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Research Paper

Role of nitrite, urate and pepsin in the gastroprotective effects of saliva

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ABSTRACT

Dietary nitrate is now recognized as an alternative substrate for nitric oxide ($\bullet$NO) production in the gut. This novel pathway implies the sequential reduction of nitrate to nitrite, $\bullet$NO and other bioactive nitrogen oxides but the physiological relevance of these oxidants has remained elusive. We have previously shown that dietary nitrite fuels an hitherto unrecognized nitrating pathway at acidic gastric pH, through which pepsinogen is nitrated in the gastric mucosa, yielding a less active form of pepsin in vitro. Here, we demonstrate that pepsin is nitrated in vivo and explore the functional impact of protein nitration by means of peptic ulcer development. Upon administration of pentagastrin and human nitrite-rich saliva or sodium nitrite to rats, nitrated pepsin was detected in the animal's stomach by immunoprecipitation. $\bullet$NO was measured in the gastric headspace before and after nitrite instillation by chemiluminescence. At the end of each procedure, the stomach's lesions, ranging from gastric erosions to haemorrhagic ulcers, were scored. Nitrite increased gastric $\bullet$NO by 200-fold ($p < 0.05$) and nitrated pepsin was detected both in the gastric juice and the mucosa ($p < 0.05$). Exogenous urate, a scavenger of nitrogen dioxide radical, blunted $\bullet$NO detection and inhibited pepsin nitration, suggesting an underlining free radical-dependent mechanism for nitration. Functionally, pepsin nitration prevented the development of gastric ulcers, as the lesions were only apparent when pepsin nitration was inhibited by urate. In sum, this work unravels a novel dietary-dependent nitrating pathway in which pepsin is nitrated and inactivated in the stomach, preventing the progression of gastric ulcers.

1. Introduction

Nitrate, from green leaf vegetables, is involved in a plethora of physiological mechanisms not only in the gut but also systemically [1]. The implications of nitrate for human health rely on its ability to trigger an NO-synthase independent reductive pathway leading to the formation of nitric oxide ($\bullet$NO), the *nitrate-nitrite-nitric oxide pathway* [2]. Nitrate reduction to nitrite and $\bullet$NO is translated into increases of gastric mucosal blood flow and mucus production, inhibition of inflammatory pathways and prevention of microbial infections [3–5]. Nitrate consumed in green leafy vegetables is absorbed in the small intestine and mixes in blood with the nitrate derived from endogenous $\bullet$NO generation. Then, c.a. 25% is taken up by the salivary glands and secreted into the oral cavity [6]. Here, metagenomic approaches have recently characterized symbionts that reduce nitrate to nitrite [7]. At the acidic

gastro-oesophageal junction, nitrite is non-enzymatically metabolized to different nitrogen oxides, including $\bullet$NO [8, 9]. Although most of the biological effects of nitrate have been attributed to $\bullet$NO, it is clear that a complex network of chemical reactions culminates in the production of higher nitrogen oxides, some with the capability to modify both endogenous and exogenous macromolecules [10–12]. Some of these oxides (such as nitrogen dioxide radical, $\bullet NO_2$) induce nitration, in which a nitro group ($-NO_2$) is inserted into a tyrosine residue within proteins or a fatty acid chain, yielding nitrated proteins or lipids, as recently shown [10,13]. This observation is of note as nitrated lipids can, in turn, signal to induce the expression of anti-inflammatory genes [14] and, therefore, a meal containing foods with both nitrate and oleic or linoleic acids, may fuel the production of anti-inflammatory molecules in the stomach that might also be absorbed into the circulation.

In this regard, we have also recently demonstrated that pepsinogen, the precursor of pepsin, is nitrated through a nitrate-dependent pathway in the stomach [13]. Pepsin is a gastric protease responsible for the breakdown of 15% of dietary proteins but, importantly, it is also known to erode the gastric mucosa,

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pinpointing its involvement in the development of peptic ulcer disease [15]. In this context, *in vitro* studies have shown that pepsin derived from nitrated pepsinogen has a lower proteolytic activity than pepsin derived from the non-nitrated zymogen [13], anticipating a potential gastroprotective effect of nitrated pepsin. In the present work it is shown that dietary nitrite induces pepsin nitration *in vivo* through a mechanism likely involving the generation of $\bullet NO_2$. Nitration decreases the proteolytic function of pepsin, preventing the development of acute gastric ulcers.

2. Materials and methods

2.1. Ethical approval

All experiments were performed according to European Community Council Directive for the Care and Use of Laboratory Animals (86/609/ECC) and approved by the local institutional animal care committee (ORBEA committee). Adult male Wistar rats (260–300 g) were purchased from Charles River, Barcelona and kept under 12 h cycles of light/dark for 7 days. During the period of acclimatization they were fed a standard chow and had access to water *ad libitum*. Before the experiments rats were fasted for 20 h (to minimize gastric contents) but had free access to water.

2.2. Surgical procedure

Rats were anaesthetized by the administration of a mixture of ketamine and xylazine (4:1, intraperitoneally, ip) and laid supine under a heating pad. Pentagastrin 20 $\mu g\,kg^{-1}$ was administered ip to stimulate gastric secretion 15 min before the surgery. Animals were under anaesthesia at the moment of pentagastrin administration in order to prevent discomfort or pain. A laparotomy was then performed and the hepatogastric ligament was cut to facilitate handling of the stomach. External clamps were applied in the lower oesophagus and pylorus to avoid the passage of air and juices to the adjacent compartments and luminal levels of $\bullet NO$ were measured by chemiluminescence (see bellow). Sodium nitrite (1.3 $mg\,kg^{-1}$) was then directly instilled into the gastric lumen through a thin needle and 5 min later, gastric $\bullet NO$ was again measured. Rats were sacrificed by cardiac arrest 30 min after nitrite instillation. Samples of gastric juice were collected, the stomach was dissected out and gastric lesions were evaluated. Gastric tissue and juice were then snap frozen until further analysis (see Fig. 1). In another set of experiments, the same procedure was performed but immediately before nitrite instillation, 400 μM urate was injected into the gastric lumen. Nitrite-enriched human saliva (collected after the ingestion of 90 g of lettuce) was also used instead of sodium nitrite (n=4). Typically, the volumes of the solutions added to the stomach was 1 mL except for sodium nitrite

for which small adjustments were made to ensure the administration of the same dose to all animals. In order to add the same amount of salivary nitrite, the volume of saliva administered was c. a. 4 mL were added.

3. Measurement of gastric ●NO

Gastric $\bullet NO$ concentrations were determined by using a high sensitive and specific chemiluminescence methodology, as previously described [5,16]. Briefly, after a laparotomy, 4 mL of $\bullet NO$-free air (typically less than 4 parts per billion) was injected in the stomach lumen through a thin needle, avoiding major gastric arteries. External clamps were used to prevent the spreading of injected gas to other gastrointestinal compartments. After 15 s, 4 mL of air was aspirated and immediately injected into a chemiluminescence analyser (CLD88 Exhalyzer, EcoMedics) to determine $\bullet NO$ concentration. The same procedure was performed 5 min after instillation of nitrite.

4. Collection of human saliva and determination of nitrite concentration

A sample of c.a. 10 mL of saliva was collected from a human volunteer who underwent an overnight fasting. Then, 90 g of iceberg lettuce (nitrate load) was ingested and saliva was again collected one hour later. After centrifugation (12,000 $g\times10$ min), nitrite content was determined by chemiluminescence (CLD88 Exhalyzer, EcoMedics). Briefly, 100 μL of supernatant was injected into a closed chamber connected to the chemiluminescence analyser containing a reducing mixture of 45 mM potassium iodide and 10 mM iodine in glacial acetic acid continuously bubbled with nitrogen at 56 °C. Under these conditions, nitrite is reduced to $\bullet NO$, which is quantified by the analyser following a calibration curve obtained from standard nitrite solutions.

4.1. Preparation of stomach homogenates

The glandular mucosa surrounding the gastric lesions was minced in ice-cold lysis buffer (50 mM Tris pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% NP 40, 1 mM PMSF and protease inhibitor cocktail, Sigma Aldrich; typically 100 mg of tissue was homogenized in 1 mL of buffer) with a pair of small scissors. The suspension was further triturated with a bullet blender (Labmark) and centrifuged at 12,000 g x 10 min (4 °C). The supernatant was collected and total protein was quantified by the Bradford method (Bio-Rad). Care was taken to avoid artifactual nitration due to media acidification.
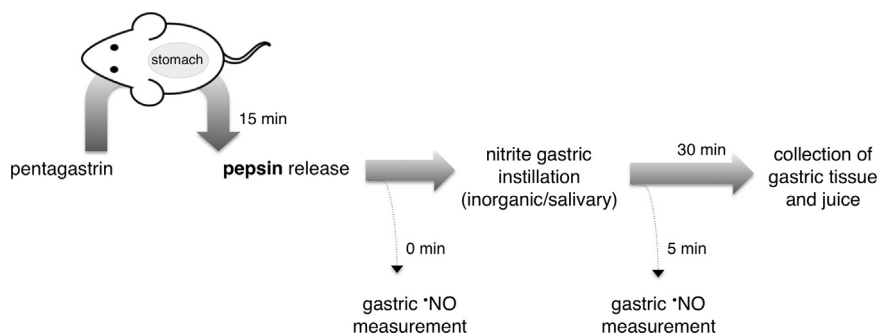

Fig. 1. Experimental design. Briefly, Wistar rats were anesthetized and pentagastrin was administered intraperitoneally to stimulate pepsin release. A laparotomy was performed 15 min later and gastric $\bullet NO$ was measured in the gastric headspace. Then, nitrite or human saliva was instilled into the stomach and, 5 min later $\bullet NO$ was again measured. After 30 min, animals were sacrificed and both gastric juice and tissue were collected.

4.2. Gastric Juice preparation

Samples of gastric juice were collected and centrifuged twice at 5000 gx5 min to precipitate debris. The supernatant was recovered and proteins were precipitated with ice-cold acetone. Four volumes of acetone were added to one volume of sample and the mixtures were incubated overnight at $-20\,°C$. Then, the samples were centrifuged at 8000 gx10 min (4 $°C$). The supernatant was removed and protein extract was resuspended with loading buffer. Samples were then analysed for nitrated proteins by western blot.

4.3. Immunoprecipitation

Solubilized proteins (800 μg) from the ulcer margin were incubated with 2 μg of a polyclonal pepsin antibody (Santa Cruz Biotechnology) for 3 h at 4 $°C$. The immuno complexes were then precipitated (1 h at 4 $°C$) with 10 μL of protein A/G Ultralink resin (Thermo Scientific) previously washed with lysis buffer through 5 consecutive centrifugations at 2000 gx1 min. The complexes were then washed with PBS (phosphate buffered saline; 2000 gx1 min, 5 times) and pepsin was eluted from the beads by adding loading buffer and heating at 95 $°C$ for 5 min. The samples were then applied and separated in SDS-12% polyacrylamide gel electrophoresis. A western blot against nitrotyrosine was then performed. Afterwards, each membrane was incubated for 5 min with 0.2 M NaOH to remove the primary and secondary antibodies and reprobed with a goat polyclonal pepsin antibody (Santa Cruz, Biotechnology) to confirm that the bands positive for nitrotyrosine were also positive for pepsin.

4.4. Immunoblotting

Nitrotyrosine was assessed by western blot in the immunoprecipitates and in the samples of gastric juice whereas myeloperoxidase (MPO) was studied in the tissue collected from the ulcer margins. Equal amounts of total protein (30 μg) were blotted to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk in TBST (Tris buffered saline, 0.1% Tween 20) for 1 h at room temperature and further incubated with a rabbit polyclonal antibody against nitrotyrosine (1:1000) overnight at 4 $°C$. Then, the membranes were washed with TBST and probed with a mouse anti-rabbit antibody (1:10000, Santa Cruz Biotechnology) for 1 h at room temperature. After another set of washings, labelling was detected by soaking with ECF for 5 min and analysed using a fluorescent image analysis system (Thyphoon, GE Healthcare). Protein loading was normalized to β-actin (1: 10,000, Santa Cruz, Biotechnology) after membrane striping and reprobing. Densitometry was performed using ImageJ software (National Institutes of Health, USA).

4.5. Scoring of macroscopic gastric lesions

The stomach was opened along the lesser curvature, gently washed with PBS, and photographed. The lesions were macroscopically graded on a 0-to-6 scale by an observer blinded to the treatment. Each grade corresponded to the following state: 0, no damage; 1, oedema; 2, reddening of mucosa; 3, petechial haemorrhages; 4, superficial erosions; 5, ulceration; 6, perforation [17].

4.6. Microscopic assessment of gastric lesions: haematoxylin & eosin staining

Samples of ulcerated regions were collected and fixed in 4% buffered paraformaldehyde. After cryopreservation with increasing sucrose gradients (10%, 20% and 30%), 10 μm slices were obtained, washed with running water and incubated with Mayer haematoxylin for 5 min, followed by 10 min with running water. Then, the slides were washed with distilled water and stained with Eosin Y for 30 s. The tissue was then washed with increasing concentrations of ethanol (80%, 90% and 100%) and immersed in xylene until permanent mounting with Permount. The preparations were then observed under a light microscope (Zeiss Axiovert 200, Carl Zeiss MicroImaging, Germany).

4.7. Statistical analysis

Two-sample comparison was performed using unpaired and two-tailed t-test whereas multi-sample analysis was performed by one-way ANOVA. A probability value ($p < 0.05$) was considered statistically significant. Values are presented as mean $\pm$ SEM of measurements in n=5 different animals, unless otherwise stated.

5. Results

5.1. Salivary nitrite increases upon nitrate intake

Saliva was diluted four- (fasting) or twentyfold (post-prandial) before nitrite quantification. Fasting nitrite concentration was 95.1 ± 2.2 μM increasing to 1182.0 ± 11.1 μM (***$p < 0.0001$) after lettuce intake (Fig. 2). These values are in agreement with data reported in the literature [18,19].

5.2. Gastric •NO production from inorganic and salivary nitrite is inhibited by urate

Gastric instillation of inorganic nitrite increased the steady state concentration of •NO from 284 ± 45 ppb to $62,812 \pm 140$ ppb (n=5; $p < 0.05$), as shown in Fig. 3. Expectedly, human saliva containing nitrite (1182 ± 11 μM) mimicked this result by increasing gastric •NO to $79,998 \pm 26,582$ ppb (n=4). Urate, naturally present in human saliva, attenuated the nitrite-induced increase in gastric •NO to 7818 ± 1877 ppb ($p=0.05$).

5.3. Salivary and inorganic nitrite induced pepsin nitration in the stomach

Gastric instillation of inorganic nitrite induced pepsin nitration from 1.1 ± 0.2 a.u. to 16.9 ± 8.2 a.u. (control vs nitrite, arbitrary units) but urate prevented the reaction (16.9 ± 8.2 a.u. to 3.7 ± 2.4 a.u., nitrite vs urate) as shown in Fig. 4A. When nitrite-enriched human saliva was injected into the stomach, nitrated pepsin was again detected (11.0 ± 0.9 a.u.). Similarly, a nitrated adduct was detected in the gastric juice of rats exposed to both pentagastrin and nitrite (Fig. 4B). The molecular weight of this nitrated adduct coincided with the one of pepsin (37 kDa) and, upon reprobing with an antibody specific for this protease, both bands matched. Under fasting conditions, and upon pentagastrin administration, the most abundant protein in the gastric lumen should be pepsin, thus we assumed that the observed band corresponded to nitrated pepsin. The intensity of this band decreased in the presence of urate and under control conditions (Fig. 4B), in agreement with the observations in the tissue surrounding the gastric lesions.

5.4. Nitrite decreases mucosal myeloperoxidase

Nitrite, both inorganic ($47.6 \pm 11.0\%$ in respect to control, $p < 0.05$) and from human saliva ($36.0 \pm 8.7\%$, $p < 0.05$) decreased MPO immunoreactivity in the gastric mucosa. A trend pointing to an increase towards control MPO levels was observed in the
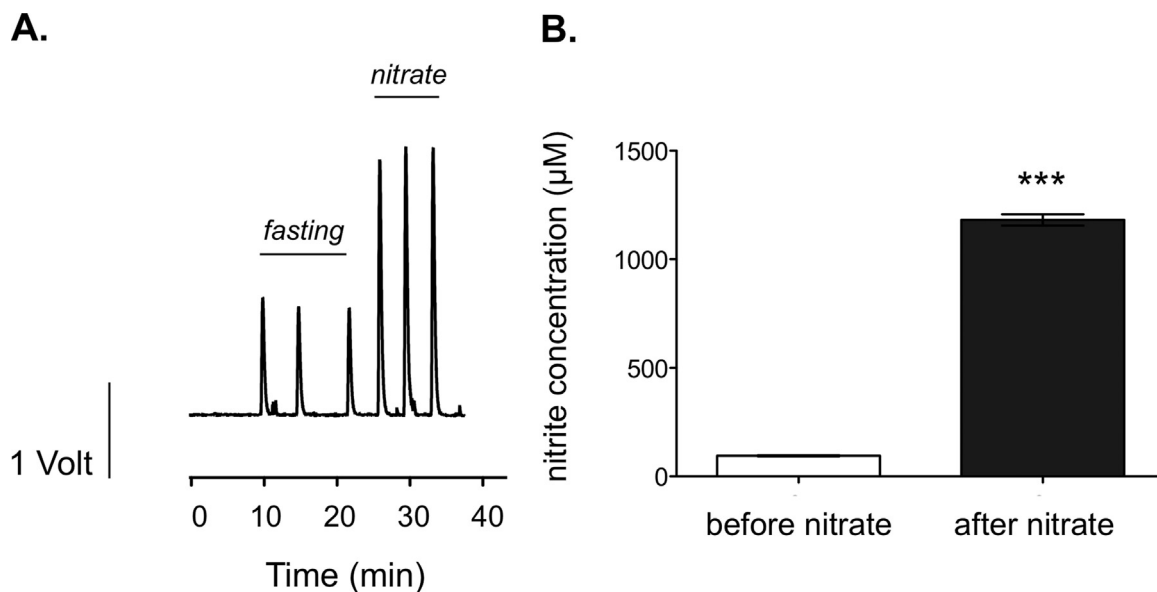
A.

B.

Fig. 2. Detection of nitrite in human saliva. (A) Recording showing nitrite in saliva samples before and after lettuce (nitrate) intake by chemiluminescence. (B) Quantification of salivary nitrite. Human saliva was diluted fourfold (fasting) and twentyfold (after nitrate) before injection into the apparatus.***p < 0.0001. These *in vitro* determinations of nitrite concentration were performed in triplicate for an n=3.
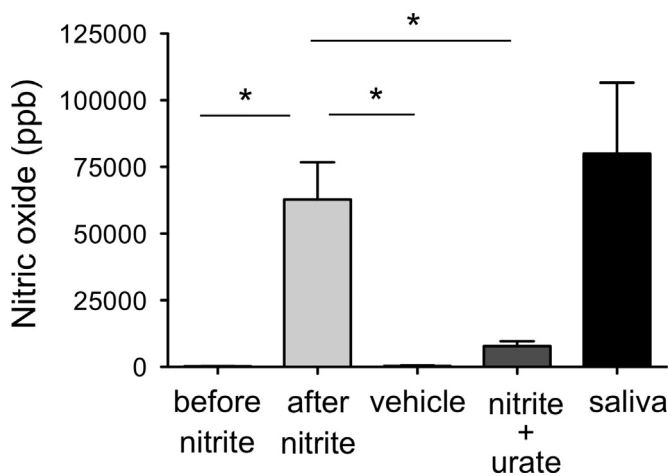

Fig. 3. Gastric generation of ●NO (ppb) from inorganic nitrite, nitrite+urate and human saliva obtained after the consumption of lettuce (nitrate load; final nitrite concentration 1182 ± 11 μM). Controls are also presented. Values are mean ± SEM; n=5, except for the groups of animals exposed to nitrite+urate or human saliva, in which n=4; *p < 0.05. Group analysis was performed by one-way ANOVA followed by Bonferroni multiple comparison test.

presence of urate but without significant differences (Fig. 5).

5.5. Evaluation of gastric injuries: the anti-ulcerogenic effect of nitrated pepsin

Pentagastrin, by triggering pepsinogen and acid release from the glandular mucosa, provoked gastric lesions, ranging from erosions to ulcers accompanied with haemorrhages (Fig. 6A). When nitrite was instilled into the stomach, a significant amelioration was observed (Fig. 6B). This observation was matched nitrite-dependent pepsin nitration (Fig. 4A). To study the physiological relevance of this effect, that is to say, the ability of salivary nitrite to participate in ulcer prevention, human saliva was instilled into the stomach. The preventive effects of inorganic nitrite were mimicked by nitrite-enriched saliva (Fig. 6B, and C) and again were accompanied by pepsin nitration. The injury index (Fig. 7) also corroborates these findings: pentagastrin induced

3.8 ± 0.7 a.u. in terms of the injury scale whereas in the presence of nitrite it declined to 2.8 ± 0.4 a.u. and to 2.9 ± 0.5 a.u. with human saliva. When pepsin nitration was prevented by the instillation of urate, the injury index increased to 4.6 ± 0.3 a.u. (p < 0.05 in comparison to nitrite instillation alone). The microscopic examination of the lesions, depicted in Fig. 8, further confirms the macroscopic observations. When urate was injected into the stomach, both the macroscopic appearance and microscopic morphology (Figs. 6–8) of the lesions worsened and were complicated by profuse bleeding (Fig. 6D). Control experiments with urate instilled into the stomach confirm that at acidic pH no damage of the gastric mucosa and macroscopic lesions were observed (not shown).

6. Discussion

Compelling evidence supports the physiological relevance of the nitrate–nitrite–nitric oxide pathway in the gut and systemically [20–22]. However, only a few studies deal with the biochemical interactions of nitrite with endogenous macromolecules in connection with post-translational modifications of proteins at acidic pH that might be translated into a functional impact. The stomach exhibits unique conditions (pH, pO_2, gaseous/liquid interface, high concentrations of reactants) that might facilitate redox reactions, notably non-enzymatic metabolism of nitrite to ●NO and other nitrogen oxides endowed with biological functions [23,12]. In this study, we show that nitrite triggers a nitrating process in the stomach that is modulated via the endogenous generation of nitrogen oxides. Such a pathway, resulting in pepsin nitration and inactivation at acidic pH, has a relevant physiological impact in terms of amelioration of peptic ulcers.

Pepsin release from the gastric mucosa was induced by pentagastrin (a gastric secretagogue) and upon nitrite instillation into the stomach, nitrated pepsin was detected both in gastric juice and mucosa (Fig. 4). Similar results were observed when inorganic nitrite was replaced with nitrite-enriched saliva, thus affording physiological significance to these observations given that saliva is the vehicle for nitrite but also for other compounds that may promote (*e.g.*, peroxidases) or inhibit (*e.g.*, thiocyanate) nitration
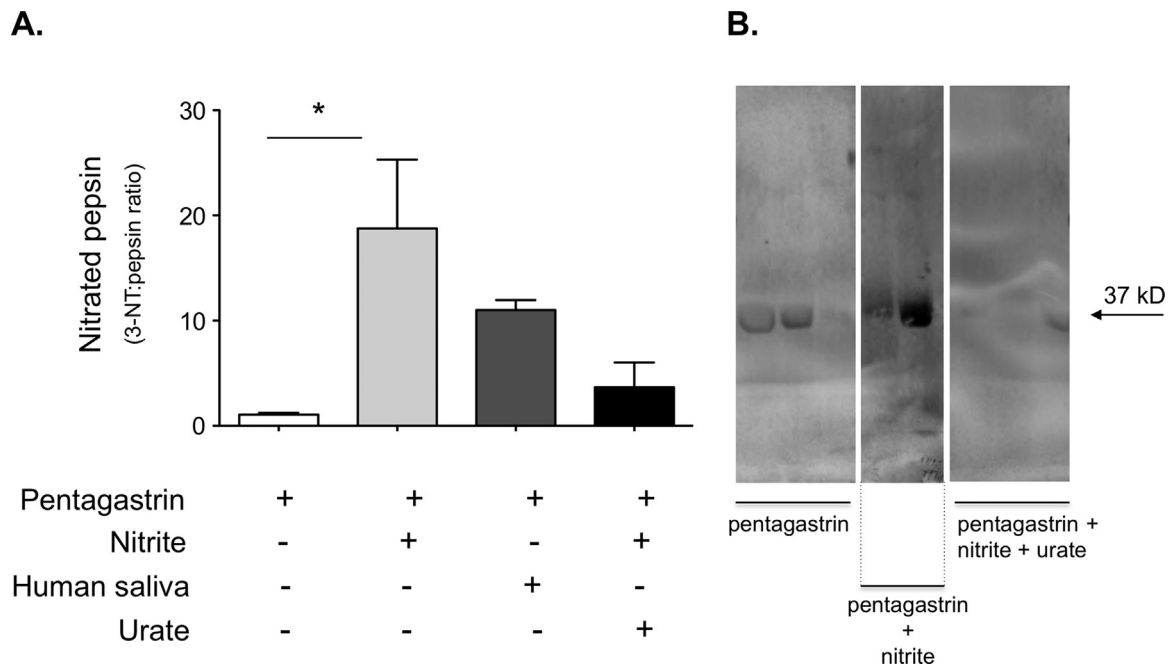
A.

B.

Fig. 4. (A) Immunoprecipitation of nitrated pepsin from the margin of pentagastrin-induced gastric ulcers; 3-NT: pepsin ratio refers to the ratio between the band intensity of the immunoprecipitates upon probing with a 3-nitrotyrosine antibody and, after stripping, with a pepsin antibody (internal control); values are mean $\pm$ SEM for $n=5$, except for the groups of animals exposed to nitrite+urate or human saliva, in which n=4;*p < 0.05. (B) Identification of nitrated pepsin in the gastric juice. Nitrated proteins were detected by western blot and the bands co-localized after reprobing with a specific pepsin antibody. Since pepsin (37 kDa) is the main gastric protease and is induced by pentagastrin, we assumed that the bands correspond to nitrated pepsin. The gastric juice of three animals per condition was used; each lane represents one animal.
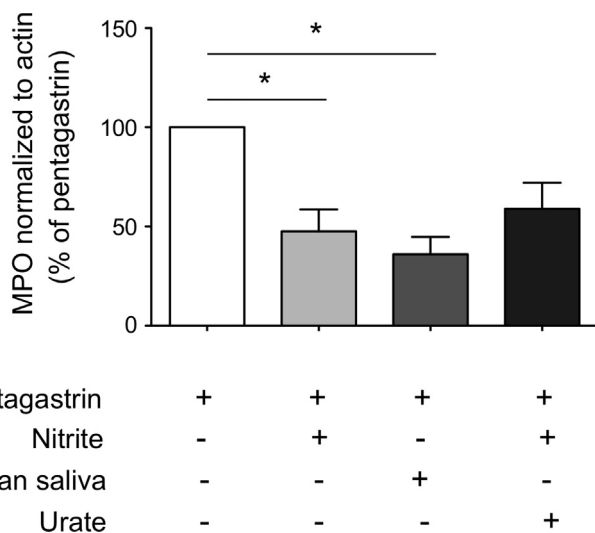

Fig. 5. Myeloperoxidase immunoreactivity in the rat gastric mucosa after instillation of nitrite, nitrite-enriched saliva or nitrite+urate. Since MPO is highly expressed in polymorphonuclear leucocytes, we used it as a marker of leucocyte infiltration in the gastric mucosa. Values are presented as mean $\pm$ SEM, n=4, *p < 0.05.

reactions in the stomach [24]. Thus, under physiological conditions and despite the contribution of other salivary compounds, nitrite generated from the enterosalivary circulation of nitrate, is able to induce pepsin nitration in the stomach. Moreover, the continuous delivery of nitrite into the stomach for 5–6 h after nitrate intake [25] ensures the long-lasting generation of nitrating agents in the gastric lumen, suggesting that pepsin nitration may occur for several hours and it is not necessarily of sporadic occurrence. This concept is in line with observations by others showing that dietary nitrite induces nitration of lipids contained in foods. Fazzari et al. have demonstrated that under gastric conditions, the lipid content of olives and olive oil is nitrated by nitrite yielding nitro-

conjugated linoleic acid and nitro-oleic acid [26]. Given that nitroalkenes induce the expression of anti-inflammatory genes and other systemic cellular adaptive responses, this and other studies suggest that once formed in the gastric lumen, nitrated lipids could be absorbed into the bloodstream and exert systemic effects [10,26]. Indeed this hypothesis was elegantly shown by a study of Delmastro-Greenwood et al. in which human volunteers consumed either N-labelled nitrate or nitrite with conjugated linoleic acid and the nitrated derivatives were detected in plasma and urine [27]. Thus, it is becoming increasingly evident that dietary nitrite fuels a nitrating pathway in the stomach, in vivo, targeting both proteins and lipids with potential implications for local and systemic physiological mechanisms. Noteworthy, it should also be highlighted that even in the absence of a dietary nitrate intake, considerable amounts of nitrate are delivered to saliva. This nitrate derives from oxidation of endogenously produced •NO. This notion has important consequences in what concerns to the physiological impact of this reaction as morphological and microscopic analyses of the gastric mucosa show that nitrated pepsin improves the prognosis of gastric ulcers. Pentagastrin provokes acute gastric ulcers accompanied by intense bleeding but upon instillation of nitrite or nitrite-enriched saliva, the lesions reversed for scattered erosions (Figs. 6–8). Taken together this data suggests that after a serving containing green-leaf vegetables, nitrite is continuously delivered into the stomach inducing the formation of nitrated pepsin that, in turn, due to a decreased proteolytic activity, prevents the erosion of the gastric mucosa and thereby the development of acute gastric ulcers. However, from a physiological viewpoint, one obvious question would be the impact on the digestion of dietary proteins. In fact, most of the proteins from nutrients are degraded by duodenal proteases, such as trypsin, chymotrypsin, carboxypeptidase and elastase, rather than by pepsin in the stomach, where just $\approx$ 15% of the proteins are digested [28]. Accordingly, no significant impact would be expected on the digestion of dietary proteins upon pepsin nitration. In turn, pepsin nitration may acquire a more obvious biological relevance under
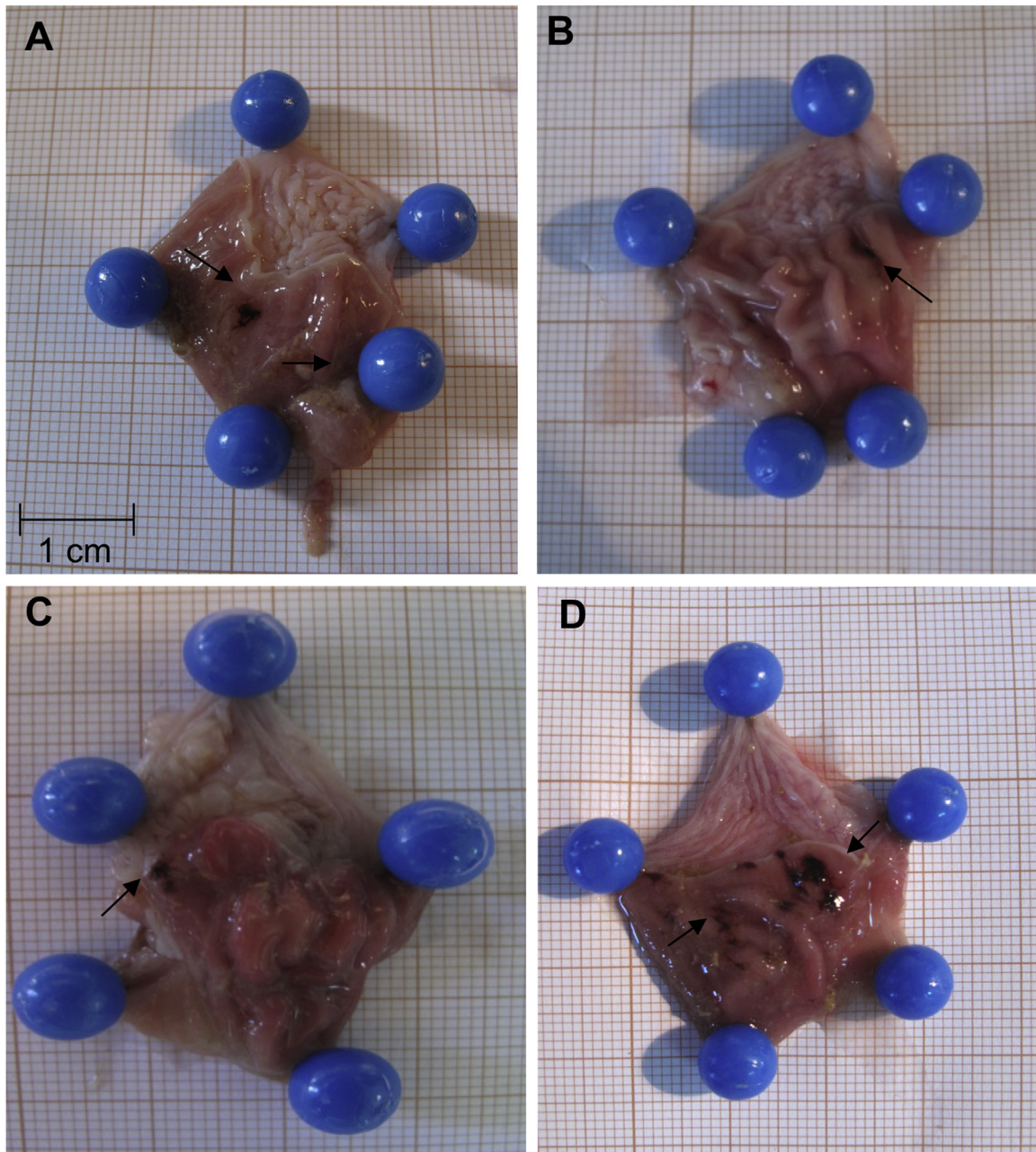
Fig. 6. Effect of nitrite on secretagogue ulcers. (A) Pentagastrin induced gastric ulcers in all animals included in this study. Typically the lesions ranged from erosions to ulcers (arrows); (B) Gastric instillation of inorganic nitrite ameliorated the prognostics of gastric damage; (C) Nitrite-enriched human saliva resembled the effect of sodium nitrite and inhibited the development of gastric ulcers; (D) Macroscopic lesions developed upon gastric instillation of urate and sodium nitrite. These are representative photographs for at least four animals per condition (n=5, except for nitrite+urate and human saliva, in which n=4).

acute inflammatory conditions, a situation in which native pepsin activity may contribute in a more critical way to worsening of mucosal damage.

From a mechanistic viewpoint, urate, a scavenger of $\bullet NO_2$ [29], inhibits protein nitration suggesting that the nitrating pathway triggered by nitrite involves this free radical. Uric acid (pKa=3.3) is an efficient scavenger of both $\bullet NO_2$ ($k=1.8 \times 10^7 \, M^{-1} \, s^{-1}$) and $\bullet NO$ ($k=1.0 \times 10^9 \, M^{-1} \, s^{-1}$) and, accordingly, we observed that it decreased significantly gastric $\bullet NO$ production and pepsin nitration (Figs. 3 and 4). Both events were paralleled by the loss of the protective effect on the progression of gastric ulcers further supporting the hypothesis that nitrated pepsin prevents mucosal erosion and ultimately ulcer progression. In addition to $\bullet NO_2$ other nitrating pathways may be operative in the gastric mucosa given the substantial infiltration of polymorphonuclear cells.

These cells express MPO, a peroxidase that triggers a cascade of oxidative reactions culminating with the production of nitrating species [30]. In this context, dietary nitrate and nitrite have been shown to inhibit leucocyte emigration into the intestinal mucosa by down-regulating the expression of ICAM-1 and P-selectin [31]. Concordantly, when nitrite is instilled into the stomach, there is a decrease of the immunoreactivity of MPO (Fig. 5) suggesting not only that the (physiological) inflammatory environment of the gastric mucosa is being attenuated, but also that $\bullet NO_2$ generated from nitrite may be the main inducer of protein nitration. Although gastric $\bullet NO$, significantly increased upon nitrite administration, may also inhibit neutrophil rolling and adhesion thereby reducing MPO immunoreactivity [32], the data herein presented suggests that the major nitrating pathway is triggered by nitrite. This observation leads to the interesting hypothesis that the
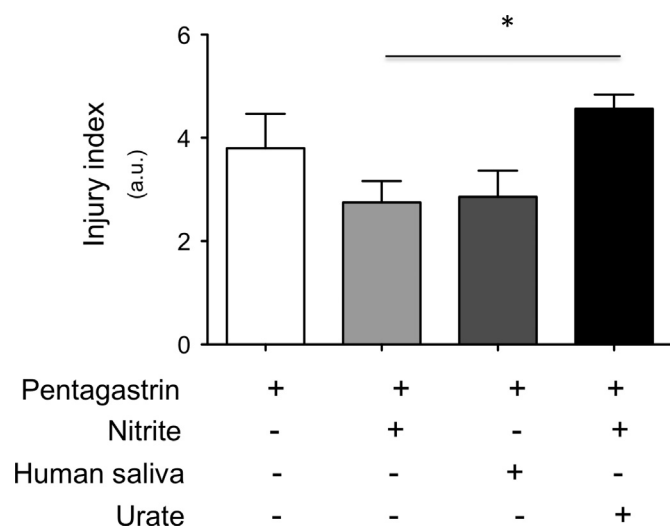
Fig. 7. Injury index of macroscopic gastric damage. Scoring is presented as arbitrary units (a.u.) and was obtained by considering different degrees of tissue damage (from normal mucosa to gastric perforation). A researcher blind to the experimental treatment performed scoring. The stomachs of five animals per condition were analysed, except for the groups exposed to nitrite + urate or human saliva, in which n=4; $^*p < 0.05$.

oxidative damage triggered by either peroxynitrite- or MPO-dependent nitration (given the production of carbonate and hydroxyl radicals or HOCl, respectively), is minimal in the novel nitrite-induced nitrating pathway described here.

Taken altogether, this work shows that the stomach provides an exquisite environment for diet-dependent redox reactions through a novel non-enzymatic pathway for •NO production, the nitrate-nitrite-NO pathway that might culminate in the modification of protein function *via* nitration. This is the case of pepsin, the major gastric protease, which, as shown here, is nitrated and inactivated *in vivo* in the stomach upon exposure to nitrite, exhibiting anti-ulcerogenic properties. Therefore, given the impact

on gastrointestinal welfare, dietary-dependent signalling pathways involving free radical generation in the gut should be further investigated. One example would be to ascertain if dietary nitrate, through the downstream generation of •NO, would impact on gut microbiome diversity. This super organism, essential to maintain gastrointestinal and systemic welfare, is bidirectionally intertwined with critical redox signalling pathways: in one hand, gastric •NO production depends on nitrate reduction to nitrite in the oral cavity by commensal bacteria [33] and, on the other, bacterial metabolites activate redox signalling pathways (*e.g.*, $NF - kB$) in the gut mucosa and thus contribute to the permanent, yet constitutive and physiological, inflammatory state of the gastrointestinal mucosa [34,35]. Accordingly, Hyde et al. have recently shown that nitrite may finely change lower taxonomic groups of mice microbiome [7]. Hence, it would be extremely appealing to evaluate as to whether nitrate consumption would impact on gut microbiome profile and ensued physiological consequences as well as how changes on gut microbiome would affect the nitrate-nitrite-NO pathway (impact not only on •NO production but also on the generation of nitrating agents that may lead to the production of physiologically active molecules such as nitrated pepsin and nitrated nitroalkenes).

Conflicts of interest

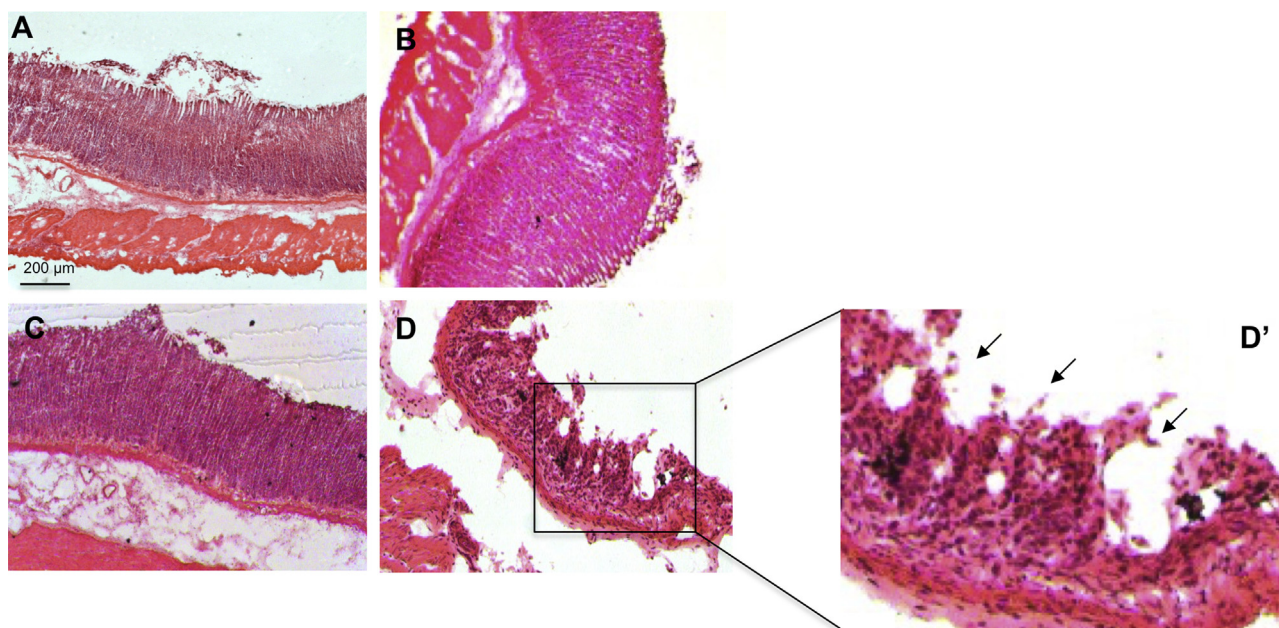Authors declare no conflicts of interest.

Acknowledgements

Fig. 8. Microscopic assessment of gastric ulcers by H&E. (A) Pentagastrin; scattered erosions and epithelial detachment is shown. (B) Inorganic nitrite, while inducing pepsin nitration and the decrease of its function, restored the normal mucosal morphology. (C) Salivary nitrite instillation into the rat stomach maintained the normal histological appearance. (D) Gastric instillation of nitrite + urate. In the inset it is shown the derangement of mucosal cytoarchitecture, with the arrows indicating places denuded of mucosa due to proteolytic digestion by pepsin (D′). These are representative photographs of at least four animals per condition (n=5, except for the groups exposed to nitrite + urate or human saliva, in which n=4).

References

[1] E. Weitzberg, J.O. Lundberg, Novel aspects of dietary nitrate and human health, Annu. Rev. Nutr. 33 (2013) 129–159.

[2] J.O. Lundberg, E. Weitzberg, M.T. Gladwin, The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics, Nat. Rev. Drug. Discov. 7 (2) (2008) 156–167.

[3] H. Bjorne, E. Weitzberg, J.O. Lundberg, Intragastric generation of antimicrobial nitrogen oxides from saliva–physiological and therapeutic considerations, Free Radic. Biol. Med. 41 (9) (2006) 1404–1412.

[4] H.H. Bjorne, J. Petersson, M. Phillipson, E. Weitzberg, L. Holm, J.O. Lundberg, Nitrite in saliva increases gastric mucosal blood flow and mucus thickness, J. Clin. Invest 113 (1) (2004) 106–114.

[5] E.A. Jansson, J. Petersson, C. Reinders, T. Sobko, H. Bjorne, M. Phillipson, E. Weitzberg, L. Holm, J.O. Lundberg, Protection from nonsteroidal anti-inflammatory drug (NSAID)-induced gastric ulcers by dietary nitrate, Free Radic. Biol. Med. 42 (4) (2007) 510–518.

[6] P.M. Bos, P.A. Van den Brandt, M. Wedel, T. Ockhuizen, The reproducibility of the conversion of nitrate to nitrite in human saliva after a nitrate load, Food Chem. Toxicol. 26 (2) (1988) 93–97.

[7] E.R. Hyde, F. Andrade, Z. Vaksman, K. Parthasarathy, H. Jiang, D.K. Parthasarathy, A.C. Torregrossa, G. Tribble, H.B. Kaplan, J.F. Petrosino, et al., Metagenomic analysis of nitrate-reducing bacteria in the oral cavity: implications for nitric oxide homeostasis, PLoS One 9 (3) (2014) e88645.

[8] N. Benjamin, F. O'Driscoll, H. Dougall, C. Duncan, L. Smith, M. Golden, H. McKenzie, Stomach NO synthesis, Nature 368 (6471) (1994) 502.

[9] J.O. Lundberg, E. Weitzberg, J.M. Lundberg, K. Alving, Intragastric nitric oxide production in humans: measurements in expelled air, Gut 35 (11) (1994) 1543–1546.

[10] G. Bonacci, P.R. Baker, S.R. Salvatore, D. Shores, N.K. Khoo, J.R. Koenitzer, D.A. Vitturi, S.R. Woodcock, F. Golin-Bisello, M.P. Cole, et al., Conjugated linoleic acid is a preferential substrate for fatty acid nitration, J. Biol. Chem. 287 (53) (2012) 44071–44082.

[11] C. Pereira, R.M. Barbosa, J. Laranjinha, Dietary nitrite induces nitrosation of the gastric mucosa: the protective action of the mucus and the modulatory effect of red wine, J. Nutr. Biochem. 26 (5) (2015) 476–483.

[12] B.S. Rocha, B. Gago, R.M. Barbosa, J.O. Lundberg, R. Radi, J. Laranjinha, Intragastric nitration by dietary nitrite: implications for modulation of protein and lipid signaling, Free Radic. Biol. Med. 52 (3) (2012) 693–698.

[13] B.S. Rocha, B. Gago, R.M. Barbosa, J.O. Lundberg, G.E. Mann, R. Radi, J. Laranjinha, Pepsin is nitrated in the rat stomach, acquiring antiulcerogenic activity: a novel interaction between dietary nitrate and gut proteins, Free Radic. Biol. Med. 58 (2013) 26–34.

[14] A. Trostchansky, H. Rubbo, Nitrated fatty acids: mechanisms of formation, chemical characterization, and biological properties, Free Radic. Biol. Med. 44 (11) (2008) 1887–1896.

[15] M.E. Smith, D.G. Morton, The Digestive System – Systems of the Body Series, Elsevier, 2010.

[16] T. Sobko, C.I. Reinders, E. Jansson, E. Norin, T. Midtvedt, J.O. Lundberg, Gastrointestinal bacteria generate nitric oxide from nitrate and nitrite, Nitric Oxide 13 (4) (2005) 272–278.

[17] S.N. Joffe, N.B. Roberts, W.H. Taylor, J.H. Baron, Exogenous and endogenous acid and pepsins in the pathogenesis of duodenal ulcers in the rat, Dig. Dis. Sci. 25 (11) (1980) 837–841.

[18] M. Govoni, E.A. Jansson, E. Weitzberg, J.O. Lundberg, The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash, Nitric Oxide 19 (4) (2008) 333–337.

[19] G.M. McKnight, L.M. Smith, R.S. Drummond, C.W. Duncan, M. Golden, N. Benjamin, Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans, Gut 40 (2) (1997) 211–214.

[20] V. Kapil, E. Weitzberg, J.O. Lundberg, A. Ahluwalia, Clinical evidence demonstrating the utility of inorganic nitrate in cardiovascular health, Nitric Oxide 38 (2014) 45–57.

[21] T. Rassaf, M. Totzeck, U.B. Hendgen-Cotta, S. Shiva, G. Heusch, M. Kelm, Circulating nitrite contributes to cardioprotection by remote ischemic preconditioning, Circ. Res. 114 (10) (2014) 1601–1610.

[22] A.L. Sindler, A.E. Devan, B.S. Fleenor, D.R. Seals, Inorganic nitrite supplementation for healthy arterial aging, J. Appl. Physiol. 116 (5) (2014) 463–477.

[23] J.O. Lundberg, E. Weitzberg, Biology of nitrogen oxides in the gastrointestinal tract, Gut 62 (4) (2013) 616–629.

[24] S.J. Farnaud, O. Kosti, S.J. Getting, D. Renshaw, Saliva: physiology and diagnostic potential in health and disease, Sci. World J. 10 (2010) 434–456.

[25] J.O. Lundberg, M. Govoni, Inorganic nitrate is a possible source for systemic generation of nitric oxide, Free Radic. Biol. Med. 37 (3) (2004) 395–400.

[26] M. Fazzari, A. Trostchansky, F.J. Schopfer, S.R. Salvatore, B. Sanchez-Calvo, D. Vitturi, R. Valderrama, J.B. Barroso, R. Radi, B.A. Freeman, et al., Olives and olive oil are sources of electrophilic fatty acid nitroalkenes, PLoS One 9 (1) (2014) e84884.

[27] M. Delmastro-Greenwood, K.S. Hughan, D.A. Vitturi, S.R. Salvatore, G. Grimes, G. Potti, S. Shiva, F.J. Schopfer, M.T. Gladwin, B.A. Freeman, et al., Nitrite and nitrate-dependent generation of anti-inflammatory fatty acid nitroalkenes, Free Radic. Biol. Med. 89 (2015) 333–341.

[28] Smith, M.E., Morton, D.G. The digestive system. Basic science and clinical conditions, 2010.

[29] E. Ford, M.N. Hughes, P. Wardman, Kinetics of the reactions of nitrogen dioxide with glutathione, cysteine, and uric acid at physiological pH, Free Radic. Biol. Med. 32 (12) (2002) 1314–1323.

[30] J.P. Eiserich, M. Hristova, C.E. Cross, A.D. Jones, B.A. Freeman, B. Halliwell, A. van der Vliet, Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils, Nature 391 (6665) (1998) 393–397.

[31] C. Jadert, J. Petersson, S. Massena, D. Ahl, L. Grapensparr, L. Holm, J.O. Lundberg, M. Phillipson, Decreased leukocyte recruitment by inorganic nitrate and nitrite in microvascular inflammation and NSAID-induced intestinal injury, Free Radic. Biol. Med. 52 (3) (2012) 683–692.

[32] C.P. Tiefenbacher, J. Kreuzer, Nitric oxide-mediated endothelial dysfunction–is there need to treat? Curr. Vasc. Pharmacol. 1 (2) (2003) 123–133.

[33] J.O. Lundberg, E. Weitzberg, J.A. Cole, N. Benjamin, Nitrate, bacteria and human health, Nat. Rev. Microbiol. 2 (7) (2004) 593–602.

[34] A. Alam, G. Leoni, C.C. Wentworth, J.M. Kwal, H. Wu, C.S. Ardita, P.A. Swanson, J.D. Lambeth, R.M. Jones, A. Nusrat, et al., Redox signaling regulates commensal-mediated mucosal homeostasis and restitution and requires formyl peptide receptor 1, Mucosal Immunol. 7 (3) (2014) 645–655.

[35] L. Eckmann, A.S. Neish, NF-kappaB and mucosal homeostasis, Curr. Top. Microbiol. Immunol. 349 (2011) 145–158.