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Nitrate/L-arginine Therapy and Nitric Oxide Levels in the Stomach and Liver of Rats

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Background: The L-arginine/nitric oxide (NO) pathway is a major defensive system in gastric mucosa. Nitrate can restore NO homeostasis when enzymatic NO production becomes dysfunctional.

Objectives: The aim of this study was to investigate the effects of nitrate/L-arginine administration on NO levels in the stomach and liver of rats.

Materials and Methods: In this interventional study, adult male Wistar rats were divided into 3 groups of control, nitrate and L-arginine (n = 8). Rats in the nitrate and L-arginine groups were administered sodium nitrate (500 mg/L) or L-arginine (2%) for one week in drinking water while those in control group consumed tap water. At the end, serum, stomach and liver NO metabolite (NO_x) concentrations were measured by the Griess method.

Results: Median (interquartile range) serum NO_x concentrations in the control group [28.2 (19.6 - 37.8) μmol/L] were significantly (P < 0.05) different to those of the nitrate [152.4 (111.4 - 180.2) μmol/L] and L-arginine [14.5 (11.2 - 21.5) μmol/L] groups. Nitrate administration increased and L-arginine administration decreased stomach and liver NO_x levels respectively. A positive correlation was observed between serum concentrations and stomach (r = 0.847, P < 0.001) and liver (r = 0.650, P = 0.006) NO_x levels.

Conclusions: Nitrate and L-arginine administration had opposite effects on NO_x levels in stomach and liver of normal rats. Increase in stomach NO_x following nitrate administration may be due to gastric nitrate absorption, while the decrease in tissue NO_x following L-arginine administration may be due to increase in arginase activity. These findings may be important considering current data on the protective roles of dietary nitrate/nitrite.

Keywords: L-arginine; Nitrate; Nitric oxide metabolites; Rat

1. Background

The L-arginine/nitric oxide (NO) pathway is a major defensive system in gastric mucosa because NO increases mucus generation and thickness and maintains adequate mucosal blood flow [1, 2]. Reduced NO synthase (NOS) activity predispose the elderly to nonsteroidal anti-inflammatory drug gastropathy [3]. It has been reported that using nitroglycerin, a drug that generates NO, is independently associated with a decreased risk of upper gastrointestinal bleeding, which is a result of both increase in blood flow of gastric mucosa and the inhibition of adherence of leukocyte to the endothelium [4].

NO is produced enzymatically from L-arginine by NOS and non-enzymatically from reduction of nitrite [5]. At least, three isoforms of NOS has been identified, i.e. endothelial NOS (eNOS), neural NOS (nNOS), and inducible NOS (iNOS) [5]. The existence of mitochondrial NOS (mtNOS) was reported in 1997 by Ghafourifar and Richter

[6]. NOS-independent NO generation was first described in the stomach [1]; furthermore neural NOS have been found in 50% of enteric nervous system neurons and in parietal cells [7]. Non-enzymatic NO production from dietary nitrate/nitrite could be a potential blood and tissue reservoir of NO [8].

Oral ingestion of inorganic nitrate generates NO in gastric lumen [9]; in addition, enterosalivary circulation of ingested nitrate provides continuous production of NO in the gastric lumen [9]. Ingested nitrate is converted into nitrite in saliva, which, when swallowed, provides a protective mechanism against ingested pathogens by increasing bactericidal activity of gastric juice and could also act as a reservoir of NO [10]. The storage form of NO in tissues is limited [11] and nitrate, as a cytoprotective element in the diet [8], can restore NO homeostasis when NO production from NOS become dysfunc-

tional [10]. L-arginine is a conditionally essential amino acid and it has been reported that L-arginine as a dietary supplement, could increase NO production [12, 13]. Despite low L-arginine doses being given as a food-additive for many years [14], some authors believe that considering the highly complex metabolism of L-arginine, exogenous arginine supplementation should be used with great caution or avoided [15].

NO levels in circulation may reflect changes in NO production by tissues [16]; however, it has not been completely clarified whether NO_x status in tissues, is reflected in the plasma [17].

2. Objectives

Considering therapeutic approaches using nitrate/nitrite and L-arginine, the aim of this study was to determine the effect of nitrate and L-arginine administrations, as possible mechanisms for increased NO availability in tissues, on the serum, stomach, and liver concentrations of nitric oxide metabolites (nitrite+nitrate = NO_x) in rats.

3. Materials and Methods

3.1. Animals and Study Protocol

In this interventional study, a total of 24 male Wistar rats (220 - 250 g and 14 weeks old) were maintained in standard conditions (temperature 22 ± 3 °C; relative humidity 50 ± 6%) with 12 hour light/dark cycles. All experiments were carried out in accordance with standards approved by the local ethics committee of the Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences. Animals, divided into 3 groups (n = 8 in each group), the control, nitrate, and L-arginine groups, had free access to standard rat chow (Pars Co., Tehran) and water during the study. Rats in the nitrate and L-arginine groups were administered sodium nitrate (500 mg/L) or L-arginine (2%) for one week in drinking water while the controls consumed tap water. At the end, after 12 - 14 hour fasting a blood sample was obtained for serum NO_x measurement and stomach and liver samples for NO_x measurement were prepared as previously described [18]; in brief, animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and after thoracotomy, organs were flushed free of blood, using EDTA/N-ethylmaleimide solution (10/2.5 mmol/L) freshly prepared in phosphate buffer saline perfused through the left ventricle for 2 minute while blood drained through the right auricle. Stomach and liver were removed, weighed and homogenized in ice-cold perfusion solution by a homogenizer (Micra D-1, Germany) and sonicated with a Microson XL 2000 sonicator (USA) while immersed in an ice-water bath.

3.2. Serum and Tissue NO_x Measurement

Serum and tissue NO_x concentrations were measured

by the Griess reaction [18]. In brief, serum samples were deproteinized by zinc sulfate (15 mg/mL), and centrifuged at 10,000 g for 10 minute; tissue homogenates were first centrifuged at 15,000 g for 20 minute, then zinc sulfate (15 mg/mL) was added and after one minute shaking, samples were recentrifuged at 15,000 g for 20 minute. For both serum and tissue samples, a 100 μL of the supernatant was transferred to a microplate well, and 100 μL vanadium (III) chloride (8 mg/mL) was added to each well to reduce nitrate to nitrite, as the Griess reaction detects only nitrite. Griess reagents [50 μL sulfanilamide (2%) and 50 μL N-ethylenediamine dihydrochloride (0.1%)] were then added and samples were incubated for 30 minute at 37 °C; absorbance was read at 540 nm using the ELISA reader (Sunrise, Tecan, Austria). NO_x concentration was determined from the linear standard curve established by 0 - 150 μmol sodium nitrate. Inter- and intra-assay coefficients of variation were 5.2% and 4.4% respectively. The sensitivity of the assay was 2.0 μmol/L and its recovery was 93 ± 1.5%. The protein content of the homogenates was determined by the Bradford method [19] and bovine serum albumin (BSA) was used as a standard; tissue NO_x levels were expressed as nmol/mg protein.

SPSS-20 was used for statistical analyses. Because of the skewed distribution of NO_x values, non-parametric statistics were used and data were presented as median (interquartile range). Kruskal-Wallis one-way ANOVA was used to compare the effects of sodium nitrate and L-arginine administration in different groups and Mann-Whitney U test was used for pairwise comparison. Spearman correlation coefficient was calculated between serum and tissue NO_x levels. Two-sided P values < 0.05 were considered statistically significant.

4. Results

In the control group, median (interquartile range) stomach NO_x was 1.02 (0.81 - 1.34) μmol/L; nitrate administration, significantly (P < 0.001) increased 5.16 (4.50 - 5.53) μmol/L and L-arginine administration significantly (P < 0.05) decreased 0.57 (0.17 - 0.87) μmol/L stomach NO_x levels (Figure 1 A). Liver NO_x in the control group was 0.52 (0.22 - 0.86) μmol/L; nitrate administration, significantly (P < 0.05) increased 1.08 (0.60 - 1.19) μmol/L liver NO_x levels while L-arginine administration decreased it to non-detectable levels (Figure 2 A). Median (interquartile range) serum NO_x concentrations in the control group 28.2 (19.6 - 37.8) μmol/L differed significantly (P < 0.05) from those of the nitrate 152.4 (111.4 - 180.2) μmol/L, and L-arginine 14.5 (11.2 - 21.5) μmol/L groups.

Spearman correlations between serum and tissue NO_x are shown in Figures 1 B and 2B. Positive correlations were observed between serum and the stomach (r = 0.847, P < 0.001) and liver (r = 0.650, P = 0.006) NO_x levels.

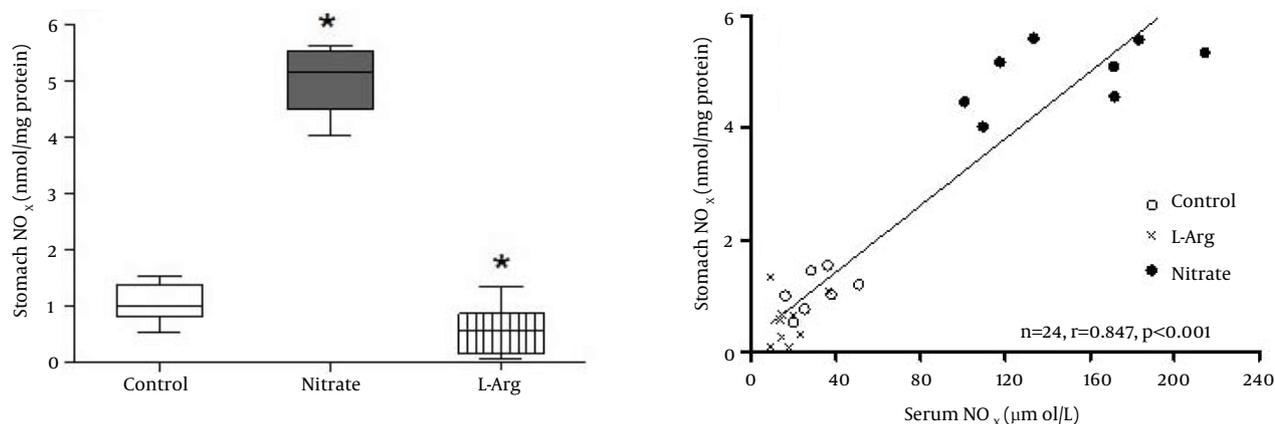


Figure 1. A, Box plots showing the effects of sodium nitrate and L-arginine administration on NO_x levels of the stomach. Median (interquartile range) stomach NO_x was 1.02 (0.81 - 1.34) μmol/L in the control group; nitrate administration significantly ($P < 0.001$) increased 5.16 (4.50 - 5.53) μmol/L and L-arginine administration significantly ($P < 0.05$) decreased 0.57 (0.17 - 0.87) μmol/L stomach NO_x levels; B, Correlation between serum and stomach NO_x content. * Significant difference compared to control group.

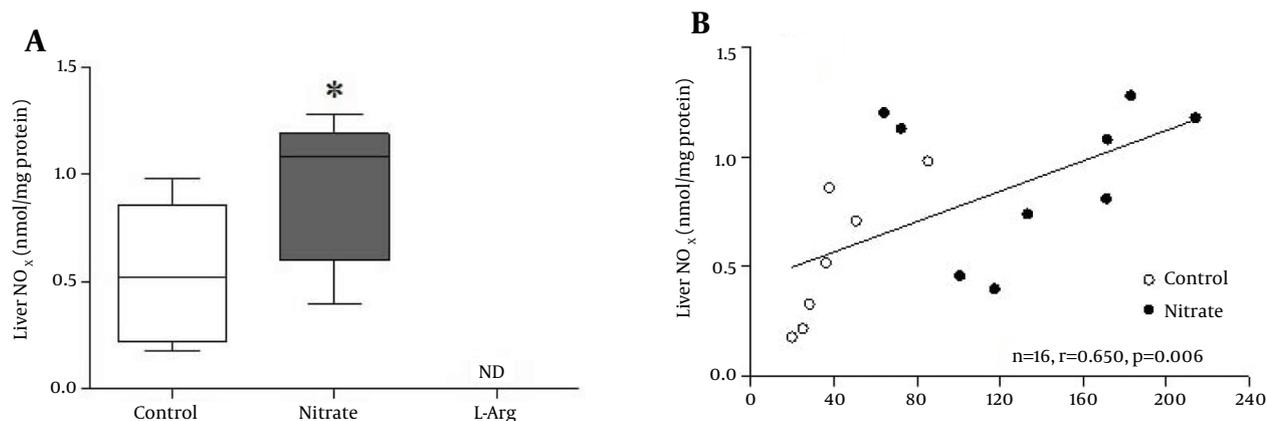


Figure 2. A, Box plots showing the effects of sodium nitrate and L-arginine administration on NO_x levels of the liver. Liver NO_x in the control group was 0.52 (0.22 - 0.86) μmol/L; nitrate administration significantly ($P < 0.05$) increased 1.08 (0.60 - 1.19) μmol/L liver NO_x levels while L-arginine administration decreased it to non-detectable levels; B, Correlation between serum and liver NO_x content. * Significant difference compared to control group; ND, non-detectable.

5. Discussion

The results of this study indicated a 4.1-fold and 1.1-fold increases in stomach and liver NO_x contents respectively of rat following one week oral nitrate administration, demonstrating the effect of dietary nitrate administration on systemic NO metabolites [18, 20] and tissue nitrite [18-21] levels. In line with our results, Jansson et al. [1] have reported 10.7 folds increase in nitrate levels of stomach following one-week administration of sodium nitrate of 1 mmol/kg in rats, a dose approximately twice that which we used in the current study (500 mg/L or 0.6 mmol/kg). Raat et al. [8] have reported 1.5-fold and 2.3-fold increases in stomach nitrate content following one-week administration of 300 mg/L and 1,500 mg/L sodium nitrite respectively. Nitrate is considered as a prodrug of nitrite [22] and similar to our results Duranski et al. [23] have re-

ported that nitrite treatment increases liver nitrite levels in mice.

The changes we observed in stomach and liver NO_x contents following nitrate administration were different; it has been reported that differences in nitrite concentrations between tissues may reflect the degree of NOS activity and the oxidation pathways of NO [24]; however, increased NO_x content of the stomach following nitrate administration may also be attributed in part to nitrate absorption from the stomach [25].

In the current study, we found relatively high correlations between serum concentrations and gastric and liver NO_x levels, a finding in line with a previous report that nitrate concentration of the blood is a major determinant of NO_x levels of the rest of the body [26]. Close

correlation between plasma and tissue nitrite after nitrite administration has been previously reported [24]. Similar to our results, Raat et al. [8] have reported a direct correlation between plasma and liver nitrite concentrations; some authors have suggested that high correlation between serum and some tissue NO_x indicates non-specific accumulation of NO_x in these organs [27] while others suggest that anion transporters aid regulated and tissue-specific transport of nitrite across cell membranes [24, 28].

Recent findings suggest that nitrate/nitrite could be considered as potential therapeutic agents [29, 30]. Following oral nitrate intake, large amounts of NO is produced in stomach, amounts greater than that required for vasodilation; the excess amount can contribute to host defense and in gastric physiology [31, 32]. One-week nitrate therapy has prevented gastric injury induced by diclofenac in rats, which may be due to increased intragastric NO formation and stimulation of mucus formation [1]. On the other hand, cancer, in particular stomach cancer, was a concern of nitrate/nitrite consumption [10, 29]. In our study, according to food and water intake measurements, rats received 13 and 51 mg/kg/day nitrate for one week in the control and nitrate groups respectively. It has been reported that sodium nitrite of 130 mg/kg in male rats for 2 years is not carcinogen [10]. Although still in doubt, it has been recently reported that old hypothesis of association between (stomach) cancer and ingested nitrate/nitrite is not supported by new data and there is no evidence implicating nitrate/nitrite as an animal or human carcinogen [10].

In the current study, L-arginine administration decreased levels of stomach NO_x by 44% and those of liver NO_x to non-detectable values. In line with our results, Ohta and Nishida [2] have reported that administration of L-arginine could prevent stress-induced increases in the gastric mucosa NO_x levels in rats. L-arginine increases arginase activity, which could decrease NO production by NOS [18] via reducing substrate availability [33]. In addition, decarboxylation of L-arginine by the arginine decarboxylase produces agmatine [34], which is a competitive inhibitor of the NOS isoenzymes [35] and could inhibit all isoforms of NOS and NO production [36, 37]. While there are several reports of the protective effect of L-arginine administration against development of gastric mucosal lesion [2], it has recently been reported that L-arginine metabolism could impair antimicrobial NO synthesis in stomach and cause *H. pylori* induced DNA damage [38]. In addition, it seems that L-arginine does not stimulate NO production *in vitro* unless during L-arginine deficiency; some of L-arginine actions *in vivo*, previously attributed to increase NO production, may be due to other mechanisms including increase in insulin secretion [39].

In conclusion, the results of this study indicate that nitrate and L-arginine administration had opposite effects on the NO_x levels in the stomach and liver of normal rats.

In addition, direct correlations were observed between serum and the tissues NO_x levels, findings which may be important considering the fast accumulating evidence on the protective roles of dietary nitrate and nitrite.

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Authors' Contributions

Fatemeh Mehrazin and Asghar Ghasemi wrote the article, Fatemeh Mehrazin, Asghar Ghasemi, and Saleh Zahediasl carried out the literature search, Asghar Ghasemi and Fatemeh Mehrazin participated in data collection, Saleh Zahediasl and Asghar Ghasemi participated in the design of the study and in the approval of the final version to be submitted. All authors read and approved the final manuscript.

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