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Active secretion and protective effect of salivary nitrate against stress in human volunteers and rats

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Abstract

Up to 25% of the circulating nitrate in blood is actively taken up, concentrated, and secreted into saliva by the salivary glands. Salivary nitrate can be reduced to nitrite by the commensal bacteria in the oral cavity or stomach and then further converted to nitric oxide (NO) in vivo, which may play a role in gastric protection. However, whether salivary nitrate is actively secreted in human beings has not yet been determined. This study was designed to determine whether salivary nitrate is actively secreted in human beings as an acute stress response and what role salivary nitrate plays in stress-induced gastric injury. To observe salivary nitrate function under stress conditions, alteration of salivary nitrate and nitrite was analyzed among 22 healthy volunteers before and after a strong stress activity, jumping down from a platform at the height of 68m. A series of stress indexes was analyzed to monitor the stress situation. We found that both the concentration and the total amount of nitrate in mixed saliva were significantly increased in the human volunteers immediately after the jump, with an additional increase 1 h later ($p < 0.01$). Saliva nitrite reached a maximum immediately after the jump and was maintained 1 h later. To study the biological functions of salivary nitrate and nitrite in stress protection, we further carried out a water-

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Appendix A. Supporting information

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immersion-restraint stress (WIRS) assay in male adult rats with bilateral parotid and submandibular duct ligation (BPSDL). Intragastric nitrate, nitrite, and NO; gastric mucosal blood flow; and gastric ulcer index (UI) were monitored and nitrate was administered in drinking water to compensate for nitrate secretion in BPSDL animals. Significantly decreased levels of intragastric nitrate, nitrite, and NO and gastric mucosal blood flow were measured in BPSDL rats during the WIRS assay compared to sham control rats ($p < 0.05$). Recovery was observed in the BPSDL rats upon nitrate administration. The WIRS-induced UI was significantly higher in the BPSDL animals compared to controls, and nitrate administration rescued the WIRS-induced gastric injury in BPSDL rats. In conclusion, this study suggests that stress promotes salivary nitrate secretion and nitrite formation, which may play important roles in gastric protection against stress-induced injury via the nitrate-dependent NO pathway.

Keywords

Stress; Salivary glands; Saliva; Nitrate; Nitrite; Nitric oxide; Gastric ulcer; Free radicals

For half a century, dietary nitrate and nitrite have been considered as the precursors of carcinogenic *N*-nitroso compounds [1–3]. However, the nitrate and nitrite anions (NO_3^- and NO_2^- , respectively) can be generated endogenously in the human body in the case of a shortage of dietary nitrate intake [4]. Thus, these anions may be essential components of the body, though their exact physiological functions are unknown. The salivary glands are important organs that mediate metabolism and dynamic nitrate balance through the enterosalivary circulation of nitrate. Dysfunction in the salivary glands has been linked to decreased nitrate secretion and increased nitrate levels in the serum and urine [5,6].

Up to 25% of circulating nitrate is actively taken up by the salivary glands, concentrated approximately 10-fold, and secreted into the saliva [7,8]. Salivary nitrate can be reduced to nitrite by the commensal bacteria in the mouth and/or stomach and then metabolized to nitric oxide (NO) in the acidic stomach [9–11]. Though most nitrate enters the systemic circulation, some salivary nitrate can be converted to nitrite and NO in other tissues [9,12]. Thus, nitrate uptake from the blood to the salivary glands seems to be a critical step in the enterosalivary circulation of nitrate. Sialin, which localizes in the lysosomes and cytoplasmic membrane of salivary glandular epithelial cells, has been shown to mediate the transport of NO_3^- , which may play an important role in the physiological regulation of systemic nitrate–nitrite–NO homeostatic balance [13,14].

The recycled salivary nitrate and nitrite absorbed into the blood can significantly influence the nitrate–nitrite–NO balance in the blood, which is important in conditions such as high blood pressure, platelet aggregation, and vascular damage [8–10]. High concentration of salivary nitrate or nitrite might be involved in host defense against oral and gastric pathogens [8,15], and dietary and recycled salivary nitrate or nitrite may also play important roles in gastric protection [16]. Salivary nitrate and nitrite that enter the stomach may enhance the bactericidal effects of gastric juice [17], increase gastric mucus blood flow and mucus thickness, and relieve chemically induced gastric ulcers [18].

However, previous studies were mainly focused on the protective effects of exogenous administration of nitrate and nitrite. In what circumstances salivary nitrate is actively secreted has not yet been determined in human beings. In this study, we investigated the effects of acute stress conditions on nitrate secretion and nitrite formation in human volunteers and the role of salivary nitrate/nitrite on stress-induced gastric injury in rats. Here, we report for the first time that the levels of salivary nitrate and nitrite are increased significantly under stress conditions and may play an important role in protection against stress-induced gastric injury via the nitrate-dependent NO pathway.

Methods

Human bungee jump protocol

Twenty-two healthy volunteers naive to bungee jumping or skydiving (14 males and 8 females, age range 21–45 years, mean age 27 years) were involved in the human stress trial. All volunteers had been living in Beijing, China, for more than 1 year and shared similar dietary habits. The trial was reviewed and approved by the ethics committees of Capital Medical University of China. Written informed consent was obtained from all subjects.

All subjects participated in a bungee jump from a height of 68 m, from a trestle platform fixed at the rock shore of the Qinlongxia Water Reservoir, Huairou District, Beijing, under the supervision and guidance of an experienced commercial bungee jump crew. The participants consumed the same food and water the day before the jump. On the morning of the study day, at 3 h before the jump, the first mixed saliva samples were collected. Subsequent saliva samples were collected immediately after the jump and 1 h after the jump. Unstimulated mixed saliva was collected in a quiet room in a germ-free centrifugal tube for 10 min; the static salivary flow rate represents the average amount of saliva per minute [19]. Saliva samples were 10,000 MW filtered and diluted before assay. The concentrations of nitrate and nitrite in the saliva were detected by high-performance liquid chromatography (HPLC) as described previously [5,6].

Before the bungee jump, the primary physical indexes (including respiratory rate, pulse rate, blood pressure) were measured just before saliva collection. These physical indexes were measured again directly after the jump. Blood samples were taken just after saliva collection before and after the bungee jump. Blood was drawn into Vacutainer tubes containing reduced glutathione–EGTA buffer for measurement of plasma noradrenalin and adrenalin. Plasma was separated and stored at –80 °C until assays were performed. Plasma noradrenalin and adrenalin were detected by HPLC [20].

Experimental animals and study design

All animal experiments were approved by the ethics committees of Capital Medical University of China. Male adult Sprague–Dawley rats (5–6 weeks of age; body weight (bw) 180–200 g) were kept under standardized conditions at 21–22 °C with 12-h light/dark cycles. The rats were allowed to adapt to this environment in mesh-bottom cages with free access to distilled drinking water and regular pellet food for at least 7 days before the experiment. The rats were randomly divided into four groups as shown in Supplementary

Fig. 1. The rats were anesthetized for each procedure with 0.4 ml/100 g bw of 10% chloral hydrate (Sigma, St. Louis, MO, USA) via intraperitoneal injection.

Bilateral parotid and submandibular gland duct ligation (BPSDL)

After the facial skin was incised, the parotid and submandibular gland ducts were surgically exposed. Bilaterally, the parotid and submandibular gland ducts were ligated to block secretion of saliva. Control rats were involved in a sham operation (sham) without any duct ligation.

Nitrate pretreatment

Half of the rats with and without BPSDL were randomly selected for nitrate supplementation (BPSDL + nitrate; sham + nitrate) and administered sodium nitrate (NaNO_3) dissolved in distilled water (5 mmol/L) for 7 days before the stress experiment. The daily dose of nitrate was approximately 1 mmol/kg bw as described previously [21]. The remaining rats with or without BPSDL were used as control groups (BPSDL; sham) and administered distilled water containing sodium chloride (NaCl) of the same dose. The baseline concentration of nitrate in the distilled water was less than 1 μM .

Measurement of gastric luminal NO

The concentration of gastric luminal NO gas in anesthetized fasting rats ($n = 6$ for each group) was analyzed before the stress experiment as described previously [22]. Briefly, the stomach was directly inflated with NO-free air ($\text{NO} < 3$ ppb) using a 5-ml syringe with a thin needle. External clamps were placed over the lower esophagus and duodenum to prevent leakage of the gas into these compartments during the sampling. After the air was incubated for 15 s, the gas was aspirated and immediately injected into a chemiluminescence analyzer (Interscan, USA) and the peak NO concentration recorded. The instrument's detection limit for NO was 10 ppb. Calibration of the instrument was performed with cylinder gas (10 ppm NO in nitrogen; AGA AB, Lidingö, Sweden).

Measurements of nitrate and nitrite in fasting gastric juice

Additional rats ($n = 6$ for each group, the grouping is described in Supplementary Fig. 1) were fasted overnight, anesthetized, and subjected to pyloric ligation. Four hours after the ligation, the animals were sacrificed, the abdomen was opened, and another ligation was placed around the distal esophagus close to the diaphragm. The stomach was removed and its contents drained into a graduated centrifuge tube, which was centrifuged at 2000 g for 15 min. NaOH (0.1 M) was added to the sample at a v/v ratio of 1/19 to prevent the nitrite from degrading. Gastric juice samples were 10,000 MW filtered and diluted before assay. The concentrations of nitrate and nitrite in the saliva were detected by HPLC as described previously [5,6].

Induction of gastric ulcer in the water-immersion-restraint stress (WIRS) assay

The WIRS assay was used in this study to induce gastric mucosal lesions [23]. Additional rats ($n = 6$ for each group) were fasted for 18–20 h before the experiment and then fixed on the rat boards and immersed in a 20 ± 2 °C water bath for 4 h up to the depth of the xiphoid

process. After general anesthesia, the abdomen was opened through a midline incision. The stomach was removed and incised along the greater curvature after being prefixed with 4% paraformaldehyde for 10 min. After a gentle wash with phosphate-buffered saline, the stomach was spread for subsequent photography. The ulcer index (UI; expressed as mm) was used to reflect the total length of gastric lesions per stomach measured by two independent researchers blinded to the protocol.

Determination of gastric mucosal blood flow

The principle of laser Doppler flowmetry (LDF) for the assessment of gastric blood flow was described previously [24]. A Moor VMS-LDF (Moor Instrument, UK) was used. The mucosal blood flow in the glandular stomach of rats during an independent stress experiment was determined as a voltage output and expressed as perfusion units (PU). An optical probe was placed gently 0.5 mm above and perpendicular to the mucosal surface of the glandular stomach. When the blood flow stabilized, three points were selected for measurement (one point for 3 min) and the average value was calculated.

Histological observation of mucosal ulcers in the glandular stomach of rats

Gastric tissue samples were fixed with 4% paraformaldehyde for 24 h. The tissue slides (4 μ m) were prepared and stained with hematoxylin and eosin. Hemorrhage spots and lines and ulcers involving the gastric mucosa lamina propria were defined as gastric surface ulcers, whereas deep ulcers touched muscularis mucosa or the submucosal and muscular layer. The total number of ulcers for six stomach samples of each group was counted. The proportion of deep ulcers was calculated to determine the severity of gastric injury.

Statistical analysis

All data are presented as the mean $\pm$ SE except the human salivary nitrate and nitrite data, which are presented as the median (range). Differences in the human physical indexes before and after the jump were evaluated by paired *t* test. Human salivary nitrate and nitrite data were evaluated by the nonparametric Kruskal–Wallis test. Differences in animal data were evaluated by one-way ANOVA. Levels of gastric nitrate, nitrite, and NO were determined by the Games–Howell test. Blood flow, UI, and percentage of deep ulcers were followed by the Fisher protected least significant difference test. *p* values of less than 0.05 were considered significant.

Results

Primary physical indexes significantly changed after acute stress

To confirm the stress status of each subject, primary physical indexes, including breath, pulse, systolic pressure, diastolic pressure, plasma adrenalin, and plasma noradrenalin were determined 3 h before and immediately after the jump. The average frequency of breaths increased from 15 ± 4 to 23 ± 6 per minute ($p < 0.001$) and the average pulse rate increased from 67 ± 8 to 114 ± 20 per minute ($p < 0.001$, Fig. 1A and 1B). Blood pressure increased in a similar fashion ($p < 0.001$, Figs. 1C and 1D). The average concentration of noradrenalin increased from 1.81 ± 0.61 to 3.13 ± 1.28 nM ($p < 0.001$) and plasma adrenalin increased

from 0.49 ± 0.10 to 0.55 ± 0.08 nM ($p = 0.040$, Figs. 1E and 1F). These data indicated that all tested subjects suffered extreme acute stress.

Levels of human salivary nitrate and nitrite significantly increased after the acute stress jump

To obtain insight into the impact of the bungee jump on levels of salivary nitrate and nitrite formation, we sequentially measured the levels of salivary nitrate and nitrite. The concentration of nitrate in mixed saliva was significantly increased immediately after the jump, with additional increases 1 h later. The concentration of nitrate gradually increased from 5.6 (median; range 1–509.8) μ M 3 h before the jump to 27.0 (0.7–938.9) μ M immediately after the jump and then to 168.8 (3–1244.8) μ M 1 h postjump ($p < 0.001$, Fig. 2A). Saliva nitrite reached a maximum immediately after the jump and was maintained 1 h later. The concentration of salivary nitrite increased from 0.64 (0.01–21.91) μ M 3 h before the jump to 4.82 (0.11–29.17) μ M postjump, which was maintained at 4.95 (0.88–15.2) μ M 1 h postjump ($p = 0.002$, Fig. 2B). Similar increase patterns were also found for the total amount of salivary nitrate and nitrite secreted in 10 min (Figs. 2C and 2D).

Block of nitrate secretion by BPSDL disturbed the gastric nitrate–nitrite–NO pathway in the stomach

To test the function of salivary nitrate/nitrite on stress-induced gastric injury, we designed the rat stress experiment, which is illustrated in Supplementary Fig. 1. Concentration and total amount of salivary nitrate in rats with bilateral parotid gland duct ligation or bilateral submandibular gland duct ligation slightly decreased, but not significant (Supplementary Fig. 2). Thus, we further tried BPSDL for each animal, which could completely block the enterosalivary circulation of nitrate. Results showed that the BPSDL treatment significantly decreased levels of gastric nitrate, nitrite, and luminal NO in the stomachs of rats. The average concentration of nitrate in gastric juice samples from fasting rats was 60% decreased by the BPSDL ($p = 0.039$, Table 1). The concentration of gastric nitrite was 66% decreased ($p = 0.050$). The gastric NO level was 62% decreased ($p = 0.026$). After nitrate supplementation in the drinking water for 1 week, the nitrate and nitrite levels in the fasting gastric juice significantly increased (1070 and 500% increase in the BPSDL + nitrate group compared to BPSDL; $p = 0.028$ and 0.032). A similar pattern was observed for the level of luminal NO, which was 1000% increased ($p = 0.004$).

Block of nitrate secretion by BPSDL aggravated stress-induced gastric ulcers

On the basis of significant changes in gastric nitrate, nitrite, and NO levels via BPSDL, we further explored the effects of these changes on stress-induced gastric ulcers. Animals in the BPSDL group had noticeably reduced mucosal blood flow, compared to the sham group (25% decreased, $p = 0.021$, Table 1). Accordingly, the average degree of gastric mucosal injury (UI) significantly increased in the BPSDL group rats (141% increase compared to sham, $p = 0.003$, Table 1 and Fig. 3). Animals in the BPSDL group displayed more severe gastric ulcers (i.e., suffered from more deep ulcers) than those in the sham group ($25.75 \pm 2.68\%$ vs $5.0 \pm 5.0\%$, $p = 0.006$, Fig. 4).

Administration of nitrate significantly reduced stress-induced gastric ulcers

To test whether administration of nitrate rescues gastric injury in rats with the BPSDL treatment, we pretreated these rats with nitrate in their drinking water. The average gastric mucosal blood flow in six rats in the BPSDL + nitrate group was restored (58% increase compared to BPSDL, $p < 0.001$) and the average UI was rescued to a level similar to that in the sham group (58% decrease to BPSDL, $p = 0.003$, Table 1 and Fig. 3). A similar increase in gastric mucosal blood flow was observed in the nitrate-treated sham rats. However, the decrease in UI in sham + nitrate rats was slight and not significant (28.2% decrease compared to sham, $p = 0.487$, Table 1 and Fig. 3). The administration of nitrate in the drinking water successfully reduced the percentage of deep ulcers to $8.12 \pm 4.93\%$ in BPSDL rats ($p = 0.016$, Fig. 4). The protective effect of nitrate was not observed in the sham and sham + nitrate groups.

Discussion

In this study, we found that the levels of salivary nitrate and nitrite were immediately increased in human volunteers after a strong acute stress caused by high-platform jumping. To explore the biological function of salivary nitrate and nitrite, we carried out a rat WIRS assay. We found that blocking nitrate secretion from the salivary glands results in a significant decrease in gastric mucosal blood flow and increased gastric ulcers in rats treated by BPSDL, which leads to $> 60\%$ decreases in the levels of gastric nitrate, nitrite, and NO. Supplementation of nitrate in the drinking water rescued the stress-induced gastric injury in the BPSDL rats, indicating that a shortage of salivary nitrate and nitrite may account for the increased gastric injuries.

Physiologically, up to 25% of dietary nitrate is actively taken up by the salivary glands and is concentrated up to 10-fold in saliva [7,8], leading to a much higher NO_3^- level in saliva than in blood [25,26]. Although it has been known for decades that salivary glands secrete nitrate positively, the specific patterns of when nitrate is actively secreted are not clear. Thus, we designed the human volunteer stress experiment and demonstrated that acute stress induced increased levels of salivary nitrate and nitrite. Both nitrate and nitrite have therapeutic effects in animal models of ischemia–reperfusion injury [27,28], reduced cellular oxygen consumption [29], reversal of metabolic syndrome, reduced oxidative stress [30], and protection against gastric ulcerations [31,32]. Short-term dietary nitrate supplementation has also been shown to control blood pressure [10,33,34]. Therefore, we speculated that the actively increased salivary nitrate and nitrite levels in humans under strong stress may provide potential protection from damage induced by persistent stress-adaptive responses such as gastric ischemia and ulcers caused by long-term vasoconstriction. The concentration and total amount of salivary nitrate gradually increased after the jump. In contrast, the concentration and total amount of salivary nitrite was maximal immediately after the jump and maintained at the same level 1 h after the jump. This finding is consistent with a previous report that the ingestion of inorganic nitrate leads to increased human salivary nitrate within 30min, whereas salivary nitrite quickly increased for 15 min and remained at this level for 30 min [35]. These findings suggest that the conversion of nitrate to nitrite in saliva is dependent on nitrate, as well as other factors. The total number of commensal

bacteria in the oral cavity that contain nitrate reductases is considered a crucial factor for determining the conversion rate of nitrate to nitrite in saliva. Because the bacteria number in the oral cavity is relatively stable in the absence of eating, the conversion of nitrate to nitrite can easily achieve saturation when salivary nitrate reaches a certain level.

We recently found a novel function for the typical lysosomal SA^-/H^+ transporter sialin (*SLC17A5*) as an electrogenic $2\text{NO}_3^-/\text{H}^+$ cotransporter in the cytoplasmic membrane in glandular epithelial cells of the salivary glands [13,14]; whether the active secretion of salivary nitrate under acute stress response is related to sialin deserves further investigation.

The dietary nitrate–nitrite–NO pathway is considered an alternative source of the endogenous formation of the biological messenger NO, which has been suggested to play a role in gastric protection [16]. In this study, we showed that both nitrate and nitrite levels in saliva are increased after stress. Completely blocking nitrate secretion by the salivary glands decreased gastric mucosal flow and increased gastric injuries. Supplement of dietary nitrate in drinking water reduced the stress-induced gastric damage in the BPSDL animals. These data indicate that nitrate secreted from the salivary glands may be physiologically involved in gastric protection against stress-induced damage. NO is suggested to have an important role in the protection of gastric mucosa [31,32]. Dietary nitrate increases gastric NO levels and potentially protects against the macroscopic injury caused by NSAID exposure [21,36]. In addition, nitrate pretreatment decreases mucosal myeloperoxidase activity and the expression of iNOS, which indicates reduced tissue inflammation [17]. Gastric NO is evidently abolished in rats after cardiac ligation, which effectively inhibits the transfer of nitrate from saliva to the stomach [31]. Together these data suggest that disruption of the nitrate–nitrite–NO pathway accounts for the decrease in gastric mucosa blood flow and increased WIRS-induced gastric ulcers. However, we did not find significant differences in the protective effect between the sham and the sham + nitrate groups. Physiological concentration of salivary nitrate may be sufficient in response to the acute stress, and additional nitrate supplementation may not show a distinctive discrepancy.

Hypofunction of the parotid glands is seen in patients with Sjögren syndrome, an autoimmune disease that mainly involves salivary and lachrymal glands [37]. The concentration of nitrate and nitrite in mixed saliva was significantly decreased, and chronic atrophic gastritis is often found in these patients [6,38]. Therefore, whether oral administration of nitrate offers guaranteed delivery of nitrite/NO into the stomach, thus providing protection against gastric injury, is worth studying.

In summary, endogenous secretion of salivary nitrate is adaptively increased in acute stress. Increased salivary nitrate and nitrite may protect against stress-induced gastric injury via the nitrate–nitrite–NO pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

WIRS	water-immersion-restraint stress
BPSDL	bilateral parotid and submandibular duct ligation
UI	ulcer index
HPLC	high-performance liquid chromatography
LDF	laser Doppler flowmetry

References

1. Tannenbaum SR, Correa P. Nitrate and gastric cancer risks. *Nature*. 1985; 317:675–676. [PubMed: 4058577]
2. Mirvish SS. Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. *Cancer Lett*. 1995; 93:17–48. [PubMed: 7600541]
3. Deng DJ. Progression of gastric cancer etiology: N-nitrosamides in the 1990s. *World J. Gastroenterol*. 2000; 6:613–618. [PubMed: 11819660]
4. Green LC, Tannenbaum SR, Goldman P. Nitrate synthesis in the germfree and conventional rat. *Science*. 1981; 212:56–58. [PubMed: 6451927]
5. Xia DS, Deng DJ, Wang SL. Destruction of parotid glands affects nitrate and nitrite metabolism. *J. Dent. Res*. 2003; 82:101–105. [PubMed: 12562881]
6. Xia DS, Deng DJ, Wang SL. Alterations of nitrate and nitrite content in saliva, serum, and urine in patients with salivary dysfunction. *J. Oral Pathol. Med*. 2003; 32:95–99. [PubMed: 12542832]
7. Pannala AS, Mani AR, Spencer JP, Skinner V, Bruckdorfer KR, Moore KP, Rice-Evans CA. The effect of dietary nitrate on salivary plasma and urinary nitrate metabolism in humans. *Free Radic. Biol. Med*. 2003; 34:576–584. [PubMed: 12614846]
8. McKnight GM, Duncan CW, Leifert C, Golden MH. Dietary nitrate in man: friend or foe? *Br. J. Nutr*. 1999; 81:349–358. [PubMed: 10615207]
9. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Discovery*. 2008; 7:156–167.
10. Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, Weitzberg E. Effects of dietary nitrate on blood pressure in healthy volunteers. *N. Engl. J. Med*. 2006; 355:2792–2793. [PubMed: 17192551]
11. Benjamin N, O'Driscoll F, Dougall H, Duncan C, Smith L, Golden M, McKenzie H. Stomach NO synthesis. *Nature*. 1994; 368:502. [PubMed: 8139683]
12. Jansson EA, Huang L, Malkey R, Govoni M, Nihlén C, Olsson A, Stensdotter M, Petersson J, Holm L, Weitzberg E, Lundberg JO. A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. *Nat. Chem. Biol*. 2008; 4:411–417. [PubMed: 18516050]
13. Qin LZ, Liu XB, Sun QF, Fan ZP, Xia DS, Ding G, Ong HL, Adams D, Gahl W, Zheng CY, Qi SR, Jin LY, Zhang CM, Gu LK, He JQ, Deng DJ, Ambudkar IS, Wang SL. Sialin (*SLC17A5*) functions as a nitrate transporter in the plasma membrane. *Proc. Natl. Acad. Sci. USA*. 2012; 109:13434–13439. [PubMed: 22778404]
14. Lundberg JO. Nitrate transport in salivary glands with implications for NO homeostasis. *Proc. Natl. Acad. Sci. USA*. 2012; 109:13144–13145. [PubMed: 22851765]
15. Shi RT, Qin LZ, Xia DS, Deng DJ, Fan ZP, Shan ZC, Xu YY, Wang SL. Increase of saliva nitrate and nitrite level in patients with oral candidacies. *Chin. J. Prev. Med*. 2009; 43:607–610.

16. Lundberg JO, Weitzberg E, Lundberg JM, Alving K. Intra-gastric nitric oxide production in humans: measurements in expelled air. *Gut*. 1994; 35:1543–1546. [PubMed: 7828969]
17. Bjorne H, Weitzberg E, Lundberg JO. Intra-gastric generation of antimicrobial nitrogen oxides from saliva—physiological and therapeutic considerations. *Free Radic. Biol. Med.* 2006; 41:1404–1412. [PubMed: 17023267]
18. Petersson J, Phillipson M, Jansson EA, Patzak A, Lundberg JO, Holm L. Dietary nitrate increases gastric mucosal blood flow and mucosal defense. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2007; 292:G718–G724. [PubMed: 17082222]
19. Wang SL, Zhao ZT, Li J, Zhu XZ, Dong H, Zhang YG. Investigation of the clinical value of total saliva flow rates. *Arch. Oral Biol.* 1998; 43:39–42. [PubMed: 9569989]
20. Jin L, Xiao SZ, Liu LS. High-performance liquid chromatographic assay of plasma adrenalin noradrenalin and dopamine. *Chin. Circ. J.* 1988; 3:107–109.
21. Jansson EA, Petersson J, Reinders C, Sobko T, Björne H, Phillipson M, Weitzberg E, Holm L, Lundberg JO. Protection from nonsteroidal anti-inflammatory drug (NSAID)-induced gastric ulcers by dietary nitrate. *Free Radic. Biol. Med.* 2007; 42:510–518. [PubMed: 17275683]
22. Sobko T, Reinders C, Norin E, Midtvedt T, Gustafsson LE, Lundberg JO. Gastrointestinal nitric oxide generation in germ-free and conventional rats. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2004; 287:G993–G997. [PubMed: 15256364]
23. Takagi K, Okabe S. The effects of drugs on the stress ulcer in rats. *Jpn. J. Pharmacol.* 1968; 18:9–18. [PubMed: 5302464]
24. Holm-Ruttil L, Berglindh T. Pentagastrin and gastric mucosal blood flow. *Am. J. Physiol. Gastrointest. Liver Physiol.* 1986; 250:G575–G580.
25. Cortas NK, Wakid NW. Pharmacokinetic aspects of inorganic nitrate ingestion in man. *Pharmacol. Toxicol.* 1991; 3:192–195. [PubMed: 2057449]
26. Wagner DA, Schultz DS, Deen WM, Young VR, Tannenbaum SR. Metabolic fate of an oral dose of ¹⁵N-labeled nitrate in humans: effect of diet supplementation with ascorbic acid. *Cancer Res.* 1983; 43:1921–1925. [PubMed: 6831427]
27. Dezfulian C, Raat N, Shiva S, Gladwin MT. Role of the anion nitrite in ischemia-reperfusion cytoprotection and therapeutics. *Cardiovasc. Res.* 2007; 75:327–338. [PubMed: 17568573]
28. Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc. Natl. Acad. Sci. USA.* 2004; 101:13683–13688. [PubMed: 15347817]
29. Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Eklöf B, Lundberg JO, Weitzberg E. Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab.* 2011; 13:149–159. [PubMed: 21284982]
30. Carlström M, Persson AE, Larsson E, Hezel M, Scheffer PG, Teerlink T, Weitzberg E, Lundberg JO. Dietary nitrate attenuates oxidative stress, prevents cardiac and renal injuries, and reduces blood pressure in salt-induced hypertension. *Cardiovasc. Res.* 2011; 89:574–585. [PubMed: 21097806]
31. Miyoshi M, Kasahara E, Park AM, Hiramoto K, Minamiyama Y, Takemura S, Sato EF, Inoue M. Dietary nitrate inhibits stress-induced gastric mucosal injury in the rat. *Free Radic. Res.* 2003; 37:85–90. [PubMed: 12653221]
32. Larauche M, Anton PM, Garcia-Villar R, Theodorou V, Frexinos J, Buéno L, Fioramonti J. Protective effect of dietary nitrate on experimental gastritis in rats. *Br. J. Nutr.* 2003; 89:777–786. [PubMed: 12828794]
33. Lundberg JO, Carlström M, Larsen FJ, Weitzberg E. Roles of dietary inorganic nitrate in cardiovascular health and disease. *Cardiovasc. Res.* 2011; 89:525–532. [PubMed: 20937740]
34. Gilchrist M, Shore AC, Benjamin N. Inorganic nitrate and nitrite and control of blood pressure. *Cardiovasc. Res.* 2011; 89:492–498. [PubMed: 20884639]
35. Govoni M, Jansson EA, Weitzberg E, Lundberg JO. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide.* 2008; 19:333–337. [PubMed: 18793740]
36. Lundberg JO, Govoni M. Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic. Biol. Med.* 2004; 37:395–400. [PubMed: 15223073]

37. Wang SL, Zou ZJ, Yu SF, Zhu JR. Recurrent swelling of parotid gland and Sjögren's syndrome. *Int. J. Oral Maxillofac. Surg.* 1993; 22:362–365. [PubMed: 8106813]
38. Sugaya T, Sakai H, Sugiyama T, Imai K. Atrophic gastritis in Sjögren's syndrome. *Nihon Rinsho.* 1995; 53:2540–2544. [PubMed: 8531370]

Fig. 1.

Physical indexes for human volunteers under acute stress conditions. (A, B) Breath and pulse frequency, (C, D) blood pressure, and (E, F) plasma noradrenalin and adrenalin concentrations increased immediately after the jump compared with 3 h before the jump. Values are presented as the mean $\pm$ SD, $n = 22$, * $p < 0.05$, ** $p < 0.01$ compared to prejump values.

Fig. 2. Changes in human salivary nitrate and nitrite levels under acute stress conditions. (A, B) The concentrations and (C, D) total amounts of salivary nitrate and nitrite in the human volunteers continuously increased immediately and 1 h after the jump. In the prejump column of (D), one dot was outside of the y-axis limit; therefore, the number of dots is 21. Values are presented as the median (range), $n = 22$, $*p < 0.05$.

Fig. 3.

Images of the gastric injury induced by WIRS in rats. Macroscopically visible ulcer lesions in the sham, sham + nitrate, BPSDL, and BPSDL + nitrate groups are shown. Rats with BPSDL suffered from more gastric injury than the sham group. Nitrate pretreatment greatly reduced the injury among BPSDL rats. No significant difference was found between the sham and the sham + nitrate groups.

Fig. 4. Proportion of deep ulcers to total ulcers. (A) Surface ulceration, (B) deep ulceration, and (C) normal gastric mucosa were observed in all groups. (D) The total number of ulcerations in six stomach samples of each group was counted, and deep ulcers accounted for a higher proportion of lesions in the BPSDL group, whereas nitrate (N) pretreatment reduced the severity of ulceration. Sham rats with nitrate pretreatment exhibited no difference compared to sham rats. Values are presented as the mean $\pm$ SD, $n = 6$, $*p < 0.05$ compared to BPSDL.

Table 1

Effects of BPSDL and nitrate pretreatment on gastric nitrate, nitrite, and NO levels; mucosal blood flow; and UI in rats subjected to WIRS.

	Gastric nitrate ($\mu\text{mol/L}$; $n = 6$)	Gastric nitrite ($\mu\text{mol/L}$; $n = 6$)	Luminal NO (ppb; $n = 6$)	Blood flow (PU; $n = 6$)	Ulcer index (mm; $n = 6$)
Sham	54.2 $\pm$ 19.9	2.05 $\pm$ 0.61	93 $\pm$ 22	47.24 $\pm$ 18.33	14.2 $\pm$ 11.7
Sham + nitrate	345.2 $\pm$ 210.7	6.37 $\pm$ 2.37	720 $\pm$ 230	69.34 $\pm$ 11.81	10.2 $\pm$ 6.9
BPSDL	21.3 $\pm$ 12.6 ^a	0.70 $\pm$ 0.26 ^a	36 $\pm$ 15 ^a	35.03 $\pm$ 10.79 ^a	34.3 $\pm$ 13.6 ^a
BPSDL + nitrate	249.3 $\pm$ 130.7 ^b	3.48 $\pm$ 1.65 ^b	404 $\pm$ 102 ^b	55.61 $\pm$ 18.41 ^b	14.7 $\pm$ 6.7 ^b

Nitrate treatment groups were given 1 mmol/kg bw of NaNO₃ in drinking water for 1 week before the WIRS experiment. The sham and BPSDL groups were given NaCl of the same dose. Each value represents the mean $\pm$ SD.

^a $p < 0.05$ compared to sham.

^b $p < 0.05$ compared to BPSDL.