Nitric oxide metabolites
Nitrate
L-arginine
Nitric oxide metabolites

Family of nitric oxide synthase (NOS) activity predi

L-arginine is a conditionally essential amino acid and it has been reported that L-arginine as a food 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 NOx status in tissues, is reflected in the plasma [17]. Considering therapeutic approaches using nitrate/nitrite and L-arginine, 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.

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 (NOx) concentrations were measured by the Griess method.

Results: Median (interquartile range) serum NOx 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 NOx 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) NOx levels.

Conclusion: Nitrate and L-arginine administration had opposite effects on NOx levels in stomach and liver of normal rats. Increase in stomach NOx following nitrate administration may be due to gastric nitrate absorption, while the decrease in tissue NOx 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.

Oral ingestion of inorganic nitrate generates NO in gastric lumen [9]; in addition, enterosalivary circulation of ingested nitrate provides consistent 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 dysfunctional [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].

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Materials and Methods

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±3ºC; relative humidity 50±6%) with 12 h 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 h fasting a blood sample was obtained for serum NOx measurement and stomach and liver samples for NOx 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 min while blood drained through the right auricle. Stomach and liver were removed, weighed and homogenized in ice-cold perfusion solution by a homogenizer (Miccre D-1, Germany) and sonicated with a Microson XL 2000 sonicator (USA) while immersed in an ice-water bath.

Serum and tissue NOx measurement: Serum and tissue NOx concentrations were measured by the Griess reaction [18]. In brief, serum samples were deproteinized by zinc sulfate (15 mg/mL), and centrifuged at 10000 g for 10 min; tissue homogenates were first centrifuged at 15000 g for 20 min, then zinc sulfate (15 mg/mL) was added and after one minute shaking, samples were recentrifuged at 15000 g for 20 min. 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 min at 37ºC; absorbance was read at 540 nm using the ELISA reader (Sunrise, Tecan, Austria). NOx 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 NOx levels were expressed as nmol/mg protein.

SPSS-20 was used for statistical analyses. Because of the skewed distribution of NOx 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 NOx levels. Two-sided p-values<0.05 were considered statistically significant.

Results

In the control group, median (interquartile range) stomach NOx 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 NOx levels (Fig. 1A). Liver NOx 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 NOx levels while L-arginine administration decreased it to non-detectable levels (Fig. 2A). Median (interquartile range) serum NOx 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 NOx are shown in figures 1B 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) NOx levels.
in mice [23]. reported that nitrite treatment increases liver nitrite levels 
and similar to our results Duranski et al. have respectively [8]. Nitrate is considered as a prodrug of
administration of 300 mg/L and 1500 mg/L sodium nitrite
increases in stomach nitrate content following one-week
mmol/kg). Raat et al. have reported 1.5-fold and 2.3-fold
which we used in the current study (500 mg/L or 0.6
mmol/kg in rats [1], a dose approximately twice that
reported 10.7 folds increase in nitrate levels of stomach
levels, B: Correlation between serum and liver NOx content
between tissues may reflect the degree of NOS activity
has been reported that differences in nitrite concentrations
contents following nitrate administration were different; it
nitrite administration has been previously reported [24].
Similar to our results, Raat et al. have reported a direct
correlation between plasma and liver nitrite concentrations [8]; some authors have suggested that high
correlation between serum and some tissue NOx indicates
non-specific accumulation of NOx in these organs [27]
while others suggest that anion transporters aid regulated-
and tissue-specific transport of nitrate 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 NOx by 44% and those of liver NOx to
non-detectable values. In line with our results, Ohta and
Nishida have reported that administration of L-arginine
could prevent stress-induced increases in the gastric
mucosa NOx levels in rats [2]. 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 synthase 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 NOx levels in the stomach and liver of normal rats.
In addition, direct correlations were observed between
serum and the tissues NOx levels, findings which may be
important considering the fast accumulating evidence on
the protective roles of dietary nitrate and nitrite.

Discussion

The results of this study indicated a 4.1-fold and 1.1-fold
increases in stomach and liver NOx 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. have
reported 10.7 folds increase in nitrate levels of stomach
following one-week administration of sodium nitrate of 1
mmol/kg in rats [1], a dose approximately twice that
which we used in the current study (500 mg/L or 0.6
mmol/kg). Raat et al. have reported 1.5-fold and 2.3-fold
increases in stomach nitrate content following one-week
administration of 300 mg/L and 1500 mg/L sodium nitrite
respectively [8]. Nitrate is considered as a prodrug of
nitrite [22] and similar to our results Duranski et al. have
reported that nitrite treatment increases liver nitrite levels
in mice [23].
The changes we observed in stomach and liver NOx
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 NOx 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 NOx levels, a finding in line with a previous report
that nitrate concentration of the blood is a major
determinant of NOx levels of the rest of the body [26].
Close correlation between plasma and tissue nitrite after
nitrite administration has been previously reported [24].

Figure 2. A: Box plots showing the effects of sodium nitrate and
L-arginine administration on NOx levels of the liver. Liver NOx 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 NOx
levels while L-arginine administration decreased it to non-detectable
levels. B: Correlation between serum and liver NOx content
* Significant difference compared to control group. ND: non-detectable.
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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.

Conflict of Interest

The authors declare no conflict of interest.

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