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# Salt and Nitric Oxide Synthase Inhibition-Induced Hypertension: Kidney Dysfunction and Brain Anti-Oxidant Capacity

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## Abstract

The specific aim of this study was to examine the effects of salt-loading on kidney function and brain antioxidant capacity. Wistar rats were divided into four groups: Control rats were given normal drinking water and no drug treatment for 2 weeks. LNNA group: rats were given normal drinking water and the nitric oxide (NO) inhibitor NG-nitro-L-arginine (L-NNA), 3 mg/kg/day. LNNA + Salt group: rats were given drinking water containing salt 2% and 3 mg/kg L-NNA. Salt group: rats were given drinking water containing salt 2% and no drug treatment. Basal blood pressure and the levels of serum BUN, creatinine, uric acid, cortisol, electrolyte, serum antioxidant capacity, and oxidative stress were measured. NO, superoxide dismutase (SOD), and catalase (CAT) levels were measured in the hypothalamus, brainstem, and cerebellum. Salt overload increased the blood pressure of the LNNA + Salt group. Salt-loading enhanced BUN, creatinine, sodium retention. High salt produced an increase in uric acid levels and a decrease in cortisol levels in serum. Additionally, the oxidative stress index in serum increased in the LNNA + Salt group. Salt-loading enhanced brain NO levels, but not SOD and CAT activity. L-NNA increased brain SOD activity, but not CAT and NO levels. In conclusion, salt-loading causes hypertension, kidney dysfunction, and enhances oxidative stress in salt-sensitive rats.

**Keywords:** kidney, hypertension, oxidative stress, superoxide dismutase, salt

## INTRODUCTION

Hypertension is a major risk factor for cardiovascular mortality and morbidity through its effect on target organs such as the heart, brain, and kidney. There is a direct, positive relationship between hypertension and salt-sensitivity. Kidney dysfunction is more often seen in salt-sensitive people and is more severe (1). Salt-loading exacerbated the renal injury via induction of oxidative stress (2). Also oxidative stress is increased in the brain in salt-sensitive hypertension (3). Accordingly, salt-sensitive hypertension should decrease brain antioxidant capacity. As a results, the specific aim of this study was to examine the effects of salt-loading on kidney function and brain antioxidant capacity. Wistar rats were given a low dose of NG-nitro-L-arginine (L-NNA) to make salt-sensitive. Chronic low-dose (subpressor) nitric oxide synthase (NOS) inhibition has no effect on blood pressure in normal salt intake but increases the salt-sensitivity in rats and thus causes volume overloading and hypertension during high salt intake (4). To induce nitric oxide (NO) deficiency, L-NNA was given to rats during high-salt intake as in previous studies (5–7). In this study, blood pressure, kidney function, and antioxidant enzyme activities in brain tissues were evaluated in rats.

## MATERIAL AND METHODS

### Animals

Thirty Wistar-albino male rats, 200–300 g, were used in the experiments. All experiments were conducted at the University of Firat ( Elazığ, Turkey). The animals were housed in quiet rooms with a 12-h light/dark cycle (7 am to 7 pm) and allowed a commercial standard rat diet (containing salt 0.8%) and water *ad libitum*. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals Department of Health, Education, and Welfare (DHEW) Publication (NIH) 8523, 1985).

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### Experimental Protocols

All rats received tap water *ad libitum* and commercially available rat chow containing sodium chloride at 0.8%. Salt (2%) was added to the drinking water for salt-loading. The amount of water that the rats received daily had been determined in a pilot study. Salt-loaded groups consumed water more than normal salt groups (Salt and LNNA + Salt vs. control and LNNA; approximately 160 ml/kg/day vs. 108 ml/kg/day). The concentration of L-NNA and salt in drinking water was calculated according to the amount of water the rats received. Rats were divided into four groups: 1. Control rats were given normal drinking water and no drug treatment (n = 7); 2. Group LNNA: Rats were given normal drinking water and the NO inhibitor L-NNA, 3 mg/dL, corresponding to a daily ingestion of approximately 3 mg/kg. This dose was previously shown to promote little or no increase in blood pressure even after several weeks of treatment (n = 7) (7); 3. Group LNNA + Salt: Rats were given drinking water containing salt 2% and 2 mg/dL L-NNA, corresponding to a daily ingestion of approximately 3 mg/kg (n = 8); 4. Group Salt: Rats were given drinking water containing salt 2% and no drug treatment (n = 8). Systolic blood pressure (SBP) was measured by the tail-cuff method (MAY BPHR 9610-PC, Commat Ltd., Ankara, Turkey). Basal blood pressure was measured for 3 consecutive days before starting the protocol and then at 3- to 4-day intervals during the 2-week study period. Body weight, food, and water intake were also recorded on each day.

After the last administration of the L-NNA and/or salt, all rats fasted about 12 h, but had free access to water. Then the rats were anesthesized with ketamine + xyslasine (60 mg/kg + 5 mg/kg, i.p.) at the end of the experiment. Blood was collected, serum was separated and used for biochemical estimations. Hypothalamus, brainstem, and cerebellum tissues were quickly removed and stored in −24° centigrade. The tissues were homogenized with prechilled physical saline in tissue homogenizer, then centrifuged at 3000 g for 10 min at 4° centigrade, and the supernatant was used for the estimation of the levels of NO, SOD, and CAT.

### Biochemical Estimations

The levels of BUN, creatinine, calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), chlorine (Cl) in serum were determined with an autoanalyzer (Olympus AU600, Olympus Optical Co., Ltd., Tokyo, Japan) by using commercial Beckman Coulter diagnostic kits (Beckman Coulter, Inc., Fullerton, CA, USA). Cortisol levels in serum were measured by enzyme-linked immunosorbent assay (ELISA). Serum total antioxidant capacity (TAC) and total oxidative stress (TOS) levels were determined using a novel automated measurement method, developed by Erel (8–10). The protein content in the hypothalamus, brainstem, and cerebellum tissues were analyzed in homogenate, supernatant, and extracted samples according to the method of Lowry et al (11). Since NO measurement is very difficult in biological specimens, tissue nitrite ($NO_2^-$) and nitrate ($NO_3^-$) were estimated as an index of NO production, and the colorimetric assay based on the Griess reaction for assessment of NO activity was used (12). Total SOD activity was determined according to the method of Sun, Oberley, and Li (13). The principle of the method is based on inhibition of Nitro Blue Tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the lysate after 1 ml of ethanolchloroform mixture (5:3, v/v) was added to the same volume of sample and was centrifuged. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. Catalase activity was determined according to Aebi's method (14).

### Chemical Reagents

Nitrite/nitrate, superoxide dismutase, and CAT diagnostic agents and L-NNA were bought from Sigma Chemical Company (St. Louis, MO). Total antioxidant capacity (TAC) and TOS diagnostic agents were bought from Rel Assay Diagnostics (Gaziantep, Turkey).

### Statistical Analysis

Data were analyzed by using a commercially available statistics software package (SPSS for Windows, verson 12.0, Chicago, IL). Distribution of the groups was analyzed with one sample Kolmogrov-Smirnov test. All groups showed normal distribution, so that parametric statistical methods were used to analyze the data. One-way ANOVA test was performed and post-hoc multiple comparisons were made using least-square differences. Results are presented as mean ± SEM; $P < 0.05$ was regarded as statistically significant.

## RESULTS

There was no difference between the weight gain of rats. Salt-loaded groups consumed water more than normal salt groups (Salt and LNNA + Salt vs. control and LNNA; approximately 160 ml/kg/day vs. 108 ml/kg/day). Table 1 summarizes SBP in all groups. Blood pressure increased significantly in the LNNA + Salt group. Salt-loading or L-NNA alone increased the blood pressure of rats minimally ($p > 0.05$). The serum cortisol, BUN, creatinine, uric acid levels are summarized

Table 1. SBP (mmHg) in all groups. Results are represented mean ± SEM

| Groups | Initially | Day 14 |
|---|---|---|
| Control | 132.9 ± 0.8 | 133.6 ± 1.1 |
| LNNA | 130.9 ± 08 | 139.7 ± 1.9 |
| LNNA + Salt | 131.2 ± 0.6 | 162.1 ± 6.1[ab] |
| Salt | 131.1 ± 0.9 | 142.1 ± 1.8 |

[a] $p < 0.001$ vs. control.
[b] $p < 0.01$ vs. LNNA and Salt groups.

in Table 2. Salt-loading reduced the serum cortisol levels in LNNA + Salt and Salt groups. The serum BUN levels increased in all groups except control and the serum creatinine levels increased in rats given L-NNA. Serum uric acid levels increased in the LNNA + Salt group ($p = 0.06$). The electrolyte levels are summarized in the Table 3. Administration of salt and L-NNA increased sodium and chlorine retention. Calcium and magnesium retention in the LNNA group was found and calcium retention in the LNNA + Salt group occurred. Salt-loading increases the retention of sodium and chlorine, but L-NNA increases the retention of calcium and magnesium in this study. Table 4 summarizes serum TAC and TOS levels and oxidative stress (OS) index in all groups. L-NNA increased the serum levels of TAC, and the serum levels of TOS and the OS index in the LNNA + Salt group also increased. NO levels in hypotalamus, brainstem, and cerebellum are shown in Figure 1. Nitric oxide levels in the brainstem of the Salt and the LNNA + Salt groups increased, but the NO level in the cerebellum of the LNNA group decreased. SOD activity in hypotalamus, brainstem and cerebellum is shown in Figure 2. Superoxide dismutase activity in the hypothalamus of the LNNA group increased. Superoxide dismutase activity in the cerebellum of the LNNA + Salt also increased. CAT activity in hypotalamus, brainstem and cerebellum is shown in Figure 3**.** Catalase activity did not change in brain tissues in any groups.

## DISCUSSION

L-NNA or salt overload alone did not significantly induce an increase in blood pressure. As expected, in rats treated with both, L-NNA and salt increased significantly blood pressure in this study as before (7,15). Normally, salt-loading does not increase blood pressure in such experiments (7). A probable reason for a partial increase of blood pressure in salt-loaded rats is mild impairment of kidney function and sodium excretion. The subpressor dose of L-NNA increased slightly blood pressure because of rat chow containing high salt, 0.8%.

We showed that the administration of L-NNA or salt alone induced renal injury in this study. It is well known that dietary salt-loading exacerbate renal injury (16) but the mechanisms responsible for this effect are not clear enough. Renal injury was more severe in the rats treated with both L-NNA and salt. Because the serum levels of blood urea nitrogen (BUN) and creatinine increased in the LNNA + Salt group. Previous studies largely support our results (7,15,17). As a result

Table 2. The serum cortisol, BUN, creatinine, and uric acid levels in all groups. Results are represented mean ± SEM

| Groups | Cortisol (μg/dL) | BUN (mg/dL) | Creatinine (mg/dL) | Uric Acid (mg/dL) |
|---|---|---|---|---|
| Control | 0.94 ± 0.04 | 13 ± 1 | 0.35 ± 0.02 | 1.21 ± 0.05 |
| LNNA | 0.93 ± 0.04 | 18 ± 1[b] | 0.44 ± 0.02[c] | 1.45 ± 0.07 |
| LNNA + Salt | 0.66 ± 0.05[a] | 20 ± 2[b] | 0.50 ± 0.03[a] | 1.57 ± 0.24[d] |
| Salt | 0.56 ± 0.06[a] | 19 ± 1[b] | 0.35 ± 0.01 | 1.17 ± 0.06 |

[a] $p < 0.001$ vs. control.
[b] $p < 0.01$ vs. control.
[c] $p < 0.05$ vs. control.
[d] $p = 0.06$ vs. control.

Table 3. The serum electrolyte levels in all groups. Results are represented mean ± SEM

| Groups | Calcium (mg/dL) | Magnesium (mg/dL) | Sodium (mEq/l) | Potassium (mEq/L) | Chlorine (mEq/L) |
|---|---|---|---|---|---|
| Control | 9.15 ± 0.25 | 2.17 ± 0.05 | 124 ± 3 | 5.33 ± 0.21 | 89 ± 2 |
| LNNA | 10.30 ± 0.36[a] | 2.58 ± 0.13[a] | 142 ± 5[b] | 5.90 ± 0.26 | 100 ± 3[a] |
| LNNA + Salt | 10.33 ± 0.48[a] | 2.47 ± 0.22 | 158 ± 6[c] | 5.40 ± 0.30 | 115 ± 5[c] |
| Salt | 9.88 ± 0.26 | 2.37 ± 0.08 | 146 ± 3[b] | 5.36 ± 0.18 | 107 ± 3[b] |

[a] $p < 0.05$ vs. control.
[b] $p < 0.01$ vs. control
[c] $p < 0.001$ vs. control.

Table 4. The serum TAC and TOS levels and OS index in all groups. Results are represented mean ± SEM

| Groups | TAC (mmol Trolox Eq./L) | TOS (μmol $H_2O_2$ Eq./L) | OS Index (Arbitrary Unit) |
|---|---|---|---|
| Control | 0.71 ± 0.02 | 14 ± 1 | 2.06 ± 0.12 |
| LNNA | 0.86 ± 0.03[a] | 19 ± 2 | 2.32 ± 0.28 |
| LNNA + Salt | 0.78 ± 0.08 | 25 ± 3[b] | 3.81 ± 0.89[a] |
| Salt | 0.78 ± 0.02 | 15 ± 1 | 2.03 ± 0.19 |

[a] $p < 0.05$ vs. control.
[b] $p < 0.01$ vs. control.

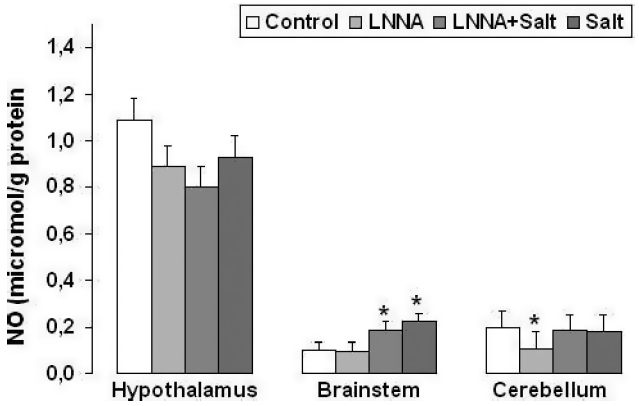


Figure 1. NO levels in hypothalamus, brainstem and cerebellum in all groups. *p < 0.01 vs. control.

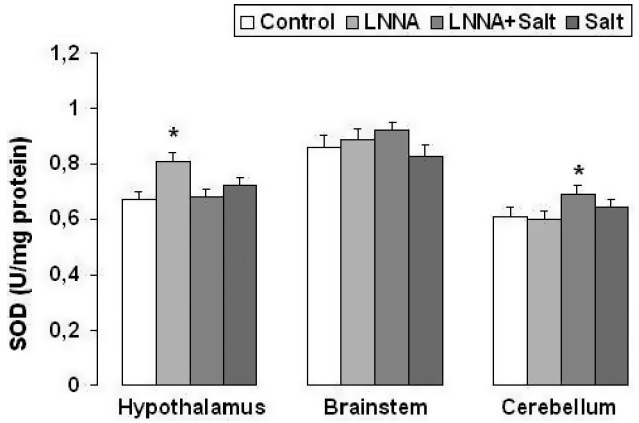


Figure 2. SOD activity in hypothalamus, brainstem and cerebellum in all groups. * p < 0.05 vs. Control.

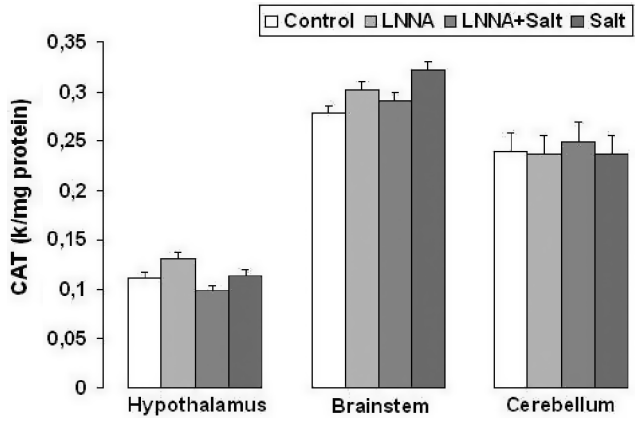


Figure 3. CAT activity in hypothalamus, brainstem and cerebellum in all groups. No significant differences between groups.

the salt load increased sodium and chlorine retention. But administration of L-NNA also induced sodium and chlorine retention. It has been demonstrated that a mild impairment of NO synthesis produces a sustained decrease in sodium excretion in a previous study (15). Serum cortisol levels decreased after salt-loading in our study. Kerstens et al. (18) reported that salt-loading induces an increase in cortisol elimination and hence a decrease in circulating cortisol in salt-resistant subjects. It has been previously demonstrated that sodium-loading raises urinary cortisol in humans (19). A second possibility is that organisms supress adrenal glands in response to an increase in renal sodium retention and thus reduces cortisol synthesis.

We found an increase in uric acid levels in the LNNA + Salt group. The level of serum uric acid is an independent risk factor for cardiovascular and renal morbidity (20). Schulman et al. (21) found a significant increase in uric acid levels of the post-menopausal, salt-sensitive and hypertensive women. Patients with target organ damage had significantly higher levels of serum uric acid as compared to those without it (20). The increase in uric acid levels in our study was accompanied by kidney dysfunction also.

An important finding of this study is the increase in serum calcium and magnesium levels in rats treated with L-NNA. Researchers reported that NO inhibits osteoclast activity and thus it would lead to a decrease in serum calcium (22). Additionally, the inhibitory effects of cytokines on osteoclast formation were blocked by the NO synthase inhibitor L-NMMA (23). According to these studies, NO inhibition should increase the levels of serum calcium. This literature supports our results. The cause of increased levels of serum magnesium in rats treated with L-NNA alone is unclear. Senturk et al. (24) reported that Mg depletion occurs in the model of chronic NO synthase inhibition-induced hypertension. Unlike our study, they administered to rats a high dose of L-NAME. They found a reduction in the level of magnesium in the tissues of hypertensive rats, but the plasma magnesium level was not changed. Magnesium excretion is not sufficient because of the impairment of kidney function and thus serum magnesium levels may be increased.

L-NNA alone induced the increase in serum TAC in our study. It is well known that nonselective NOS inhibitors induce superoxide production (25). L-NNA alone also enhanced brain SOD activity. Accordingly, NO inhibition increases the central and systemic antioxidant capacity. Oxidative stress did not become enhanced in the LNNA group because we administrated a low-dose of L-NNA to the rats. To understand the cause of the increase of TAC requires further research. In previous studies it has been shown that high-salt diet increases oxidative stress in rats (26,27). We found a similar result from the mentioned articles. Increased oxidative stress impairs vasodilation in vasculature and thus increases blood pressure (27).

Salt-loading increased NO levels in the brainstem in both the Salt and the LNNA + Salt groups, but NO levels did not change in hypothalamus. L-NNA alone decreased NO levels in the cerebellum. Salt-loaded hypertensive Dahl rats showed increased NO production

in the brainstem, paraventricular nucleus (PVN), and supraoptic nucleus (SON), but not in the diencephalon or cerebellum (28,29). Unlike our results, it has been reported that a high sodium intake decreases constitutive NOS messenger ribonucleic acid (RNA) levels in the hypothalamus (30), but they used a DOCA (deoxycorticosterone)-salt model of hypertension in the rats. Local perfusion of the PVN region with hypertonic saline increases a local release of NO (31). These results suggest that salt-loading enchances brain NO levels in rats with or without developed hypertension.

Researchers reported that the SOD and CAT enzyme activities decrease in the rostral ventrolateral medulla (RVLM) of spontaneously hypertensive rats (SHR) (19,32). But L-NNA enhanced SOD activity in the hypothalamus of the LNNA group and the cerebellum of the LNNA + Salt group in our study. Catalase activity did not change in the brain tissue of rats. Oxidative stress increased in the cerebral cortex, hippocampus, and brain of salt-loaded hypertensive rats (3,33). We did not measure oxidative stress in brain tissues in this study. Probably, to scavenge the increased superoxide radicals in the brain tissues, SOD enzymes might be activated.

In conclusion, salt-loading causes hypertension, kidney dysfunction, and enhances oxidative stress in salt-sensitive rats. Further research should investigate the role of the kidney and the antioxidant system in salt-sensitive hypertension.

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