Efficacy of Dietary Arginine: Lysine Ratio on Cardiometabolic and Nephrological Hypertension Transcriptional Marker in Wistar Rats

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Keywords: endothelial nitric oxide synthase (eNOS), dimethylarginine dimethyl hydroxylase 2 (DDAH2), angiotensin-1 converting enzyme (ACE1), Neutrophil gelatinase-associated lipocalin (NGAL), hypercholesterolemia

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Abstract
The research looked at the effects of high arginine and high lysine on the expression of transcriptional cardiometabolic indicators and nephrological hypertension markers in Wistar rats. Hypertensive Wistar rats were administered diets supplemented with two dietary amino acids, arginine and lysine. Biochemical indicators, as well as molecular indicators of vasodilator, nephrotoxic, and lipid-lowering, were studied. After 6 weeks of supplementing with high arginine (HA), the group’s systolic blood pressure dropped by 25.0 mmHg. The effects of high arginine and high lysine treatment were measured using mRNA of vasodilator markers nephrotoxic markers, and lipid-lowering markers in Wistar rats. Our findings show that dietary supplementation with high arginine reduces nephrotoxicity and influences the metabolic pathways of cardiometabolic markers, hypercholesterolemia markers, and high lysine markers. Hypertension is the multifactorial ailment that affects over one billion individuals, cardiovascular disease, roughly one-third of world population, and chronic kidney disease (CKD) is an ignored medical basis of resistant hypertension with roughly 490 million CKD patients. In our present investigation, we hypothesise a profound effect of the dietary amino acids, arginine, and lysine, on hypertension, nephrotoxicity, and hypercholesterolemia. Hypercholesterolemia would result from an increase in lysine in the hepatic indicators of cholesterol, fatty acids, and TAG homeostasis. The high arg: lys ratio has a prominent influence in the effective regulation of the hypertension molecular markers in combating systolic blood pressure and controlling the hypercholesterolemia, and biomarkers of renal impairment recovery.

Introduction
Hypertension is the multifactorial disorder that affects over one billion people and that will rise to 1.5 billion by 2020 (Düsing, 2010). Hypertension is an important worldwide public-health challenge because of its high frequency and concomitant risks of cardiovascular and kidney disease (Kearney et al., 2005). Globally, approximately 17 million deaths a year accounts because of cardiovascular disease, nearly one-third of the total deaths (WHO, 2011). Chronic kidney disease (CKD) is an overlooked medical basis of resistant hypertension with approximately 490 million CKD patients (Mills et al., 2015). Hypertension is influenced by genetic or environmental factors (including age, weight, and diet), or their interactions (Wang et al., 2016). It has been noted as the main cause of death and is the third biggest contributor to disability-adjusted life years. Numerous reports on the incidence of hypertension in various parts of the world (Kearney et al., 2005). A large number of candidate genes for hypertension have now been widely studied, the genes Nitric oxide synthase (NOS), and Dimethylarginindimethylaminohydrolase enzymes (DDAH) are few among them. NOS is essential for nitrergic transmission, which generates nitric oxide (NO) from L-arginine (Abhary et al., 2010). DDAH enzyme was encoded by DDAH1 and DDAH2 genes that remove asymmetric dimethylarginine (ADMA) from the kidneys and liver (Teerlink et al., 2009). ADMA, as an endogenous inhibitor of nitric oxide (NO) synthase, decreases NO and thus regulates endothelial function. Detection of endothelial dysfunction at the initial stage of hypertension, NOS has a considerable clinical impact on hypertension (Wang et al., 2016). These genes influence hypertension since they play a fundamental role in maintaining endothelial function.
In clinical trials, pharmacological inhibition of the renin-angiotensin system (RAS) with angiotensin-1-converting enzyme (ACE1) inhibitors slow the progression of renal disease, thus confirming the role of angiotensin II (Ang II) in the pathophysiology of chronic renal injury (Noris & Remuzzi, 2002). The urinary kidney biomarkers such as kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), cystatin C, clusterin, and trefoil factor-3 (TFF-3) are the most sensitive evidence of nephrotoxicity. These were the advanced nephrotoxic biomarkers compared to the previous serum creatinine and blood urea nitrogen parameters (Zhang et al., 2014). These genes regulate hypertension as they are responsible markers in maintaining endothelial function.

The liver is involved in cholesterol, fatty acid, and triacylglyceride (TAG) homeostasis. Most of the lipogenic enzymes, including acetyl-CoA carboxylase α (ACCα), fatty acid synthase (FAS), are regulated at the transcription level in the liver, mediated by sterol regulatory element-binding protein-1c (SREBP-1c), a transcription factor (Oh et al., 2003). Lipoprotein lipase (LPL), a water-soluble enzyme hydrolyzes TAG into free fatty acids and acylglycerol molecule, mainly seen in chylomicrons and very-low-density lipoproteins (Mead, 2002). Low-density lipoprotein receptor (LDLr) functions in the hepatic uptake and clearance of plasma cholesterol (Brown & Goldstein, 1986). The plasma and liver were loaded with cholesterol and other lipid molecules cause hypercholesterolemia and further leads to hypertension. L-arginine treatment prevents an increase in the blood pressure in animals prone to hypertension and directs renal microvascular vasodilation with an increase in renal blood flow and glomerular filtration rate (Venho et al., 2002). L-Lysine has been reported to have a hypercholesterolemic effect and also an important essential amino acid, which is the first limiting amino acid in animals (Hevia et al., 1980).

In our present study, we hypothesize a profound effect of the dietary amino acids, arginine, and lysine, on hypertension, nephrotoxicity, and hypercholesterolemia. With an increase in lysine, the hepatic markers in cholesterol, fatty acids, and TAG homeostasis would lead to hypercholesterolemia. Our study will focus on the effect of supplementation of high arginine, to restore the hypotensive effect in the liver. Effect of arginine on hypertensive markers involved in signaling pathways of CKD and vasodilatory effect in RAS, NOS-DDAH pathway will be investigated. Thus, the effect of arginine (the protagonist) and lysine (antagonist) amino acids will be examined in transcriptional cardiometabolic markers and nephrological markers of hypertension and hypercholesterolemia pathways.

Materials and Methods

Animals and Maintenance

The animal experiment was conducted after obtaining approval from Institutional animal ethics committee (IAEC) (IEAE no. 222/12) as per the guidelines laid down by the Committee for the purpose of control and supervision of experiments on animals (CPCSEA) by the Government of India, Ministry of Social Justice and Empowerment. Male Wistar rats (Rattusnorvegicus), weighing 180 to 200 g (6 weeks old), were obtained for hypertension study from the animal house facility of Central Food Technological Research Institute, Mysuru, India. The rats were housed individually in stainless steel cages at 25°C, lights
were maintained on a 12-h light/dark cycle. The animals were acclimatized to these conditions for 1 week and given free access to water and control diet (AIN-93M). The final composition of a diet supplemented with high arginine of 6:1 (arg: lys) and high lysine of 6:1 (lys: arg) with direct substitution to the casein protein (Table 1) were prepared and fed to Wistar rats in high cholesterol diet (HCD) (diet, with 1% cholesterol). The rats have sacrificed at the end of the experiment after being anesthetized with isoflurane, and the organs were harvested and stored at -80°C for further analysis.

**Experimental design**

The Wistar rats were randomly divided into two major groups namely; control and High cholesterol diet (HCD) with 6 animals in each group (n = 6). The groups were further classified as Control with a normal diet with casein, High cholesterol diet (HCD) with a normal diet containing 1% cholesterol, HCD lysine (HL), and HCD arginine (HA) as shown in Table 1a. The detailed composition of the diets of each group and the composition of the amino acids varied in the diet is shown in Table 1b. Where groups control, HCD: High diet cholesterol, HL: high Lysine and HA: high arginine. Values are mean ± SD of 6 animals. Hypertension was induced through hypercholesterolemia in Wistar rats (7 weeks) and was supplemented with diets (high arginine, high lysine) for the next 6 weeks. Measurement of systolic blood pressure (SBP) undertaken thrice a week by the tail-cuff method and the average of three values was considered. (Electro-sphygmomanometer, Model 179, Blood pressure analyzer IITC, Woodland Hills, CA, USA).

**Biochemical parameters**

In plasma, the blood urea nitrogen (BUN), plasma total cholesterol (TC), TAG, LDL-C were quantified by following Agapee diagnostic kits manufacturer’s protocol (Kerala, India). Liver (100mg) samples harvested were homogenized in 400 mM Tris buffer (pH 7.2) containing protease inhibitors. Liver lipid profiles such as TC and TAG were measured in the liver (Folch, Lees & Stanley, 1957: Fletcher, 1968). Homogenization of kidney tissue (100 mg) was done in ice-cold 50mM phosphate-buffered saline (PBS, pH 7.2) with 5µM phenyl methyl sulfonylfluoride, 10µM dithiothreitol as protease inhibitors. The ADMA and homocysteine assays were performed as per the manufacturer’s protocol (My BioSource, California, USA).

**Histopathological studies**

The vital organs harvested from each rat were washed with saline, blotted, weighed and preserved in 10% (v/v) phosphate-buffered formalin, at pH 7.0. The preserved organs were processed to examine liver morphology, tissues were sliced, embedded in paraffin wax, 5µM thick sections were cut and exposed to hematoxylin and eosin (H&E) staining, and Picrosirius red staining (Pas) for detailed microscopic examination. The tissue slides were scored for fat droplets using a conventional light microscope (200×) (Hsieh et al., 2014).

**Gene expression of molecular markers**

Total RNA extraction from frozen liver and kidney tissues were homogenized using TRI Reagent (1.0mL) (Sigma-Aldrich, USA). The specific primers of particular genes were designed and synthesized were
procured from Sigma-Aldrich (Mumbai, India) with details listed in (Table 1b). Hypertensive markers; ACE1: Angiotensin-I converting enzyme, Args1: Arginase1, Args 2: Arginase2, AT1R: Angiotensin II subtype 1 receptor, DDAH1: Dimethylargininedimethylamino hydroxylase 1, DDAH2: Dimethylargininedimethylamino hydroxylase 2, iNOS: induced nitric oxide synthase, eNOS: endothelial nitric oxide synthase. NGAL: Neutrophil gelatinase-associated lipocalin, TFF3: Trefoil factor 3, KIM-1: Kidney injury molecule-1, CaMKII:Ca/CaM-dependent Protein Kinase, Cys-C: Cystatin C. Hypocholesterolemic markers; HMG CoAR: 3-hydroxy-3-methylglutaryl CoA reductase, Cyp7A1: Cholesterol-7-hydroxylase, LDLr: Low-density lipoprotein receptor, PPAR γ: Peroxisome proliferator-activated receptors γ, LPL: Lipoprotein lipase, ACC2: Acetyl-CoA carboxylase 2, SREBP1c: Sterol regulatory element-binding protein 1, SREBP2: Sterol regulatory element-binding protein 2.

For cDNA synthesis, total RNA (20µg) is used as a template to generate cDNA using a cDNA archive kit (Fermentas-Thermo fisher scientific). In qPCR quantitation, SYBR green PCR core reagents (Fermentas-Thermo fisher scientific), forward and reverse primers (1pmol/L) were mixed and the PCR reaction was carried out in CFX manager sequence detection system (Biorad) with 40 cycles of 95°C for 30s at 60°C for 30s each. The reaction mixture without cDNA was placed as the negative control to check the primer dimerization. β-actin was used as an internal control. The housekeeping gene β-actin was used for normalization. The relative mRNA quantities of β-actin and gene of interest were measured by qPCR using 2^−∆∆Ct Livak method.

Western blot analysis of hypertensive markers

Frozen kidney samples were homogenized in RIPA buffer Sigma (Mumbai, India), and then centrifuged at 12,000 rpm for 20 min at 20°C. Supernatants were collected, and the protein concentrations were determined by Lowry’s method (Lowry et al., 1951). The western blotting assay was performed against specific antibodies of rat polyclonal antibody against AT1R (sc-1173-G) (Santacruz), ACE1 (ab134709), and polyclonal rabbit antibody against β-actin (ab8227) (Abcam). HRP-conjugated secondary antibody (sc-2030) was obtained from Santa Cruz Biotechnology as previously described (Vallabha et al., 2016).

Statistical analysis

All the results were represented as the mean ± standard deviation and were analyzed with Microsoft Excel 2016. One-way analysis of variance (ANOVA) with post-hoc test was applied. A value of P < 0.05 was considered statistical significance.

Results

Arginine is a known vasodilator whereas, lysine though an essential amino acid but in excess leads to hypercholesterolemia. Our work aims to evaluate the efficacy of two amino acids, arginine and lysine. It is now essential to maintain a specific concentration ratio of lysine that would prevent hypercholesterolemia-inducing hypertension. The effect of dietary supplementation of high arginine and high lysine has been evaluated in the hypertensive Wistar rat model induced through
hypercholesterolemia. For the first time, this study highlights the importance of high arginine and high lysine ratios, which may result in an intense effect on hypertension and hypercholesterolemia.

**Effect on hypertensive markers regulating RAS and NOS-DDAH pathway**

Systolic blood pressure (SBP) significantly decreased by ($p < 0.001$) 25 mmHg in a diet supplemented with high arg:lys amino acids (HA group) compared to the High cholesterol diet group (HCD group) (Fig. 1a). The qRT-PCR analysis of renal hypertensive markers showed a significant decrease in the expression levels of vasoconstrictors, ACE-1, and AT1R by ($p < 0.001$) 6.7 folds and ($p < 0.001$) 11.4 folds respectively in the HA group compared to HCD group (Fig. 1d). Another western blot method also showed a significant decrease ($p < 0.001$) in the gene expression of ACE-1 and AT1R as shown in (Fig. 1b and c). Similarly, the renal mRNA expression of vasodilator, eNOS was effectively regulated with a significant increase ($p < 0.02$) (0.65 fold) boosting vasodilation after dietary supplementation of arginine (HA group) compared to HCD group [Figure 1d]. In the NOS-DDAH pathway, the DDAH2 mRNA expression levels increased by ($P < 0.03$) 1.11 folds in the HA treatment group compared to the HCD group (Fig. 1d). Both the cardiometabolic markers like ADMA ($P < 0.001$) (1.4 fold) and homocysteine ($P < 0.001$) (2.1 fold) considerably decreased concurrently in the HA group compared to HCD group (Table 2). Overall, the data shows hypotensive evidence after dietary supplementation of high arg with vasodilatory effect in the NOS-DDAH pathway and prevents vasoconstriction in the renin-angiotensin system (RAS).

**Biomarker of ADMA, NGAL associated with Signaling pathways and chronic kidney disease (CKD)**

The biomarker signaling associated with kidney impairment in CKD improved after dietary arginine supplementation. In CKD condition, eNOS activation via upregulation of Calcium/calmodulin-dependent protein kinase (CaMKII), which shows an increase by ($P < 0.001$) 0.1 folds in HA group compared to HCD group with a decrease in ADMA mediating increase in nitric oxide production ($p < 0.001$) (Fig. 2a). The mRNA levels of urinary markers, kidney injury marker-1 (KIM-1) showed ($P < 0.001$) 1.75 fold decrease and Cystatin-C (Cys-C) decreased by ($P < 0.001$) 3.5 fold after dietary supplementation of high arginine (HA group) compared to HCD group indicated lowering of nephrotoxicity (Fig. 2b). The blood urea nitrogen (BUN) levels show a significant decrease ($P < 0.001$) (7.6 fold) in the HA group (Table 2).

**Effect on hepatic hypercholesterolemic markers regulating cholesterol and TAG homeostasis**

The mRNA expression of liver the SREBP2 regulates the synthesis of LDLr upregulated by ($P < 0.8$) 2.30-folds with a subsequent increase in the LDLr levels by ($P < 0.012$) 0.58-folds in the HA group compared to HCD group respectively (Fig. 3a). Whereas HMG-CoAR involved in cholesterol metabolism was significantly decreased ($P < 0.001$) in the HL group compared to HCD group (Fig. 3a). These results indicate that the high arginine dietary supplementation involved in the decrease in lipid levels in liver.
tissue. Dietary supplementation of highly: arg (6:1) maintained hypercholesterolemia whereas, high arg: lys (6:1) restored to the normal liver condition in the induced group. In plasma, TC (P < 0.001) (1.84 fold) and TAG (P < 0.001) (1.47 fold) levels were decreased significantly in the HA group compared to the HCD group. A similar effect is observed with the lowering of lipid levels in hepatic TAG in the HA group compared to the HCD group (P < 0.001) (18.17 folds) [Table 2]. These results indicate that the high arginine supplementation regulates cholesterol and TAG homeostasis exhibiting an anti-hypercholesterolemic effect in the HCD group.

The hepatic mRNA expression levels of ACCβ involved in β-oxidation showed (P < 0.001) 0.55-fold decrease with high arg supplementation, whereas antagonist effect was observed with high lys (P < 0.001) (2.2-folds increase) compared to HCD group (Fig. 3b). The mRNA levels of FAS were down-regulated (P < 0.001) (1.87-folds) in association with the up-regulation of LPL (P < 0.001) (0.58-folds) in HA group compared to HCD group (Fig. 3b). TAG levels were considerably decreased concurrently in the HA group compared to the HCD group (Table 2). These results are supported by histopathological pieces of evidence in liver tissues where the lipid accumulation is remarkably reduced in the HA, whereas no effect was observed in the HL (Supplementary, Figure S1a, and S1b).

Discussion

Overall, the data shows hypotensive evidence after dietary supplementation of high arg with vasodilatory effect in the NOS-DDAH pathway and prevents vasoconstriction in the renin-angiotensin system (RAS). According to previous studies, in hypertension and hypercholesterolemia patients, the increased ADMA is associated with reduced DDAH activity due to oxidative stress (Boger, 1998). Several mechanisms contribute to impaired nitric oxide (NO) production which is coordinated with decreased NOS expression during hypercholesterolemia condition (Jobgen et al., 2006). The cholesterol feeding reported decreasing the NOS activity in the erythrocytes from rats. The mouse ATR1A functions controlling vascular and hemodynamic responses to angiotensin II and change in expression of the ATR1 gene has marked effects on blood pressure (Crowley, Gurley & Coffman, 2007). Dietary nitrate has shown to reduce blood pressure, and restore endothelial function (Zand et al., 2011). Furthermore, the combing of the experimental model to the analysis of nephrological damage used induced Wistar rats. Chronic kidney disease (CKD) leads to progressive damage of the renal cells and the loss of nephrons, which cause chronic renal injury (Viau et al., 2010). The above mentioned nephrological biomarkers innovations are better for appropriate diagnosis, to predict the severity and outcome, and to observe the proximal tubule injury in CKD. Urinary KIM-1, NGAL, and serum Cys-C levels were significantly higher in AKI patients (Lei et al., 2018). Kajimoto et al. explored the novel mechanism of ADMA signaling link with impaired endothelial function in CKD (Kajimoto et al., 2012).

In the consequence of hypercholesterolemic homeostasis, It was reported that there was a statistically significant reduction in 72% of patients with TAG levels (P < 0.05) (150 mg/dL) after 30 days with the nitric oxide (NO) dietary supplement compared to control. This suggests that dietary supplement of L-
arginine restore NO homeostasis and reduce TAGs, thereby reducing the overall burden of risk of the
development of CVD (Zand et al., 2011).

LPL catalyzes the hydrolysis of TAG contained in lipoproteins and chylomicrons that release the free fatty
acids. The LPL also regulates the LDL-C levels in plasma and liver. Our results also implicate that the
uptake of the LDL-C by LDLr regulated the cholesterol levels in the blood.

Conclusion

In conclusion, the physiological changes exhibited a decrease in systolic blood pressure, TAG, and TC
levels in both liver and blood plasma with high arginine supplementation. The hepatic genes SREBP2,
ACCβ, and LPL were upregulated indicating a decrease in lipid levels after high arginine supplementation.
The hypertensive markers ACE, and AT1R responsible for renal hemodynamics were effectively regulated
and upregulation of eNOS exert vascular protective effect after high arginine supplementation.
Downregulation of KIM-1, and NGAL after arginine supplementation indicating lowering of renal
nephrotoxicity. The high arg: lys ratio has a prominent influence in the effective regulation of the
hypertension molecular markers in combating systolic blood pressure and controlling the
hypercholesterolemia, and biomarkers of renal impairment recovery.

Declarations

Conflict of interest statement

There are no conflicts of interest to be declared by the authors.

Authors Contribution

Vishwanath Singanodi Vallabha- designed, performed the experiments and prepared the manuscript,
Purnima Kaul Tiku- conceptualized, designed and analyzed the data, and Varun Kumar- performed the RT-
PCR experiment for genes expression, and Shinde Vijay Sukhdeo help in animal experiment.

Acknowledgements

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work in the institute.

Ethical Approval: Institutional animal ethics committee (IAEC) (IEAE no. 222/12) was taken.

References


Tables
<table>
<thead>
<tr>
<th>Group Labels</th>
<th>Group Names</th>
<th>Diet Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>20% Casein protein</td>
</tr>
<tr>
<td>HCD</td>
<td>High cholesterol diet</td>
<td>20% Casein protein + 1% cholesterol</td>
</tr>
<tr>
<td>HL</td>
<td>HCD induced lysine (lys: arg= 6:1)</td>
<td>10% Casein protein + 10% lysine + 1% cholesterol</td>
</tr>
<tr>
<td>HA</td>
<td>HCD induced arginine (arg: lys= 6:1)</td>
<td>10% Casein protein + 10% arginine + 1% cholesterol</td>
</tr>
</tbody>
</table>

**Table 1A.** Randomization of groups of Wistar rats based on their blood pressure into control, hypercholesterolemia induced hypertensive groups (HCD).

<table>
<thead>
<tr>
<th>Markers</th>
<th>Primer</th>
<th>Sequence</th>
<th>Size</th>
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</thead>
<tbody>
<tr>
<td>DDAH2</td>
<td>Forward</td>
<td>5'-AAACGGCACAAAAAGCAAT-3'</td>
<td>18</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCGTCTGGAGATGGTGCTA-3'</td>
<td>20</td>
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<tr>
<td>eNOS</td>
<td>Forward</td>
<td>5'-ACACAGCAAGACACTGAA-3'</td>
<td>17</td>
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<td></td>
<td>Reverse</td>
<td>5'-GCCGCGCTCTGTAACCTCCT-3'</td>
<td>20</td>
</tr>
<tr>
<td>ATR1</td>
<td>Forward</td>
<td>5'-ACAGAAGTACATCGTCATAT-3'</td>
<td>20</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-AATTTTTCCCAGAAAAACC-3'</td>
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<tr>
<td>ACE1</td>
<td>Forward</td>
<td>5'-CAACGCGGAAGGCTGCTCT-3'</td>
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<td></td>
<td>Reverse</td>
<td>5'-CAACAGAGACTGACACCTGCTGTC-3'</td>
<td>25</td>
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<tr>
<td>β-Actin</td>
<td>Forward</td>
<td>5'-CTAAGGGCAACCCGTGAA-3'</td>
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<td></td>
<td>Reverse</td>
<td>5'-TCCGCTGGAACCTGTCCTCA-3'</td>
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<tr>
<td>CamKII B</td>
<td>Forward</td>
<td>5'-AGGAAAGTCCAGTCCAG-3'</td>
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<td>Reverse</td>
<td>5'-CAGAGATTAGTGGCATGATG-3'</td>
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<td>NGAL</td>
<td>Forward</td>
<td>5'-ACACGTACCTAGATACAGAGCTA-3'</td>
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<td>5'-CATTTGCTGCAATGGATGTGCGT-3'</td>
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<tr>
<td>Cystatin C</td>
<td>Forward</td>
<td>5'-TGCGTACCCACAAGCCG-3'</td>
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<td></td>
<td>Reverse</td>
<td>5'-CCATCTCCACATCCAATATAAT-3'</td>
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<td>KIM-1</td>
<td>Forward</td>
<td>5'-GGGCGTGTGAGATCTCAGA-3'</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-GCGACGCCAGATCCACATA-3'</td>
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<tr>
<td>HMGCoAR</td>
<td>Forward</td>
<td>5'-AACGGGGCGCGGCAAAAGCAATC-3'</td>
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<td>Reverse</td>
<td>5'-ATACGCGCAGAAAAAGAGAAACCATAAT-3'</td>
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<td>SREBP2</td>
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<td>LKL</td>
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<td>5'-CGAAAATGCTAAAGATTGCGG-3'</td>
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<td>ACCβ</td>
<td>Forward</td>
<td>5'-CATCAATGATGTGCAATTTGGC-3'</td>
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<td></td>
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<td>5'-GGTGGGCGGGATGGTTTCTC-3'</td>
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<td>FAS</td>
<td>Forward</td>
<td>5'-GCCGAGATCCCTGGAACGTTAA-3'</td>
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<td>LDLr</td>
<td>Forward</td>
<td>5'-AGAAGCGGGGCGGCGAAGAAGC-3'</td>
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<td>5'-AAACCGGTGGGACATAGCCACTGAC-3'</td>
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**Table 1b.** Primer sequences of hypertensive and hypercholesterolemic markers.
<table>
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<th>Control</th>
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<th>HL</th>
<th>HA</th>
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<tr>
<td><strong>Plasma</strong></td>
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<tr>
<td>TC (mg/dL)</td>
<td>74.8 ± 8.0</td>
<td>155.5 ± 37</td>
<td>91.1 ± 5*</td>
<td>83.6 ± 8*</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>121.1 ± 51</td>
<td>605.6 ± 99</td>
<td>264.9 ± 61*</td>
<td>292.5 ± 53*</td>
</tr>
<tr>
<td>TAO (mg/dL)</td>
<td>4.7 ± 0.3</td>
<td>29.1 ± 4.2</td>
<td>24.6 ± 4.7</td>
<td>19.7 ± 3.2*</td>
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<tr>
<td>BUN (mg/dL)</td>
<td>25.5 ± 11</td>
<td>36.6 ± 3</td>
<td>50.8 ± 11*</td>
<td>14.8 ± 2*</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
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<td></td>
<td></td>
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<tr>
<td>TC (mg/g)</td>
<td>54.8 ± 4.0</td>
<td>122.3 ± 16</td>
<td>81.3 ± 6*</td>
<td>73.6 ± 4*</td>
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<tr>
<td>TAG (mg/g)</td>
<td>6.4 ± 1.2</td>
<td>42.7 ± 4.3</td>
<td>30.6 ± 0.7</td>
<td>23.5 ± 3.4*</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
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<tr>
<td>ADMA (μM/mg)</td>
<td>11.0 ± 0.9</td>
<td>18.5 ± 0.6</td>
<td>15.0 ± 0.4</td>
<td>13.2 ± 0.6*</td>
</tr>
<tr>
<td>Homocysteine (mM/mg)</td>
<td>3.4 ± 0.4</td>
<td>5.2 ± 0.4</td>
<td>4.8 ± 0.4</td>
<td>2.4 ± 0.4*</td>
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</table>

*Table 2.* Groups of Wistar rats showing changes in plasma parameters Total cholesterol (TC), Low-density lipoprotein–cholesterol (LDL-C), Triacylglyceride (TAG), Blood Urea Nitrogen(BUN), and liver lipid profiles such as TC, TAG, and kidney ADMA, homocysteine levels in the hypertensive group. Values are mean ± SD of 6 animals. * indicates the (p<0.001) value is significantly different compared to the HCD group.

**Figures**
Figure 1. (A) Effect of amino acid supplementation on Systolic blood pressure (mmHg) in Wistar rats fed with high cholesterol diet to induce hypertension through hypercholesterolemia (B) Protein expression of renal marker ACE1 quantified by showing their relative density using western blot method from each group (C) Protein expression of renal markers AT1R quantified by showing their relative density using western blot method from each group (D) The mRNA expression of renal hypertensive markers AT1R, ACE1, DDAH2, and eNOS by qRT-PCR analysis were normalized using β-actin housekeeping gene and quantified by ΔΔCt Livak method. Fold change in the expression of genes are relative to the control as shown. Data points are presented as mean ± SD in triplicate experiments (n = 6). * indicates the (p<0.05), and (p<0.001) value are significantly different compared to the HCD group. The groups were classified into control (●), HCD: high cholesterol diet (○), HL: HCD lysis (■), and HA: HCD extract (▲).

Figure 1

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Figure 2. (A) qRT-PCR analysis of mRNA expression of renal nephrological markers, NGAL, CaMKII, TFF3 were normalized using β-actin housekeeping gene and quantified by ΔΔ²⁰C Livaq method (B) qRT-PCR analysis of mRNA expression of renal nephrological markers, urinary nephrological markers CystatinC, KIM-1 were normalized using β-actin housekeeping gene and quantified by ΔΔ²⁰C Livaq method. Fold change in the expression of genes are relative to the casein diet as shown. Data points are presented as mean ± SD in triplicate experiments (n =6). * indicates the (p<0.001) value is significantly different compared to the HCD group. The groups were classified into control (■), HCD: high cholesterol diet (□), HL: HCD lysine (●), and HA: HCD arginine (○).

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Figure 3. (A) qRT-PCR analysis of mRNA expression of hypercholesterolemic markers SREBP2, HMG CoAR and LDLr of cholesterol metabolism were normalized using β-actin and quantified by ΔΔ^CT Livaq method. (B) ACCβ, LPL and FAS of TAG metabolism were normalized using β-actin housekeeping gene and quantified by ΔΔ^CT Livaq method. Fold change in the expression of genes are relative to the casein diet (=1.00) as shown. Data points are presented as mean ± SD in triplicate experiments (n =6). * indicates the (p<0.8, p<0.001, p<0.012) value is significantly different compared to the HCD group. The groups were classified into control (■), HCD: high cholesterol diet (□), HL: HCD lysine (●), and HA: HCD arginine (●).

See image above for figure legend

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- Suplimetry.docx