


L-arginine Supplementation Increased Only Endothelium-Dependent Relaxation in Sprague-Dawley Rats Fed a High-Salt Diet by Enhancing Abdominal Aorta Endothelial Nitric Oxide Synthase Gene Expression

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ABSTRACT

BACKGROUND: Abnormal vascular reactivity and reduced expression of endothelial nitric oxide synthase (*eNOS*) gene are hallmark of salt-induced hypertension in rats. Although L-arginine is an established vasodilator, the mechanism by which it modulates vascular reactivity in salt-induced hypertension is not clearly understood.

OBJECTIVES: This study was designed to investigate the mechanism by which oral L-arginine supplementation modulates vascular reactivity and *eNOS* gene expression in Sprague-Dawley rats fed a high-salt diet.

METHODS: Forty-eight weaned male Sprague-Dawley rats of weight range 90 to 110g were randomly divided into 6 groups of 8 rats per group. Group I was fed normal rat chow *ad libitum* and served as the Normal Diet group. Group II was fed a diet that contained 8% NaCl. Groups III and IV took normal and high-salt diet, respectively, and then received oral L-arginine supplementation (100mg/kg/day), while groups V and VI took normal and high-salt diet, respectively, and then were co-administered with both L-arginine and L-nitro-arginine methyl ester (L-NAME; 100mg/kg/day and 40mg/kg/day, respectively) orally. At the end of 12-week experimental period, the animals were sacrificed to assess vascular reactivity and gene expression level.

RESULTS: Our results show that high-salt diet significantly reduced ($P < .05$) endothelium-dependent relaxation response to acetylcholine and qualitatively reduced *eNOS* gene expression in the abdominal aorta of the rats. However, L-arginine supplementation improved the impaired endothelium-dependent relaxation and nitric oxide level while ameliorating the reduced *eNOS* gene expressions.

CONCLUSION: This study suggests that oral supplementation of L-arginine enhances endothelial-dependent relaxation in rats fed a high-salt diet by ameliorating *eNOS* gene expression in the abdominal aorta of the rats.

KEYWORDS: L-arginine, eNOS—endothelial nitric oxide synthase, high-salt diet hypertension, vascular reactivity, SD, Sprague-Dawley rats

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Background

Sustained increase in blood pressure is arguably the most important cardiovascular risk factor.¹ High dietary salt intake has been shown to lead to this sustained increase in blood pressure in different animal species and humans.^{2–4} Vascular function impairment is known to be part of the key mechanisms by which high-salt diet leads to the increase in blood pressure.^{5–7} In the endothelial cells, endothelial nitric oxide synthase (*eNOS*) helps in the production of citrulline and nitric oxide from L-arginine.⁸ The *eNOS* mRNA expression has, however, been reported to be a key modulator of vascular tone, and its uncoupling has been shown to contribute to development of hypertension.^{9,10} For example, in the *ApoE* Knock-Out mouse model, the deficient *eNOS* system was reported to be mainly responsible for causing the hypertension.¹⁰ In some other studies, mice disrupted of the

eNOS gene were essentially absent of acetylcholine-induced relaxation and subsequently become hypertensive.¹¹

L-Arginine is found abundantly in dietary proteins.¹² It is not only a necessary substrate in the nitric oxide (NO) pathway but also an inhibitor of the renin angiotensin system (RAS). It has also been reported to be partially involved in the regulation of insulin secretion.¹² Although L-arginine is produced endogenously, exogenous intake through diet has been shown not to only contribute to the body's supply but also address alterations in the metabolism of L-arginine. To buttress this point, several studies in animals and humans show that L-arginine supplementation ameliorates sustained increase in blood pressure and cause improvement in vascular function.^{13–15}

Although L-arginine is an established vasodilator¹⁴ and has been shown to possess antioxidant properties,¹⁶ the precise role



of exogenous L-arginine in the vascular reactivity response to salt loading is not clearly understood. Precisely, there is dearth of information on the direct effect it has on the *eNOS* gene expression in salt-induced hypertension. A study to ascertain the precise endothelial effect of exogenously administered L-arginine in salt-induced hypertension is therefore germane at this point to understand the interplay between exogenous and endogenous L-arginine in the modulation of vascular reactivity in salt-induced hypertension. This study thus sought to determine the mechanism by which exogenous L-arginine supplementation alters vascular reactivity and *eNOS* gene expression in Sprague-Dawley (SD) rats fed a high-salt diet.

Methods

Experimental animals

Forty-eight (48) weaned male SD rats of weight range 90 to 110g were used for this study. The rats were divided into 6 groups of 8 rats per group (Oloyo et al., 2011) by simple random sampling such that the mean difference in weight across the groups was not statistically significant ($P > .05$). These rats were obtained from the Laboratory Animal Center, College of Medicine of the University of Lagos. The rats were acclimatized for 2 weeks before the commencement of the study. All through the period of the study, depending on the groups, they were either fed with normal rat chow containing 0.3% salt or high-salt diet containing 8% salt (Sofola et al., 2002; Oloyo et al., 2011; Oloyo et al., 2016; Adejare et al., 2017). The rats were housed in transparent cages where they had free access to food and clean water. They were maintained in a well-ventilated environment under standard environmental conditions (28°C-30°C, 12-hour light/12-hour dark cycle).

Ethical statement

This protocol was approved by the Health Research Ethics Committee of the College of Medicine of the University of Lagos (Approval number CM/HREC/12/16/080). All procedures for this study were carried out in strict adherence to the National Institutes of Health Guide for the care and use of laboratory animals.¹⁷

Study design

Rats were randomly divided into 6 groups containing 8 rats per group by an observer unaware of the treatment groups. Group I rats were fed normal rat chow *ad libitum* and served as the Normal Diet group. Group II rats were fed a diet that contained 8% NaCl.^{6,7} Groups III and IV rats took normal and high-salt diet, respectively, and then received oral L-arginine supplementation (100mg/kg/day), while groups V and VI took normal and high-salt diet, respectively, and then were co-administered with both L-arginine and L-nitro-arginine methyl ester (L-NAME; 100mg/kg/day and 40mg/kg/day,

Table 1. Grouping of animals.

GROUPS	RAT DIET (N=8)
Group I	Normal diet
Group II	High-salt diet
Group III	Normal salt + L-arginine
Group IV	High-salt diet + L-arginine
Group V	Normal salt diet + L-arginine and L-NAME
Group VI	High-salt diet + L-arginine and L-NAME

Abbreviation: L-NAME, L-nitro-arginine methyl ester.

respectively) orally.¹⁵ The grouping is as illustrated in Table 1. At the end of 12-week experimental period, the animals were sacrificed to assess vascular reactivity and gene expression level.

Experimental procedure

Preparation of abdominal aortic rings. Rats were sacrificed by cervical dislocation. Immediately after dislocation, the abdominal region was opened and the abdominal aorta cut out and placed in a Petri dish containing cold Physiological Salt Solution (PSS) at 4°C. The abdominal aorta was freed of fat and connective tissues. The aorta was then cut into ring segments of about 2 to 3 mm. The ring was thereafter mounted between one long and one short stainless steel hooks. The small S-shaped hook was attached to the base of the organ bath, while the long L-shaped rod was attached to the isometric force transducer (top force transducer MLT 050/D; AD Instruments, Bella Vista, Australia) that was attached through MLAC11 Grass adapter cable to a computerized data acquisition system with LabChart-7 pro software (Power Lab-4/24T, model MLT844/P; AD Instruments Pty Ltd., Castle Hill, Australia). During this procedure, special care was taken to avoid rubbing the endothelial surface of the rings. The 20 mL organ bath contained PSS with the composition: 119.0 mol/L NaCl, 4.7 mol/L KCl, 1.2 mol/L KH₂PO₄, 1 to 2 mol/L MgSO₄, 24.9 mol/L NaHCO₃, 1.6 mol/L CaCl₂, and 11.5 mol/L glucose at 37°C. The pH of the PSS was adjusted to 7.4 and the set-up gassed with 95% O₂:5% CO₂ mixture.⁶

For each ring, a passive tension of 1.5 g was applied and the ring was allowed to equilibrate for 90 minutes in the PSS during which at 30 minutes interval, it was subjected to a dose of 10⁻⁶ M noradrenaline. The ring was rinsed after each stimulations. This 90 minutes stabilization was necessary to ensure a consistent response during the experiment. After this stabilization period, the relaxation response to graded doses of acetylcholine (10⁻⁹-10⁻⁴ M) and sodium nitroprusside (SNP; 10⁻¹⁰-10⁻⁴ M) was assessed separately in the absence and presence of L-nitro-arginine-methyl-ester (L-NAME, 10⁻⁴ M) and methylene blue (MB, 10⁻⁵ M) following pre-contraction

Table 2. PCR primers and PCR protocols.

PRODUCT SIZE	PCR PRIMER SEQUENCES	PCR PROTOCOLS
GAPDH	Forward: 5'-TCCCTCAAGATTGTCAGCAA-3'	94°C/30 s 57°C/40 s 72°C/minutes } 28 cycles
308 bp	Reverse: 5'-AGATCCACAACGGATACATT-3'	
eNOS	Forward: 5'-CATGTTTGTCTGCGGTGATGT-3'	94°C/30 s 57°C/40 s 72°C/min } 32 cycles
96 bp	Reverse: 5'-GCTTCATCCAGCTCCATGCT-3'	

Abbreviations: eNOS, endothelial NOS; GAPDH, glyceraldehyde-3-PO₄ dehydrogenase; PCR, polymerase chain reaction.

with 10⁻⁷M NA. In the presence of the inhibitors, the incubation lasted for 30 minutes before pre-contraction and application of graded doses of acetylcholine or SNP. Endothelium-intact rings were used for acetylcholine-induced relaxations, while endothelium-denuded rings were used for SNP-induced relaxations. All experiments took place under the same environmental temperature and pressure.

Gene expression of eNOS

Dissection. Following cervical dislocation of the rats for gene expression studies, the abdominal region was opened and the abdominal aorta excised. The abdominal aorta was placed on a phosphate-buffered saline (PBS) pre-cooled plate. The adherent connective tissues were then gently removed and the tissue frozen in liquid nitrogen (-170°C) for subsequent analysis. During dissection, special care was taken to avoid unnecessary stretching of the abdominal arteries.

RNA isolation. Ribonucleic acid (RNA) extraction was carried out in the cut segments of the abdominal aorta using AurumTM Total RNA Mini Kit (Catalog #732-6820; Bio-Rad Laboratories Inc., Hercules, CA, USA). The extracted RNAs were then treated with RNase-free DNase and the concentrations determined by ultraviolet (UV) light absorbance with wavelength set at 260 nm.

Reverse transcription polymerase chain reaction. With the aid of an Oligo-dT primer, reverse transcription polymerase chain reaction (RT-PCR) was carried out to synthesize cDNA from the extracted RNA using an Omniscript RT Kit. The synthesized complementary DNA (cDNA) fragments were then amplified using HotStar HiFidelity Polymerase Kit. The primer sequence used for the amplification is as illustrated in Table 2. A thermal cycler (iCycler; Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the operating conditions set at 5 minutes denaturation, 30 seconds at 94°C; annealing, 40 seconds at 57°C; and a 1 minute extension step at 72°C was used to perform the PCR cycles. A final extension step of 5 minutes at 72°C followed the last cycle to complete the procedure. To check for genomic DNA contamination, samples were subjected to the same PCR procedure in the absence of the reverse transcriptase enzyme. The process was finalized by separating

the PCR products by gel electrophoresis. The gel prepared consisted of 1.5% agarose. The products were visualized by ethidium bromide staining under ultraviolet light. The house-keeping gene used for the study was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and all results were presented relative to its expression. Prior to the commencement of the study, database search of GenBank was performed with BLASTN.

Experimental outcomes

The experimental outcomes for this study include the maximum relaxation to acetylcholine or SNP, -Log EC₅₀, and expression level of the eNOS gene in each group. Relaxation responses following pre-contraction with noradrenaline were calculated and used to assess vascular reactivity. Abdominal aortic rings were obtained from each animal and used for vascular reactivity. Results from the first 8 viable rings from the 6 animals were used in the statistical analyses. Abdominal aortic rings that were not viable were discarded.

Statistical analyses

The collected data were expressed as means ± standard error of mean (SEM) and were analyzed using one-way analysis of variance (ANOVA; independent group is categorical vs dependent group, ie the outcome that is continuous) followed by Student-Newman-Keuls post hoc test (because there were mean differences in some groups). A *P*-value less than .05 (*P* < .05) was considered significant. For the vascular reactivity part, GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to generate -Log EC₅₀ values, which were subsequently subjected to statistical analysis.

Results

Effect of salt-load and L-arginine supplementation on relaxation response to acetylcholine

Relaxation responses were significantly reduced (*P* < .01) in salt-loaded group (maximal response of 32.24% ± 2.74%) compared to Normal Diet (maximal response of 81.29% ± 4.92%). There was a significant (*P* < .01) increase in -Log EC₅₀ value in the salt-loaded group compared to normal diet (Table 3). In the groups supplemented with oral L-arginine, the relaxation

Table 3. -Log EC₅₀ and percent maximum relaxation response of abdominal aortic rings to acetylcholine with or without L-NAME.

GROUPS	-LOG EC ₅₀		% MAXIMUM RELAXATION	
	ACH	ACH + L-NAME	ACH	ACH + L-NAME
Normal diet	7.98 ± 0.35	7.43 ± 0.67	81.29 ± 4.92	32.31 ± 2.75
Salt-loaded	6.47 ± 1.23**	–	32.24 ± 2.74‡‡	23.53 ± 1.36
ND + L-arginine	8.08 ± 0.12	7.76 ± 0.31	84.13 ± 2.79	26.93 ± 1.70
SD + L-arginine	7.32 ± 0.21**	7.03 ± 0.34	53.91 ± 2.76‡	26.82 ± 1.25
ND + L-arginine + L-NAME	8.01 ± 0.08	8.97 ± 0.01	78.56 ± 2.44	20.06 ± 1.77
SD + L-arginine + L-NAME	6.89 ± 0.09**	6.47 ± 1.00	44.47 ± 1.71‡	18.21 ± 1.28

Abbreviations: ND, normal diet; SD, salt diet.

Data are presented as mean ± SEM.

Significantly higher (** $P < .01$) compared with corresponding normal diet group. Significantly lower (‡ $P < .05$, ‡‡ $P < .01$) compared with corresponding normal diet group (n=6 rings).

response to acetylcholine was significantly reduced in SD + L-arginine group (maximal response of 53.91% ± 2.76%) compared to ND + L-arginine group (maximal response of 84.13% ± 2.79%). There was also a significant ($P < .01$) reduction in the -Log EC₅₀ in the SD + L-arginine group compared to ND + L-arginine group. In the groups supplemented with L-arginine and L-NAME concomitantly, the relaxation response to acetylcholine was significantly reduced (Figures 1 and 2). It is worthy of note that oral supplementation with L-arginine did not significantly ($P > .05$) change the maximal relaxation response (81.29 ± 4.92 vs 84.13 ± 2.79) to acetylcholine in ND + L-arginine group. However, oral supplementation with L-arginine significantly improved the percent maximal relaxation response to acetylcholine in the rings of the SD + L-arginine group. These effects of L-arginine were attenuated by concomitant administration of L-NAME with it.

Effect of salt-load and L-arginine supplementation on relaxation response to acetylcholine in the presence of L-NAME

Figures 3 and 4 show the relaxation responses to acetylcholine following incubation in L-nitro-arginine-methyl-ester (L-NAME). There was no significant change ($P > .05$) in the maximum relaxation response to acetylcholine in the groups. There was, however, a significant ($P < .05$) increase in -Log EC₅₀ in the SD + L-arginine group when compared with ND + L-arginine group (Table 3).

Effect of salt-load and L-arginine supplementation on relaxation response to acetylcholine in the presence of methylene blue. Relaxation responses were not significantly different ($P > .05$) in the salt-loaded group (maximal response of 15.44% ± 2.50%) compared to the Normal Diet group (maximal response of 11.11% ± 1.10%). In the groups supplemented with oral L-arginine, the relaxation response to acetylcholine was not

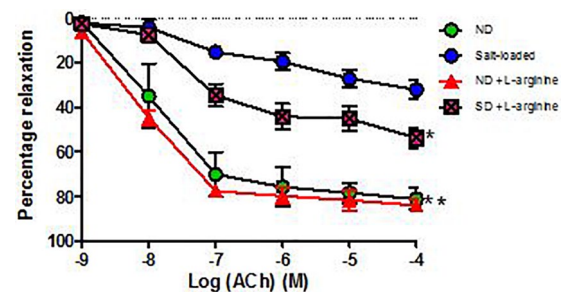


Figure 1. Percentage relaxation response of aortic rings to acetylcholine (ACh) in Groups 1 to 4 (6 rings). Relaxation responses are expressed as percentage of decrease in submaximal contraction elicited by NA (10-5M). Each point on the graph represents mean ± SEM. L-NAME indicates L-nitro-arginine-methyl-ester; NA, noradrenaline; ND, normal diet; SD, salt diet.
**Significant increase ($P < .01$) compared with control.

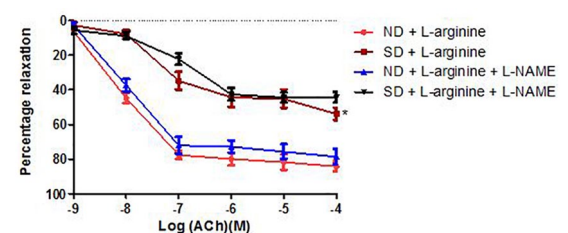


Figure 2. Percentage relaxation response of aortic rings to acetylcholine in Groups 3 to 6 (6 rings). Relaxation responses are expressed as percentage of decrease in submaximal contraction elicited by NA (10-5M). Each point on the graph represents mean ± SEM. L-NAME indicates L-nitro-arginine ethyl-ester; NA, noradrenaline; ND, normal diet; SD, salt diet.
*Significant increase ($P < .05$) compared with SD + L-arginine + L-NAME group

significantly different in the SD + L-arginine group (maximal response of 16.65% ± 1.43%) compared to the ND + L-arginine group (maximal response of 15.26% ± 2.00%; Figures 5 and 6 and Table 4). This pattern was also observed in the groups supplemented with L-arginine and L-NAME. Because

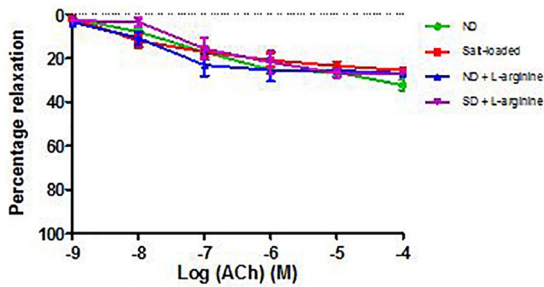


Figure 3. Relaxation response of abdominal aortic rings to acetylcholine (ACh) in the presence of L-NAME across Groups 1 to 4 (6 rings). Relaxation responses are expressed as percentage of decrease in submaximal contraction elicited by NA (10-5M). Each point on the graph represents mean \pm SEM. L-NAME indicates L-nitro-arginine-methyl-ester; NA, noradrenaline; ND, normal diet; SD, salt diet.

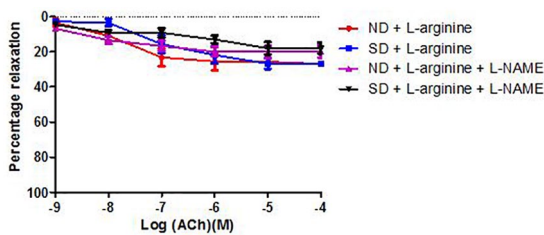


Figure 4. Relaxation response of abdominal aortic rings to acetylcholine (ACh) in the presence of L-NAME across Groups 3 to 6 (6 rings). Relaxation responses are expressed as percentage of decrease in submaximal contraction elicited by NA (10-5M). Each point on the graph represents mean \pm SEM. L-NAME indicates L-nitro-arginine-methyl-ester; NA, noradrenaline; ND, normal diet; SD, salt diet.

of the high degree of inhibition in virtually all the groups to acetylcholine-induced relaxation response in the presence of methylene blue, the $-\text{Log EC}_{50}$ of the rings to acetylcholine could not be computed.

Effect of salt-load and L-arginine supplementation on relaxation response to SNP

The maximal relaxation response to SNP (10^{-10} - 10^{-4}) in NA-precontracted endothelium-denuded abdominal aortic rings was not significantly different ($P > .05$) in the aortic rings from salt-loaded group when compared with Normal Diet. Oral L-arginine supplementation caused no significant change in maximal relaxation response to SNP in all groups. Co-administration of L-arginine with L-NAME had no significant effect on these relaxations. This is illustrated in Figures 7 and 8.

Effect of salt-load and L-arginine supplementation on relaxation response to SNP in the presence of L-NAME

There was no statistically significant difference ($P > .05$) in the maximal relaxation response to SNP in the presence of L-NAME across the groups. Oral L-arginine supplementation

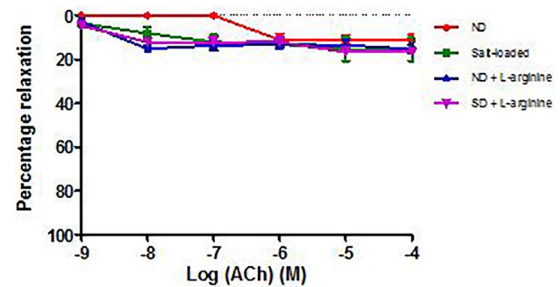


Figure 5. Relaxation response of abdominal aortic rings to ACh in the presence of Methylene Blue (MB) across groups 1 to 4 (6 rings). Relaxation responses are expressed as percentage of decrease in sub-maximal contraction elicited by NA (10-5M). Each point on the graph represents mean \pm SEM.

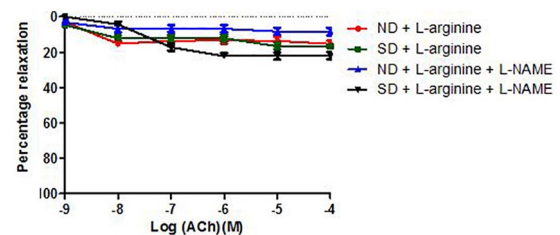


Figure 6. Relaxation response of abdominal aortic rings to ACh in the presence of Methylene Blue (MB) across groups 3 to 6 (6 rings). Relaxation responses are expressed as percentage of decrease in sub-maximal contraction elicited by NA (10-5M). Each point on the graph represents mean \pm SEM of 5 rings from different animals. $*p < 0.05$ compared with Control rats.

slightly increased the vasodilatory responses to SNP in only the salt-loaded group, but the increase was not statistically significant ($P > .05$). Co-administration of L-NAME with L-arginine did not significantly change the relaxation responses to SNP. This is illustrated in Figures 9 and 10 and Table 5.

Effect of salt-load and L-arginine supplementation on relaxation response to SNP in the presence of methylene blue

There was no statistically significant difference ($P > .05$) in the maximal relaxation response to SNP in the presence of MB across the groups. Oral L-arginine supplementation also caused no statistically significant ($P > .05$) difference in the maximal relaxation responses. Co-administration of L-NAME with L-arginine did not significantly change the relaxation responses to SNP. This is illustrated in Figures 11 and 12 and Table 6.

Effects of salt-loading and oral L-arginine supplementation on nitric oxide level

Nitric oxide concentration appears to be lower in the salt-loaded group compared to normal diet but not significantly ($P > .05$). Nitric oxide level was significantly higher ($P < .05$) in the ND + L-arginine group when compared with the normal diet group. Nitric oxide level was also significantly higher

Table 4. $-\text{Log EC}_{50}$ and percent maximum relaxation response of abdominal aortic rings to acetylcholine with or without methylene blue (MB).

GROUPS	$-\text{LOG EC}_{50}$		% MAXIMUM RELAXATION	
	ACH	ACH + MB	ACH	ACH + MB
Normal diet	7.98 ± 0.35	–	81.29 ± 4.92	11.11 ± 1.11
Salt-loaded	$6.47 \pm 1.23^{**}$	–	$32.24 \pm 2.74^{\ddagger\ddagger}$	15.44 ± 2.50
ND + L-arginine	8.08 ± 0.12	–	84.13 ± 2.79	15.26 ± 2.00
SD + L-arginine	$7.32 \pm 0.21^{**}$	–	$53.91 \pm 2.76^{\ddagger}$	16.65 ± 1.43
ND + L-arginine + L-NAME	8.01 ± 0.08	–	78.56 ± 2.44	9.36 ± 2.10
SD + L-arginine + L-NAME	$6.89 \pm 0.09^{**}$	–	$44.47 \pm 1.71^{\ddagger}$	22.89 ± 1.62

Abbreviations: ACh, acetylcholine; MB, methylene blue; ND, normal diet; SD, salt diet (n=6 rings).

Data are presented as mean \pm SEM.

Significantly higher (** $P < .01$) compared with corresponding normal diet group. Significantly lower ($^{\ddagger}P < .05$, $^{\ddagger\ddagger}P < .01$) compared with corresponding normal diet group.

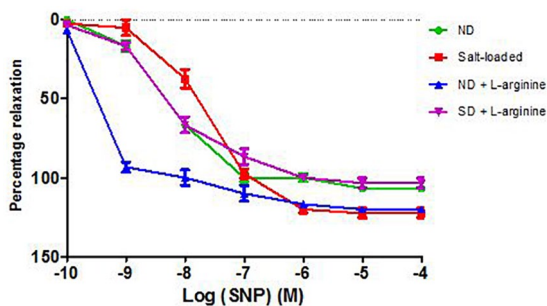


Figure 7. Percentage relaxation response of aortic rings to sodium nitroprusside (SNP) in Groups 1 to 4 (6 rings). Each point on the graph represents mean \pm SEM. ND indicates normal diet; SD, salt diet.

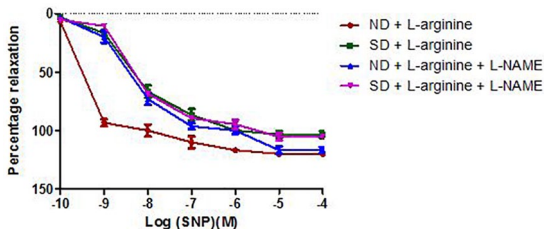


Figure 8. Relaxation response of abdominal aortic rings to sodium nitroprusside (SNP) in Groups 3 to 6 (6 rings). Relaxation responses are expressed as percentage of decrease in submaximal contraction elicited by NA (10-5M). Each point on the graph represents mean \pm SEM. L-NAME indicates L-nitro-arginine ethyl-ester; NA, noradrenaline; ND, normal diet; SD, salt diet.

($P < .001$) in the SD + L-arginine group when compared with the salt-loaded group. These effects of L-arginine were reversed by concomitant co-administration with L-NAME. This is illustrated in Figure 13.

Effects of salt-load and L-arginine supplementation on eNOS gene expression

For all the above-mentioned effects to occur, there must have been changes in the expression of a lot of genes in the

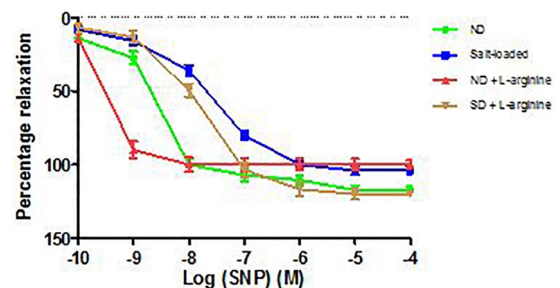


Figure 9. Relaxation response of abdominal aortic rings to sodium nitroprusside (SNP) in Groups 1 to 4 (6 rings) in the presence of L-NAME. Relaxation responses are expressed as percentage of decrease in submaximal contraction elicited by NA (10-5M). Each point on the graph represents mean \pm SEM. L-NAME indicates L-nitro-arginine ethyl-ester; NA, noradrenaline; ND, normal diet; SD, salt diet.

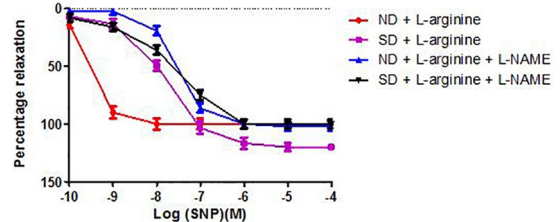


Figure 10. Relaxation response of abdominal aortic rings to sodium nitroprusside (SNP) in Groups 3 to 6 (6 rings) in the presence of L-NAME. Relaxation responses are expressed as percentage of decrease in submaximal contraction elicited by NA (10-5M). Each point on the graph represents mean \pm SEM. L-NAME indicates L-nitro-arginine ethyl-ester; NA, noradrenaline; ND, normal diet; SD, salt diet.

endothelium.^{13,18} Of all these genes, arguably, the most important one is the gene that codes for the eNOS enzyme responsible for conversion of L-arginine to nitric oxide in the endothelium. The expression of eNOS mRNA was lower in the salt-loaded group compared to normal diet group (as illustrated in Figure 14). The reduced expression of eNOS was improved by treatment with L-arginine. Concomitant use of L-NAME with the L-arginine blunted the effect of L-arginine.

Table 5. $-\text{Log EC}_{50}$ and percent maximum relaxation response of abdominal aortic rings to SNP with or without L-NAME.

GROUPS	$-\text{LOG EC}_{50}$		% MAXIMUM RELAXATION	
	SNP	SNP + L-NAME	SNP	SNP + L-NAME
Normal diet	8.28 ± 0.06	8.51 ± 0.07	106.7 ± 3.98	120.40 ± 5.28
Salt-loaded	$7.61 \pm 0.06^*$	$6.69 \pm 0.08^*$	122.50 ± 9.39	104.23 ± 2.70
ND + L-arginine	—	—	121.4 ± 2.83	118.2 ± 3.16
SD + L-arginine	$8.29 \pm 0.11^*$	$7.79 \pm 0.05^*$	104.6 ± 3.05	113.1 ± 7.7
ND + L-arginine + L-NAME	8.35 ± 0.13	7.52 ± 0.07	120.00 ± 2.08	101.20 ± 1.03
SD + L-arginine + L-NAME	8.24 ± 0.06	$6.75 \pm 0.13^*$	107.6 ± 5.23	108 ± 1.80

Abbreviations: L-NAME, L-nitro-arginine ethyl-ester; ND, normal diet; SD, salt diet (n=6 rings).

Data are presented as mean \pm SEM.

Significantly higher (* $P < .05$, ** $P < .01$) compared with corresponding normal diet group.

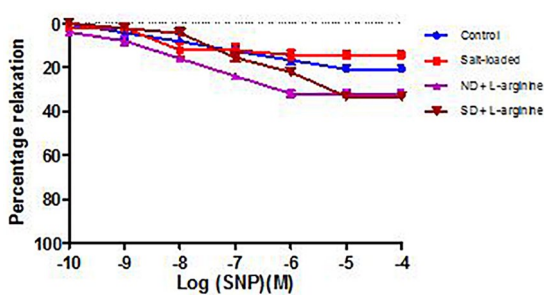


Figure 11. Relaxation response of abdominal aortic rings to SNP in the presence of Methylene Blue (MB) across all the groups (6 rings). Relaxation responses are expressed as percentage of decrease in sub-maximal contraction elicited by NA (10^{-5} M). Each point on the graph represents mean \pm SEM.

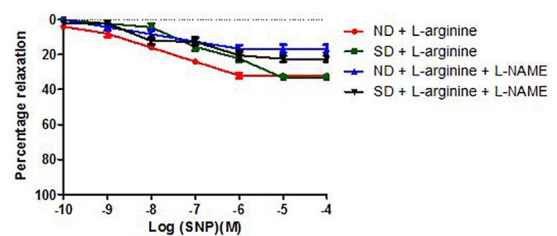


Figure 12. Relaxation response of abdominal aortic rings to SNP in the presence of Methylene Blue (MB) across groups 3 to 6 (6 rings). Relaxation responses are expressed as percentage of decrease in sub-maximal contraction elicited by NA (10^{-5} M). Each point on the graph represents mean \pm SEM.

Adverse events

The 6 (n = 6 out of 8) animals reported in each group for vascular reactivity were the animals that completed all the treatments. Those that died were replaced and treated separately and accordingly. For the gene expression part, the remaining 2 (n = 2 out of 8) animals were used and 3 rings were cut out from each animal to make 6 rings per group. These rings were then used in the qualitative conventional PCR reactions. Likely influence of female sex hormones was removed by using male rats in the study.

Discussion

In this study, the relaxation response to acetylcholine in the salt-loaded rats was greatly reduced compared to the Normal Diet rats. The sensitivity of the rings to acetylcholine as measured by the $-\text{Log EC}_{50}$ values was also observed to be significantly reduced in the salt-loaded rats. This corresponds with a reduced expression of the *eNOS* gene in the abdominal aorta of the rats. This implies that high-salt diet impairs vascular relaxation response to acetylcholine, which depends on the presence

of an intact endothelium possibly by suppressing the expression of the *eNOS* gene. These vascular relaxation responses to acetylcholine occurred without significant interference with relaxation responses to SNP. Oloyo *et al.* made similar observations in the abdominal aortic rings from SD rats fed a high-salt diet.^{6,7} In line with the results of this study, in Dahl salt-sensitive hypertensive rats, a high-salt diet was reported to impair endothelium-dependent relaxations induced by a variety of vasodilators.^{19,20} This impairment could best be explained by reduced activity and expression of the eNOS mRNA which results in reduced conversion of the substrate, L-arginine present in the plasma to usable nitric oxide to cause vasodilation.²¹⁻²³ Also, previous studies have demonstrated clearly that vascular functions and more importantly endothelium-dependent relaxations are impaired following elevated dietary salt intake in animals and humans.^{6,7,24} Many mechanisms have been proposed for this impairment part of which is failure of nitric oxide from the L-arginine-nitric oxide pathway in the endothelium to cause vasodilation^{6,24} and alteration in *eNOS* gene expression.

Administration of exogenous L-arginine in this study significantly increased acetylcholine-induced relaxation and caused improvement in the sensitivity of the abdominal aortic

Table 6. $-\text{Log EC}_{50}$ and percent maximum relaxation response of abdominal aortic rings to SNP with or without methylene blue (MB).

GROUPS	$-\text{LOG EC}_{50}$		% MAXIMUM RELAXATION	
	SNP	SNP + MB	SNP	SNP + MB
Normal diet	8.28 ± 0.06	7.79 ± 1.13	106.7 ± 3.98	20.80 ± 1.50
Salt-loaded	$7.61 \pm 0.06^*$	8.27 ± 1.56	122.50 ± 9.39	14.29 ± 1.80
ND + L-arginine	—	7.79 ± 0.18	120.4 ± 2.83	32.00 ± 1.5
SD + L-arginine	$8.29 \pm 0.11^*$	$6.65 \pm 0.20^{**}$	103.6 ± 3.05	33.33 ± 1.53
ND + L-arginine + L-NAME	8.35 ± 0.13	8.22 ± 0.75	120.00 ± 2.08	16.67 ± 2.21
SD + L-arginine + L-NAME	8.24 ± 0.06	$7.65 \pm 0.50^{**}$	104.6 ± 5.23	22.31 ± 1.82

Abbreviations: SNP, sodium nitroprusside; MB, methylene blue; ND, normal diet; SD, salt diet (n=6 rings).

Data are presented as mean \pm SEM.

Significantly higher ($*P < .05$, $**P < .01$) compared with corresponding normal diet group.

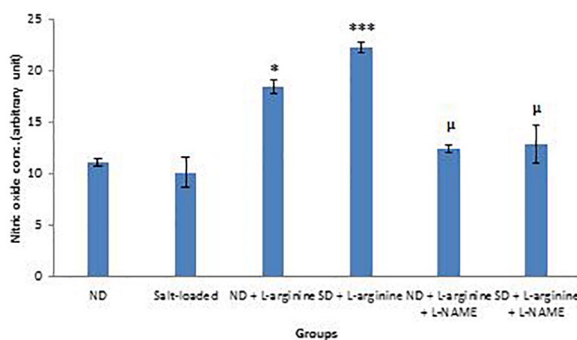


Figure 13. Nitric oxide level in Groups 1 to 6. Data are presented as mean \pm SEM (n=8). Significantly higher ($*P < .05$, $***P < .001$) compared with salt-loaded group.

rings to acetylcholine. There was also an appreciable increase in the expression of the *eNOS* gene. This implies an improvement in vascular function. Administration of exogenous L-arginine had also been reported to ameliorate vasomotor dysfunction in vascular injury models²⁵ and pathological disorders in which it is reduced. The result of this study is also in agreement with the report by Cooke and Dzau who also observed an improvement in endothelium-dependent relaxation following L-arginine supplementation in hypertensive rats.²⁶ In line with this report also, there was an improvement in endothelium-dependent relaxation following supplementation with L-arginine in rats with metabolic syndrome.²⁷ The increase in the maximum percent relaxation response in the salt-loaded group supplemented with L-arginine shows the ability of L-arginine to restore endothelium-dependent relaxation, and it could thus be inferred that L-arginine supplementation also attenuated the vascular function-impairing effect of a high-salt diet by ameliorating its *eNOS* mRNA expression effect. The downstream effect on nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway is to cause dilation of the arteries by causing closure of some calcium channels.²⁸

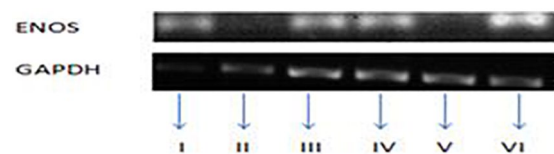


Figure 14. Expression of *eNOS* mRNA in the aorta from each group of rat as assayed by qualitative conventional PCR (6 rings). Group I = Control, Group II = Salt-loaded, Group III = Supplemented with L-arginine, Group IV = Salt-loaded + L-arginine, Group V = co-admin. with L-arginine and L-NAME, Group VI = Salt-loaded and co-admin. with L-arginine and L-NAME. L-NAME indicates L-nitro-arginine ethyl-ester; PCR, polymerase chain reaction; mRNA, messenger RNA.

It is worthy of note that oral supplementation with L-arginine had no significant effect on vasodilatation to SNP, in both the normotensive and hypertensive abdominal aortic ring types. Our results also showed that the endothelium-independent relaxation response to SNP was similar in rings from Normal Diet and L-arginine-treated rats. In close association with these results, other reports had earlier shown no effect of L-arginine on endothelium-independent relaxations to SNP in hypertensive experimental animals and humans.²⁹ The nonsignificance of the results obtained from methylene blue inhibition not only showed that vascular function impairing effect of high-salt diet on the abdominal aorta is not cGMP dependent but also the modulatory effect of L-arginine acts not through cGMP as the sole pathway.

Conclusion

Results from this study suggest that oral supplementation of L-arginine ameliorates reduced endothelium-dependent relaxation in rats fed a high-salt diet by restoring toward normal, the reduced *eNOS* gene expression in the abdominal aorta of the rats. This result may partly explain the beneficial effect of exogenous L-arginine in the maintenance of vascular homeostasis and possible usage as adjunct therapy in the management of salt-induced hypertension.

Limitation

Only male rats were used in this study to rule out the possible influence of female hormones and thus the conclusion made may not be applicable to female rats with salt-induced hypertension. This is thus a subject of another study. Also, only *eNOS* gene expression was studied here, and many other genes may, however, be involved in the control.

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Author Contributions

AA conceived the idea, carried out the bench work, did the statistical analysis and drafting of the manuscript for publication.

AO helped to develop the original idea, participated in the bench work, provided the materials and equipment used for the work, participated in the drafting of the manuscript for publication.

CA helped to develop the original idea, read through the manuscript and supervised the research work.

SJ contributed to the development of the original idea, read through the manuscript and supervised the research work.

Disclosure

All authors have approved the final article.

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