Original Article
Long-term dietary inorganic nitrate increases gastric mucosal blood flow in Mongolian gerbils

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Abstract: Inorganic nitrate is rich in human diet especially in some vegetables. Dietary inorganic nitrate can be absorbed into the blood, recycled in salivary glands, reduced to nitrite by oral bacteria, and further reduced to bioactive nitrogen oxides in the acidic stomach for maintaining the systemic nitrate (NO₃⁻)-nitrite (NO₂⁻)-NO homeostatic balance in the body [6-8]. These dietary nitrate exerts multiple physiological functions in the body such as reduction in blood pressure [9], vasodilatation [10], and improvement in brain perfusion [11]. Notably, recent studies show that short-term administration of nitrate could increase gastric mucosal blood flow to maintain the gastric mucosal integrity [12, 13]. It is well established that adequate mucosal blood flow plays an important role in protecting the gastric mucosa from injuries [14]. A stable mucosal blood flow helps to carry bicarbonate to neutralize acid and removes toxic compounds. Our previous study showed that short-term administration of nitrate significantly restored the average gastric mucosal blood flow and reduced stress-induced gastric ulcers in rats [15]. However, whether long-term delivery of dietary inorganic nitrate plays roles in gastric mucosal blood flow and hence helps to enhance the mucosal defense safely is unknown. Since the gastric pathological changes are similar to those observed in human patients [16], Mongol-
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Lian gerbils were used as experimental animal model to investigate the long-term effects of dietary nitrate on gastric mucosal blood flow. Here we provide evidences that long-term nitrate supplementation in diet is beneficial for maintaining gastric mucosal blood flow.

Materials and methods

Animals

Male Mongolian gerbils (6 weeks old, 40-50 g) were provided by Capital Medical University. All animals were kept under standardized conditions at 21-22°C with 12 h light/dark cycles. The animals were allowed to adapt to this environment with free access to distilled drinking water and regular pellet food for at least 7 days before the experiment. All animal works were conducted in accordance with the Animal Care and Use Committee of the Capital Medical University.

Nitrate supplementation

A total of 36 qualified Mongolian gerbils were randomly divided into 2 groups (n=18 each): the nitrate treated group and the sodium chloride treated (control) group. The nitrate treated Mongolian gerbils were administered 1 mM sodium nitrate (NaNO₃) dissolved in distilled water for 6 months. The daily dose of nitrate intake in Mongolian gerbils was approximately 0.2 mmol·kg⁻¹ as described previously [15]. The remaining animals were used as control groups and administered distilled water containing sodium chloride (NaCl) of the same dose.

Determination of blood flow in gastric mucosal

The blood flow of gastric mucosal in Mongolian gerbils was detected by Laser Doppler Flowmetry (LDF) at 2, 4, and 6 months administration of dietary nitrate as described previously [17]. For details, a Moor VMS-LDF with an attached vascular monitor system (Moor Instrument, UK) was used. The mucosal blood flow in the glandular stomach of Mongolian gerbils 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.

Determination of nitrate and nitrite level in serum

Blood samples were collected after 6 months supplementation of dietary nitrate. Serum was obtained by centrifugation of the blood samples at 3000 r/min for 20 minutes at room temperature. Serum samples were 10,000 MW filtered and diluted prior to assay. The concentrations of nitrate in these samples were detected by high-performance liquid chromatography (HPLC) system as described previously [18, 19].

Determination of gastric mucosal PGE₂

PGE₂ levels in stomach tissues, which were obtained from Mongolian gerbils treated by dietary nitrate or sodium chloride for 6 month, were detected by enzyme labeled immunosorbent assay as described previously [20]. For details, the stomach tissue was homogenized and centrifuged, and then the supernatant was used for determination of PGE₂ by using an enzyme labeled immunosorbent kit (R&D Systems, Abingdon, UK). The optical densities were measured at 450 nm and the results were expressed as ng/g protein.

Histologic analysis

For histological evaluation, Stomach tissues were removed after 6 month dietary nitrate or...
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Figure 2. Long-term nitrate supplementation increased the nitrate and nitrite concentrations in blood. Nitrate and nitrite levels in blood were significantly increased after 6 month delivery of dietary nitrate. Student’s t test was used to determine statistical significance. Data represent mean ± SD, *P < 0.05 compared with control group.

Figure 3. The level of gastric mucosal PGE$_2$ content in gastric mucosa was significantly increased after 6 month delivery of dietary nitrate. Student’s t test was used to determine statistical significance. Data represent mean ± SD, *P < 0.05 compared with control group.

sodium chloride administration and fixed in 4% paraformaldehyde. After being dehydrated with gradient ethanol, the samples were embedded in paraffin, and then sectioned at 4 μm thickness. The sections were stained with hematoxylin and eosin (HE) for the evidence of histologic changes.

Statistical analysis

All data were analyzed with SPSS 13.0 statistical software. Data were presented by mean ± standard deviation (SD). Student t-test was used to compare the difference of blood flow, serum nitrate and nitrite and PGE$_2$ level between two groups. Statistical significance was set at P < 0.05.

Results

Gastric mucosal blood flow significantly improved by long-term dietary nitrate

To test whether long-term administration of nitrate affects the gastric mucosal blood flow in Mongolian gerbils, nitrate was added in the drinking water. After 2, 4, and 6 months supplementation of dietary nitrate, the gastric mucosal blood flow detected by LDF was significantly improved compared to sodium chloride treated group (P < 0.05) (Figure 1).

Serum nitrate and nitrite significantly increased after long-term dietary nitrate

To detect the nitrate and nitrite levels in blood after long-term delivery of dietary nitrate, we collected blood samples in Mongolian gerbils after treated by 6 month nitrate. As showed in Figure 2, the concentration of serum nitrate in the nitrate treated group was higher than that of the control group (101.65±41.84 μmol/L versus 52.29±18.85 μmol/L, P < 0.05). Meanwhile, the nitrate treated group had higher serum nitrite levels compared to the control group (2.22±0.71 μmol/L versus 1.36±0.18 μmol/L, P < 0.05).

Mucosal PGE$_2$ level significantly increased after long-term dietary nitrate

To further examine whether administration of dietary nitrate has correlation with PGE$_2$ level in gastric mucosa, which is crucial in maintaining the cellular integrity via improving blood flow in the gastric mucosa [21], we measured PGE$_2$ levels in stomach tissues after 6 month administration of dietary nitrate. As showed in Figure 3, significant increase of PGE$_2$ level was found in gastric mucosa by long-term dietary nitrate treatment (P < 0.05).

No histologic change in gastric mucosa of Mongolian gerbils after long-term dietary nitrate

To determine whether long-term administration of nitrate has any detrimental effect on gastric mucosa, histologic performers of stomach were
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Discussion

In the present study, we investigated the effect of long-term nitrate administration on the gastric mucosal blood flow in Mongolian gerbils. A long-term significant increase of gastric mucosal blood flow was found, as well as increased PGE₂ level in gastric mucosal. Notably, histological analysis showed no abnormalities of the gastric mucosa after the long-term nitrate treatment.

The gastric mucosa is continuously exposed to plenty of aggressive irritants. To protect the gastric mucosa from aggressive factors, a complex defense system has evolved, which includes increase the production of bicarbonate in surface mucus, regulate the gastric mucosal blood flow, and accelerate the epithelial regeneration [22]. Among all the defense system, gastric mucosal blood flow plays key role in mucosal defense. Sufficient mucosal blood flow helps the gastric mucosa recover from injuries as soon as possible. Clinically, most patients who develop stress ulcers have experienced an episode of shock from hemorrhage, sepsis, or cardiac dysfunction [23]. In this study, we demonstrated that gastric mucosal blood flow in Mongolian gerbils was significantly enhanced by long-term dietary nitrate compared to sodium chloride of the same dose, indicating that long-term NO₃ delivery may serve as protective factor for the mucosal defense system.

The increased nitrate and nitrite levels in blood may serve as NO reservoir for maintaining the systemic NO₃-NO₂-NO homeostatic balance in the body. NO plays key roles in the regulation of cardiovascular function, cellular energetics, neurotransmission, and immune function [24]. NO-mediated vasodilation also plays essential role in gastric mucosal defense [25]. Thus, long-term dietary nitrate may protect against diseases associated with gastric mucosal blood flow deficiencies by regulating NO homeostasis in the body. In addition, it is reported that Helicobacter pylori is killed by nitrite under acidic conditions [26]. Thus, protective effect of nitrate on gastric ulcer development may occur through inhibition of Helicobacter pylori in stomach.

Mucosal PGE₂ is one of crucial protective factors involved in maintaining gastric mucosal defense. PGE₂ acts via improving blood flow to maintain the cellular integrity in the mucosa [27], increasing mucus secretion, and bicarbonate and sulfhydryl compounds to strengthen the mucosal defense [28]. Our results showed that PGE₂ production in the long-term nitrate treated group was significantly up regulated in Mongolian gerbils. Dietary nitrate may play an important role in promoting angiogenesis via up regulation of PGE₂ production.

Figure 4. Histologic analysis of gastric mucosa by hematoxylin and eosin staining. A. Represents gastric mucosa from sodium chloride treated animals; B. Represent gastric mucosa from nitrate treated animals. No abnormalities were noticed on the gastric mucosa after long-term administration of nitrate.
To further explore the biological safety of long-term dietary nitrate on gastric mucosa, we carried out histologic analysis in gastric mucosa of Mongolian gerbils. We found that there were no abnormalities in the gastric mucosa after long-term administration of dietary nitrate. Our daily diet represents a major source of inorganic nitrate, and some vegetables are particularly rich in nitrate, which typically contain between 1,800 and 4,500 mg/kg nitrate [29]. In this study, the daily dose of nitrate supplementation employed in Mongolian gerbils is 0.2 mmol·kg⁻¹, which is a relatively low concentration employed in animal experiments. It acted in accordance with what is readily achievable in human through a high intake of nitrate-rich diet. Thus, the dose of nitrate supplementation employed in Mongolian gerbils suggests a safe and even beneficial role in the body.

In conclusion, this study provides evidences that long-term nitrate supplementation could increase gastric mucosal blood flow, and may serve as a beneficial factor for enhancing mucosal defense. These findings suggest an important physiological role of nitrate in the diet.

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Disclosure of conflict of interest

None.

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