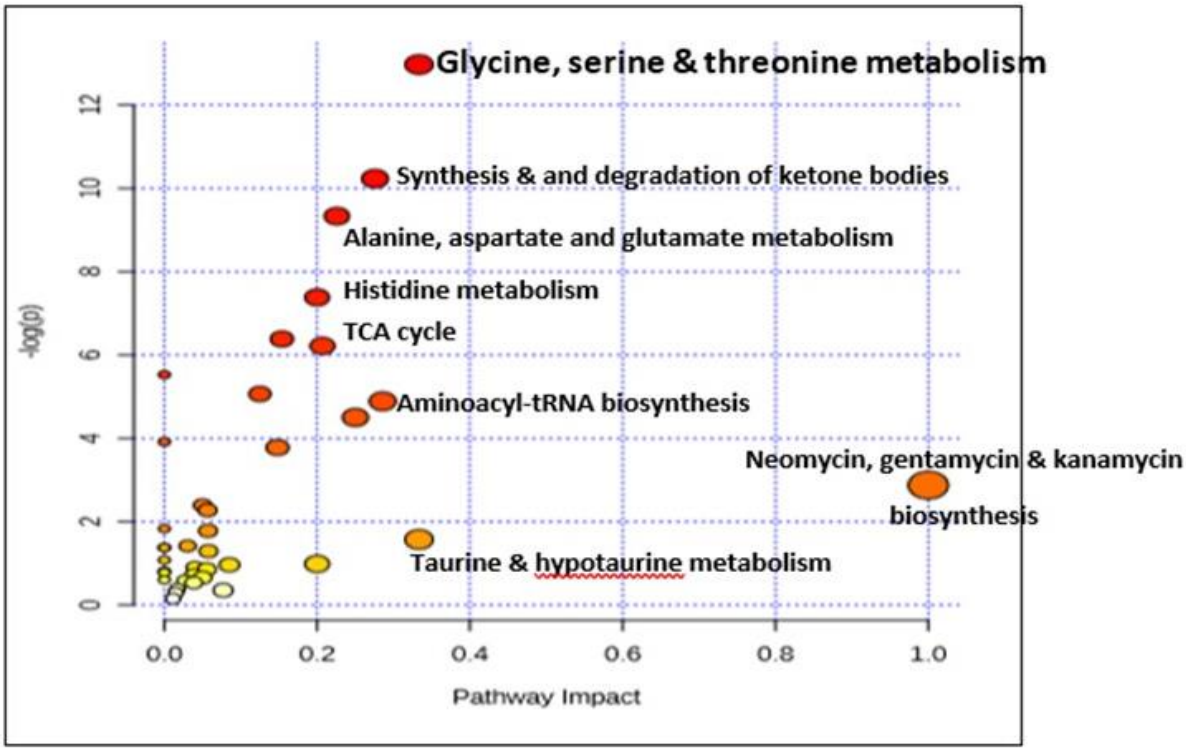

Figure 5 The pathway impact analysis

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DISCUSSION

In the current study, the well-known blood pressure lowering effect of losartan was clearly evident in SHR after 4 weeks of treatment. Treatment with losartan did not cause a change in body weight, food intake, urine output or in urinary and serum electrolyte concentrations in SHR. The serum levels of various components of RAAS also remained unaffected except for angiotensinogen level that was somewhat higher after treatment with losartan. Metabolomic studies showed a clear distinction in the pattern of urinary metabolite excretion between losartan and vehicle-treated rats.

The effect of losartan on blood pressure as observed in the current study, is consistent with that in previous reports that used the same hypertensive rat model as well as in other rat strains [12-14]. Losartan binds to the AT_1 receptor, hence preventing binding of Ang II to AT_1 receptor. This blocks the vasoconstrictive effects of Ang II resulting in vasorelaxation and a reduction in blood pressure. In addition, by inhibiting the binding of Ang II, losartan also inhibits the secretion of aldosterone, leading to reduction in renal sodium and water reabsorption, adding further to its blood pressure reducing effect.

In the current study, losartan did not cause significant changes in body weight, food intake and urine output compared to control SHR (Table 1). In this regard, earlier studies have also shown variable results. Deoxycorticosterone acetate (DOCA)-salt hypertensive rats treated with losartan via cerebroventricular injection (1mg/4uL) for 2 weeks showed no significant difference in their body weight compared to that of the control group [15]. However, when losartan was given for 4 weeks, the body weight was significantly lower compared to the control rats. On the other hand, Wistar rats fed with high-fat diet (HFD) with and without losartan (20 mg / kg BW) showed no difference in the mean body weight gain after 2 months of treatment [16]. Hence it is likely that the dose, duration of treatment and the type of rat strains used might influence the effects of losartan on the body weight.

The lack of differences in food intake, urine output and creatinine clearance between control and losartan-treated rats observed in the current study are also in line with results of previous studies [17-22]. Interestingly, we did not observe any differences in serum concentrations and urinary excretions of the electrolytes, albumin and protein between controls and losartan-treated rats (Tables 2 and 3), in both groups. Although there is a case report on losartan-induced severe hyponatremia [23], this study did not find a similar finding.

Looking at the trend of the urinary Na^+ excretion values of losartan-treated rats at week 4 (Table 2), it is possible that we might see some significant changes if the treatment is continued for more than 4 weeks. The findings in the current study are, nevertheless in line with recent findings by Salve et al (2019) who showed no changes in the serum concentration of sodium, potassium and calcium after 6 months of losartan therapy in patients with essential hypertension [24].

The current study used SHR that were 12-14 weeks old and at this age SHR are yet to develop significant renal damage, which only becomes apparent sometime after 40 weeks of age. Hence, a further change in urinary protein excretion despite the known reno-protective effects of losartan was not evident after 4 weeks of treatment in the current study.

Notably in the current study, we did not observe any significant changes in components of RAAS in the serum after treatment with losartan except for an increase in the angiotensinogen level (Table 4). The reason for the increased angiotensinogen level in serum after treatment with losartan remains unclear although previous studies have shown that losartan may reduce excretion of angiotensinogen and enhance its expression [25, 26]. The effect of losartan on Ang II concentration remains unclear or equivocal. A reduction in serum Ang II level following more than two weeks of losartan treatment at a dose of 100 mg has been reported. Another study did not find a significant effect of losartan on Ang II levels at doses of 25 mg [27]. Telmisartan did not cause any changes in plasma Ang I, Ang II and aldosterone levels after 12 months of therapy but plasma renin activity and plasma ACE activity were significantly higher [28]. Studies have also reported an increase in serum concentration of ACE2 and plasma Ang 1-7 after treatment with losartan [29]. The reason for these differences is unclear but

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maybe it is related to the duration of treatment or perhaps even the dose of losartan that was used. Suffice to say that losartan when given orally at 10mg kg^{-1} per day for 4 weeks to SHR does not significantly alter the components of RAAS except perhaps for the serum concentration of angiotensinogen.

This study demonstrated clear differences in the urinary metabolomic profile between losartan-treated and control SHR. Some of the metabolites in the urine of the losartan-treated SHR that were significantly different or lower from the control SHR included dimethylamine (DMA), citrate, succinate, betaine, trimethylamine-N-oxide (TMAO), choline, methylamine (MA) and creatine (Figure 4). Based on the pathway analysis (Figure 5), glycine, serine and threonine metabolism are one of the highest impact pathways, which might be involved in the antihypertensive action of losartan. There is emerging evidence linking TMAO with cardiovascular diseases [30-32] and the finding of a lower urinary TMAO is in accordance with the well-known cardioprotective effect of losartan. TMAO is one of the gut microbiota-derived metabolites. It is a product of oxidation of trimethylamine (TMA). As reviewed by Janeiro et al., (2018) both TMAO and TMA can be converted to DMA [33]. Choline and betaine were also found to be lower in the losartan-treated SHR (Figure 4). Since TMA is derived from choline, betaine and L-carnitine [34], it is possible that less amount of choline and betaine caused lesser production of TMA, which subsequently resulted in a lower level of DMA in the losartan-treated SHR (Figure 4). Other than TMAO, the main source of DMA is asymmetric dimethylarginine (ADMA), the endogenous inhibitor of nitric oxide synthesis. ADMA has been found to increase blood pressure in a randomized, double blind, placebo-controlled study in healthy individuals [35]. Losartan has been shown to inhibit ADMA-induced monocytic adhesion to endothelial cells [36], which is the key event leading to vascular inflammation. It is possible that the BP lowering effect of losartan also involves inhibitory effects towards the action of ADMA. ADMA and L-NAME levels in the renal tissue of L-NAME and salt-induced hypertension is reduced following treatment with losartan [37]. In addition to that, positive correlation between ADMA and DMA was found in the urine of patients suffering from coronary artery diseases [38], linking these metabolites with cardiovascular events. Additionally, losartan has been shown to also produce antioxidant effect by increasing the plasma level of nitric oxide and reducing superoxide in the kidney and aorta of Dahl-salt sensitive rats on high salt diet [39]. These findings suggested that the BP lowering effect of losartan involves not just its antagonistic action on the AT_1 receptor, but also its antioxidant effect.

Other than serine and threonine, choline is an important source of glycine [40]. In contrast to L-serine, glycine was found to cause pressor response in SHR and L-NAME treated rats [41]. It is possible that low urinary glycine excretion in the losartan-treated SHR is due to low level of choline (Figure 4), as losartan has been shown to attenuate the uptake of choline into the H9c2 myoblast cells, resulting in inhibition of phosphatidylcholine biosynthesis [42]. This could be the basis for the lower urinary choline excretion in the losartan group (Figure 4). Phosphatidylcholine consumption is linked positively with cardiovascular mortality in the US population [43]. It can be speculated that losartan, by reducing the amount of phosphatidylcholine generated might provide a protective effect from cardiovascular diseases, including hypertension. Involvement of glycine, serine and threonine metabolism in the regulation of blood pressure has been reported recently [44]. Further studies may reveal the potential of using choline and glycine, if not all metabolites, as markers, together with the blood pressure readings, to assess the cardioprotective effects of losartan.

CONCLUSION

In conclusion, it appears that the mechanism of the antihypertensive effect of losartan might also involve changes in serine, glycine and threonine metabolism apart from its AT_1 receptor antagonism. Further studies are needed to provide more information on the precise roles of these metabolites and their pathways in the antihypertensive action of losartan.

Conflict of Interest

Authors declare none

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Authors' Contributions

Norasikin Ab Azis, Mohd Salleh Ahmad Kamal, Zurain Radjeni were involved in the design, experimentation, data collection, analysis and preparation of the manuscript.

Renu Agarwal was responsible for the planning, designing, supervising, analysis of data and preparation of the manuscript.

Ahmed Mediani was responsible for the preparation of the extract.

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