

**Diffusion of cytotoxic concentrations of nitric oxide generated luminally at the gastro-oesophageal junction of rats**

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**Abbreviations:** DETC, diethyldithiocarbamate; EPR, electron paramagnetic resonance;

GE, gastro-esophageal; NO, nitric oxide; NOS, nitric oxide synthase.

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## **ABSTRACT**

**Background:** In human, high concentrations of nitric oxide are generated luminally at the gastro-esophageal junction through the entero-salivary re-circulation of dietary nitrate.

**Aim:** To investigate whether luminal nitric oxide can diffuse into the adjacent digestive tissue and alter the tissue integrity.

**Methods:** We designed an animal model using Wistar rats in which physiological concentrations of nitrite and acidified ascorbic acid were administered separately so that the two reactants first meet to form nitric oxide at the gastro-esophageal junction. The luminal or tissue concentration of nitric oxide was measured with an electrode or an electron paramagnetic resonance spectrometer, respectively. The concentration of glutathione in the tissue was measured as a marker of nitrosative stress.

**Results:** High concentrations of luminal nitric oxide were generated locally at the gastro-esophageal junction of the nitrite-administered rats, reproducing a phenomenon observed in human. High levels of nitric oxide were also detected largely in the superficial epithelium of the gastro-esophageal junction. The concentration of tissue glutathione at the gastro-esophageal junction was significantly lower in nitrite-administered rats compared to control rats, whereas that in the distal stomach was similar between the two rat groups.

**Conclusions:** Using an animal model, this study demonstrated that nitric oxide generated in the lumen diffuses into the adjacent gastric tissue to a substantial degree, leading to localized consumption of glutathione in the tissue. Nitrosative stress induced by this mechanism may be involved in the high prevalence of inflammation and metaplasia, and subsequent development of neoplastic disease at that site.

## INTRODUCTION

Nitric oxide (NO) is an important radical that mediates a wide range of physiologic and pathologic events. It is generated at low concentrations by the enzyme constitutive nitric oxide synthase (NOS) to modulate neuromuscular and vascular functions. Higher concentrations are generated by the inducible form of the enzyme as a part of the immune and inflammatory response. Sustained generation of NO by inducible NO synthesis has been implicated in the aetiology of the mutagenesis and neoplasia related to chronic inflammation.[1][2][3]

The highest concentrations of NO occurring in the body are not the result of enzymatic synthesis but rather by chemical reactions within the lumen of the stomach,[4][5][6][7] especially in the most proximal part of it.[8] This arises from the entero-salivary re-circulation of dietary nitrate. Ingested nitrate as ingredient of food is absorbed from the small intestine, and 25% of this is taken up by the salivary glands and re-secreted into the mouth.[9][10][11][12][13] Bacteria on the dorsum of the tongue then reduce about 30 % of this nitrate to nitrite. [9][10][11][12][13] Under a fasting condition, the salivary nitrite concentration is approximately 50  $\mu$ M, and this rises to as high as 2 mM after ingesting food with a high nitrate content such as green lettuce.[7] [14] When salivary nitrite enters the stomach, the combination of the acidity and ascorbic acid content of the gastric juice converts the nitrite to NO.[15][16][17] Since this reaction between nitrite and ascorbic acid at acidic pH is very rapid,[18][19][20] the intraluminal concentration of NO generated by the reaction is expected to be maximal at the gastro-esophageal (GE) junction and cardia, where the nitrite in saliva first encounters gastric acid. Indeed, this was confirmed by a recent study in healthy volunteers which reported that at these anatomical locations substantial amounts of NO are generated following nitrate ingestion, in some case in excess of 50  $\mu$ M.[8]

The entero-salivary re-circulation of dietary nitrate is sustained for several hours,[7] [14] [21] during which period the adjacent epithelium of the GE junction is exposed to abundant amounts of NO generated in the lumen. Membranes in the tissues are not barriers to the diffusion of NO because of its gaseous and lipophilic properties.[22] Therefore, the NO produced in the lumen should readily diffuse into the surrounding epithelium, where NO may be accumulated to a level sufficient to exert some influence on the integrity of the tissue. In the present experiment, we developed an animal model in which high concentrations of NO were generated luminally at the GE junction by the administration of nitrite plus acidic ascorbic acid. Using this model, we investigated whether such high concentrations of NO could diffuse into the adjacent digestive tissue and, if so, at what level in the tissue by means of electron

paramagnetic resonance (EPR) spectroscopy. In addition, we also investigated whether diffused NO could cause localized consumption of a major antioxidant, glutathione, as an index of nitrosative stress in the tissue.

## **METHODS**

### **Animals**

Male Wistar rats, weighing approximately 250g (CLEA Japan Inc, Tokyo, Japan) were used in all of the experiments. Approval of the Animal Welfare Committee at the institute was obtained for all studies.

### **The animal model**

A model was developed to simulate the interaction of nitrite swallowed in saliva with gastric hydrochloric acid (HCl) and ascorbic acid in the human gastric lumen, which occurs at the GE junction of human.[8]

Rats were deprived of food for 24 hours but allowed free access to tap water. They were anesthetized with an intraperitoneal injection of urethane (1.25 g/kg) (Tokyo-Kasei, Tokyo, Japan), and were laid supine on a board with their heads elevated at a gradient of 30 degrees. A fine polyethylene tube (0.61 mm in diameter, BECTON DICKINSON, USA) was placed orally in the stomach and was used to administer 10 mM ascorbic acid dissolved in pH 2.0 HCl aqueous solution. An another metallic tube (0.9 mm in diameter, 50 mm in length, Natsume, Tokyo, Japan) was inserted orally so that the tip could be placed within the lower part of the rat esophagus and was used for the continuous administration of sodium nitrite aqueous solution (0.5 mM - 2 mM) or water alone as a control, together with 1 mM sodium thiocyanate aqueous solution. Thiocyanate, which is actively secreted in human saliva, is known to be an important catalyst in the chemistry of nitrite under an acidic condition.[19] [23] Ascorbic acid solution (0.5 mL) was administered in the stomach, and then the infusion of nitrite solution was started at a rate of 0.8 mL/hour. The infusion rate of nitrite solution was fixed at this level in all experiments, and corresponded approximately to the delivery rate of human saliva per kg of body weight. In this model, the nitrite administered into the esophagus was acidified on entering the GE junction and reduced to NO promptly at that site by the reaction with the ascorbic acid pooled in the stomach (fig 1). In addition, in another group of rats, famotidine (kindly donated by Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan) (2.5 mg/kg) was administered subcutaneously 2 hours before the experiments and ascorbic acid was prepared with pH 7.4 phosphate buffer, and then the same procedure was repeated.

### **Luminal NO concentration**

An NO sensor (ISO-NO, WPI Inc., Sarasota, USA) was used for monitoring the local concentrations of NO within the gastric lumen in rats.[19][20] The electrode was calibrated by placing it in a solution of 0.1 mol/L H<sub>2</sub>SO<sub>4</sub>/KI with various concentrations of potassium nitrite.

Under urethane anesthesia, the abdomen was opened by a midline incision and two electrodes were inserted into the gastric lumen via each incision in the forestomach, so that the tips of the electrodes could be placed at the GE junction and at the distal stomach, respectively. Then, infusion of the test solution was started and the concentrations of NO generated in the lumen were monitored by the two electrodes simultaneously at each point (fig 1).

### **Direct measurement of NO by EPR Spectrometry**

The concentrations of NO in the gastric tissue of the rats were measured by using the NO trapping technique combined with EPR spectroscopy. Spin traps react with unstable free radicals such as NO to form a relatively stable radical adduct. This long-lived adduct formation results in accumulating a steady-state formation of these free radicals, and thus, the resultant radical adduct can be detected readily by EPR spectroscopy. Here, we used a Fe-diethyldithiocarbamate (DETC) complex[24][25] as a trapping agent to quantify the NO levels in the gastric tissues of the rats.[26][27] A DETC 3H<sub>2</sub>O solution (400 mg/kg; Aldrich Chemical Co., Milwaukee, WI) and a Fe-citrate mixture (40 mg/kg of FeSO<sub>4</sub> 7H<sub>2</sub>O and 200 mg/ml of sodium citrate; Wako Pure Chemicals, Osaka, Japan) were injected intraperitoneally and subcutaneously, respectively. Because Fe-DETC complex formed internally is lipophilic and has a high specificity for NO,[25] it possesses strong affinity for gastric tissue and the ability to trap NO to form NO-Fe-DETC complex, which could be detected with EPR spectroscopy. The concentration in tissues was determined 30 min after the injection of NO trapping reagent and was expressed in the unit of nanomoles per gram of tissue per 30 min.[25][26][27]

Thirty min after administration of the trapping reagent, the stomach was removed and the tissue was resected selectively from both the GE junction and the distal stomach, the approximate weights of which were 100 - 120 mg (fig 1). The tissue from each area was minced separately and was subjected to the immediate measurement of NO by EPR spectroscopy. X-band (~9.5 GHz) EPR spectra were measured at room temperature with a JEOL TE-200 EPR spectrometer (Tokyo, Japan). Typical instrument settings are shown in the legend of Figure 3. In some experiments, Na<sup>15</sup>NO<sub>2</sub> (99.2 % <sup>15</sup>N; Prochem, UK) in place of Na<sup>14</sup>NO<sub>2</sub> (Sigma, St. Louis, USA) was used as nitrite. In others, the tissue of the stomach was scratched by a slide glass to separate the mucosa from the muscular layer.

### **Measurements of reduced glutathione concentration in the tissue**

Nitrite solution (2 mM) or water as a control was injected continuously in the esophagus over a period of 4 hours with a bolus administration of 0.5 mL ascorbic acid solution (pH 2.0) in the

stomach every 30 min. Subsequently, the stomach was removed and tissue was resected from similar parts of the stomach as in the above EPR experiment. The resected tissues were washed in normal saline and weighed. They were minced and homogenized in ice-cold metaphosphoric acid solution. The samples were mixed and centrifuged for 10 min at  $3000 \times g$ . The resulting supernatants were used for the assay. The reduced glutathione concentrations were determined by colorimetric assay (BIOXYTECH GSH-400<sup>TM</sup>, OxisResach, USA).[29]

### **Histology**

At the end of 4-hour administration of 2mM nitrite solution, as in the above experiment, the stomach was removed and opened along the greater curvature. These tissues were fixed in 10 % buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin.

### **Statistics**

Data are expressed as the mean  $\pm$  S.D. of the values from five rats in each group. Statistical analyses were performed using a one-way analysis of variance followed by the unpaired *t*-test. Values associated with a probability (p value) of  $< 0.01$  were considered significant.

## RESULTS

### Luminal NO concentration

High spike peaks of luminal NO were observed intermittently at the GE junction during the continuous administration of 1 mM nitrite solution. In the meantime, there were synchronized but much lower peaks of NO detected at the distal stomach (fig 2). The intermittent transit of nitrite into the GE junction due to peristalsis of the esophagus or transient opening of the GE junction could be responsible for such peaks in the luminal NO concentrations observed during the continuous injection of the nitrite solution into the esophagus. The NO generation in the lumen was completely inhibited by maintaining the gastric lumen at neutral pH with famotidine injection (data not shown).

### X-band EPR spectroscopy

A signal with high intensity was observed in the 1 mM nitrite-administered rats compared with the control rats, and was more evident in samples from the GE junction (fig 3). With administration of  $^{15}\text{N}$ -nitrite in place of  $^{14}\text{N}$ -nitrite, a characteristic two-line EPR signal was observed as shown in fig 4. These splittings of signals originate from the hyperfine interaction of an unpaired electron with the  $^{14}\text{N}$  ( $I = 1$ ) or  $^{15}\text{N}$  nucleus ( $I = 1/2$ ) in NO.[28] This observation definitely demonstrates that NO trapped by the Fe-DETC complex was derived from the exogenously administered nitrite. In the nitrite administered rats, the mean NO level in nanomoles per gram tissue per 30 min was  $1.8 \pm 0.2$  ( $n = 5$ ) at the GE junction, and was significantly higher than that at the distal stomach. In the same group of rats, manipulation to eliminate acid from the gastric lumen by famotidine administration lowered the NO level to  $0.7 \pm 0.3$  ( $n = 5$ ) at the GE junction, leading to a level similar to that of the distal stomach (fig 5). The high signal intensity of NO-Fe-DETC complex was largely confined to the mucosal layer of nitrite-administered rats, leaving only a small signal in the inner muscular layers (fig 6). The NO level in the mucosal layer increased depending on the concentration of nitrite administered, while maintaining the constant distribution of the NO level within the stomach: that of the GE junction was higher than that of the distal stomach. The NO level reached up to  $6.1 \pm 1.0$  ( $n = 5$ ) in the mucosal layer of the GE junction by administration of 2 mM nitrite. In contrast, the NO level in the muscular layer was little affected by the administration of nitrite, except for a small increase seen in the GE junction at the highest concentration administered (fig 7). In some DETC experiments with 2 mM nitrite administration, the liver, kidney, and esophagus were also resected from the rats and subjected to EPR determinations. In these organs, there were no discernible differences in the EPR signals compared with the respective controls, suggesting



that, because of its lipophilic property, NO-Fe-DETC complex did not go into the circulation and remained for at least 30 min in the tissue where the complex was formed (data not shown).

In the experiment described above, our samples from the distal stomach consisted of columnar mucosa alone whereas those of the GE junction contained a small portion of squamous mucosa from the forestomach as well. Hence, an additional experiment was performed using a Lucite chamber system (Tamura Seisakusho, Kyoto, Japan) [26] to check if the type of the gastric mucosa per se could affect the results of the estimation of the NO level by EPR spectrometry. Both columnar and squamous mucosa samples were exposed simultaneously to various concentrations of NO formed in the chamber lumen by the reaction of nitrite and ascorbic acid in the presence of pH 2.0 HCl. Consequently, the NO level was similar between samples from both types of mucosa, suggesting that the NO determination by EPR spectrometry was not influenced by the type of the gastric mucosa (data not shown).

#### **Reduced glutathione concentration in the tissue**

Four-hour exposure to the luminal NO generated by continuous infusion of 2 mM nitrite resulted in a significant decrease in the reduced glutathione concentration at the GE junction of the rats compared with that of water administered rats as a control, while such a change was not observed in the distal stomach (fig 8).

#### **Histology**

Four-hour exposure to the high level of luminal NO did not induce any mucosal injury both macroscopically and microscopically in this animal model.

## DISCUSSION

In the current study, we designed an animal model in which a high level of luminal NO could be formed mainly at the GE junction in order to reproduce the phenomenon observed in human.[8] A major finding of this study is that NO generated in the lumen can diffuse into the adjacent gastric epithelium to a substantial degree, leading to local consumption of reduced glutathione at that site. As we applied a concentration of nitrite that has been observed in human saliva after the ingestion of a high nitrate meal, this finding may be applicable to the GE junction in human.

Previous studies demonstrated that at an acidic pH such as pH 1.5 or 2.5 in the presence of ascorbic acid, most nitrite is converted to NO within a few seconds.[18][19][20] In the current animal model, nitrite and acidified ascorbic acid were administered separately so that the generation of NO from nitrite could be maximal at the GE junction where the reactants first meet. Elimination of acid from the gastric lumen completely inhibited luminal NO generation in this model, which is consistent with previous reports on the essential role of the presence of acid in the reaction between nitrite and ascorbic acid.[18][19] Using this animal model enabled us to investigate *in vivo* the influence of NO generated luminally on the integrity of the adjacent gastric tissue.

Since NO is known to have dual effects within the tissue, i. e. cytoprotective and cytotoxic depending on the gas level, determination of the NO level is required to evaluate its function in the tissue. However, it is practically difficult to know the actual amounts of NO in the tissue because NO is rapidly metabolized to nitrite, nitrate or other metabolites and transferred to the circulation. To overcome this difficulty, we applied an EPR spectroscopy technique with a Fe-DETC complex as an NO trapping agent to estimate the level of NO in the tissue.[25][26][27] By direct measurement of the NO concentration in the tissue, this technique clearly demonstrated that NO generated in the lumen could actually diffuse into the adjacent gastric tissue. The technique also clarified the distribution of NO within the stomach, that is, the level of the gas diffusion into the tissue was higher in the GE junction in our model, where nitrite and acidified ascorbic acid first meet to form NO luminally, than in the distal stomach.

In our previous study with EPR spectroscopy, the concentration of NO derived from the constitutive form of NOS in the rat stomach was as small as 0.2 - 0.3 nmole/g-tissue,[26] whereas it reached a level beyond 1.0 nmole/g-tissue when NO was generated by the inducible form of the enzyme in lipopolysaccharide-treated rat stomach.[27] Therefore, the high level of NO, as much as 6.0 nmol/g-tissue, detected in the mucosal layer at the GE junction of nitrite administered rats was similar to the level derived from inducible NOS, which can induce

cytotoxic effect on living cells.

In oxygen-containing fluid, the NO would react with dissolved oxygen to form the nitrosating species  $N_2O_3$ , which can be hydrolyzed to nitrite.[30] In the acidic gastric lumen, nitrite thus formed will be rapidly reduced back to NO by ascorbic acid[18][19][20] to maintain the NO concentration. However, once the gas is diffused into the tissue at neutral pH, NO will be irreversibly converted to nitrite. The rate of NO oxidation is proportional to the square of the NO concentration,[31] which indicates that the lifetime of NO is inversely proportional to its concentration. This means that, at high concentrations as in gastric lumen, the half-life of NO in the presence of oxygen is as short as less than a second.[31][32] In addition, this reaction is 300 times faster within a hydrophobic media, including the lipid cell membrane, than within an aqueous media because both NO and oxygen are more soluble in the former than in the latter.[22] Hence, the highest concentration of the nitrosating species  $N_2O_3$  arising from the luminal NO will occur within the hydrophobic lipid membrane of the superficial epithelial cells and this highly reactive species will immediately react with surrounding molecules. Consequently, the majority of NO arising from the lumen will be exhausted within the superficial epithelial layer, which is consistent with the current result using EPR spectroscopy in which the NO was largely confined to the superficial mucosal layer.

Interestingly, a small but significant amount of NO was detected even in the inner muscular layer of the GE junction at highest concentration of nitrite administered, suggesting that a small portion of NO arising from the lumen could escape from autoxidation occurring within the superficial layer, reaching the inner tissue. Therefore, the abundant NO formed in the lumen at the GE junction may be sufficient to penetrate the epithelium and then affect the function of the inner smooth muscle cell in the lower esophageal sphincter.[33]

A high flux of NO arising from the lumen at a sustained concentration could have the potential for causing nitrosative stress on the adjacent epithelial cells. The toxicity of NO is largely related to its reaction with oxygen to form higher oxides of nitrogen such as  $N_2O_3$ .[32] Because of the high affinity of  $N_2O_3$  for sulfhydryl-containing peptides such as glutathione[34][35] and the abundant contents of glutathione in the gastric tissue,[36] glutathione can become one of the intracellular targets of NO. In this study, the reduced glutathione concentration in the tissue was significantly decreased only at a site exposed to high concentrations of luminal NO, suggesting that NO arising from the lumen reacted with the glutathione in the adjacent tissue via the formation of the intermediate. This is consistent with previous *in vitro* studies showing that treatment with NO donors caused a decrease in

intracellular reduced glutathione level in a variety of cultured cells,[37][38] including a gastric cell line.[39] Glutathione is known to play a central role as an antioxidant in the protection against cell damage. It has been demonstrated that glutathione-deficient cells were more susceptible to the damaging effects of oxidizing species[40][41] or to NO-mediated cytotoxicity.[38][39] Therefore, the decreased level of reduced glutathione seen in the gastric tissue exposed to high concentrations of luminal NO may render gastric epithelial cells susceptible to oxidative or ongoing nitrosative stress. Although the histological examination of the current study failed to show any histological abnormalities in the gastric tissue after 4-hour exposure to NO derived from nitrite, further studies are required to investigate if such treatments could alter the susceptibility of the gastric epithelium to subsequent challenge with luminal irritants such as bile acids. In addition, a long-term study is also required to investigate the role of luminal NO generation in epithelial neoplasia. Intriguingly, previous studies have demonstrated that administering nitrite to rats along with ascorbic acid continuously over 50 weeks could induce tumors in the proximal stomach.[42][43][44] Luminal NO generation by the administration of nitrite plus ascorbic acid may be involved in the mechanism of this tumor induction.

In human, it has been shown that a high level of salivary nitrite is sustained over several hours after the ingestion of a high nitrate meal.[7] [14] [21] Nitrite in swallowed saliva has been recently shown to be rapidly converted to NO at the GE junction.[8] Using an animal model, we have extended this recent work by showing that a substantial amount of NO actually diffuses from the lumen into the adjacent tissue. Therefore, the human GE junction is likely to be a region of high nitrosative stress. It has been reported in a recent endoscopic study of asymptomatic adults that there was a high incidence (around 30%) of inflammation and intestinal metaplasia, both of which were confined mostly to the GE junction and most proximal cardia region.[45][46][47][48][49][50][51] Nitrosative stress induced by luminal NO generation may be involved in these isolated findings observed in healthy acid-secreting stomach, which could also be responsible for the subsequent development of epithelial mutagenesis at this anatomical site.

In conclusion, using an animal model, this study demonstrated that NO generated in the lumen diffuses into the adjacent gastric tissue to a substantial degree at a level comparable to that of iNOS derived-NO production, leading to local consumption of reduced glutathione in the tissue. Nitrosative stress induced by such a mechanism can actually occur in the human body, that is, in the epithelium of the GE junction, and may be involved in the high prevalence of

inflammation and metaplasia, and subsequent development of neoplastic disease at that site.

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#### **FOOTNOTES**

Conflict of interest: None declared.

## FIGURE LEGENDS

### **Figure 1. Schematic diagram of animal model in this study.**

A. Both fine polyethylene and metallic tubes for administrating the reagents described in the text were inserted orally into the rat stomach. After each treatment, the gastric tissues were obtained from each diagonal area, the GE junction and the distal stomach, and then were employed for various measurements. B. In other experiments, two electrodes inserted through incisions in the forestomach were placed in the lumen of both the GE junction and the distal stomach to monitor the NO concentrations simultaneously.

### **Figure 2. A representative profile of the NO concentrations monitored simultaneously at the two points within the stomach.**

After placing 0.5 mL of 10 mM ascorbic acid (pH 2.0) in the stomach, continuous injection of 1 mM sodium nitrite was started at a rate of 0.8 mL/hour. The luminal NO concentrations were monitored by the two electrodes at the GE junction and distal stomach simultaneously. The solid line represents the data from the GE junction, and the dotted line represents the data from the distal stomach. The generation of luminal NO was considerably higher at the GE junction compared to the distal stomach in this animal model.

### **Figure 3. Tracings of the three-line spectra at $g = 2.04$ to represent the nitric oxide adduct of Fe-DETC (NO-Fe-DETC) complex in the rat gastric tissue.**

After placing 0.5 mL of 10 mM ascorbic acid (pH 2.0) in the stomach, continuous injection of 1 mM sodium nitrite or water as a control was started at a rate of 0.8 mL/hour. Thirty minutes after administering the NO-trapping agent (Fe-DETC), the gastric tissue was resected selectively from both the GE junction and the distal stomach. The signal height is proportional to the amount of NO trapped. The level of NO-Fe-DETC complex was estimated by comparing it with the signal height of a standard solution of a chemically synthesized NO complex: A: EPR spectra in the GE junction with nitrite administration. B: in the distal stomach of nitrite administration. C: in the GE junction of control. D: in the distal stomach of control. The instrument settings were as follows: center field, 331 mT; field scan, 4 mT; sweep time, 2 min; time constant, 0.3 s; modulation amplitude, 0.32 mT; modulation frequency, 9.44 GHz; microwave power, 60 mW.

### **Figure 4. Tracings of two-line spectra with administration of $^{15}\text{N}$ -nitrite in place of $^{14}\text{N}$ -nitrite in the rat gastric tissue.**

The experimental conditions were similar to that in Figure 3 except that  $^{15}\text{N}$ -nitrite was used in place of  $^{14}\text{N}$ -nitrite. The characteristic two-line EPR signal definitely indicated that the NO

trapped by the Fe-DETC complex derived from nitrite administered exogenously. The instrument settings were similar to those in Figure 3.

**Figure 5. NO concentrations in the rat gastric tissue estimated by X-band EPR spectrometry.**

The experimental conditions were similar to those in Figure 3. The NO levels were elevated in the nitrite-administered rats compared to control rats, particularly at the GE junction. Famotidine administration abolished the predominance of NO level at the GE junction observed in nitrite-administered rats. Mean values of 5 rats are represented by the horizontal bars. ++ indicates significant difference from control ( $p < 0.01$ ), \*\* indicates significant difference from nitrite administration ( $p < 0.01$ ), and ## indicates significant difference from the distal stomach ( $p < 0.01$ ), respectively.

**Figure 6. Tracings of EPR spectra in the mucosal and muscular layer of the rat stomach.**

The experimental conditions were similar to those in Figure 3. Samples resected from the GE junction and the distal stomach were separated into mucosal and muscular layers, respectively. A, B, C, D: 1 mM nitrite administration; E, F, G, H: control. A, B, E, F: the GE junction; C, D, G, H: the distal stomach. A, C, E, G: the mucosal layer; B, D, F, H: the muscular layer. The instrument settings were similar to those in Figure 3.

**Figure 7. NO concentrations in the mucosal and muscular layers of the rat stomach as a function of the concentrations of administered nitrite.**

The experimental conditions were similar to those in Figure 6 except for the various concentrations (0 - 2 mM) of sodium nitrite administered. The majority of NO was detected in the superficial mucosal layers from each resected site, with little NO in the inner muscular layers. The NO concentrations dose-dependently increased as a function of the concentrations of nitrite administered in the mucosal layer of the GE junction as well as in that of the distal stomach, although the former was consistently higher than the latter. The values represent means  $\pm$  S.D. of 5 rats. \*\* indicates significant difference from respective control ( $p < 0.01$ ).

**Figure 8. Concentrations of reduced glutathione in the gastric tissue resected from the GE junction and distal stomach.**

Sodium nitrite (2 mM) or water as a control was continuously injected over a period of 4 hours with a bolus administration of 0.5 mL ascorbic acid solution (pH 2.0) every 30 min. Thereafter the tissue was obtained from the same parts of the stomach as in the DETC experiment. The reduced glutathione concentration was reduced by nitrite administration at the GE junction while it remained unchanged at the distal stomach. Mean values of 5 rats are represented by the

horizontal bars. \*\*:  $p < 0.01$ , n.s.: not significant.



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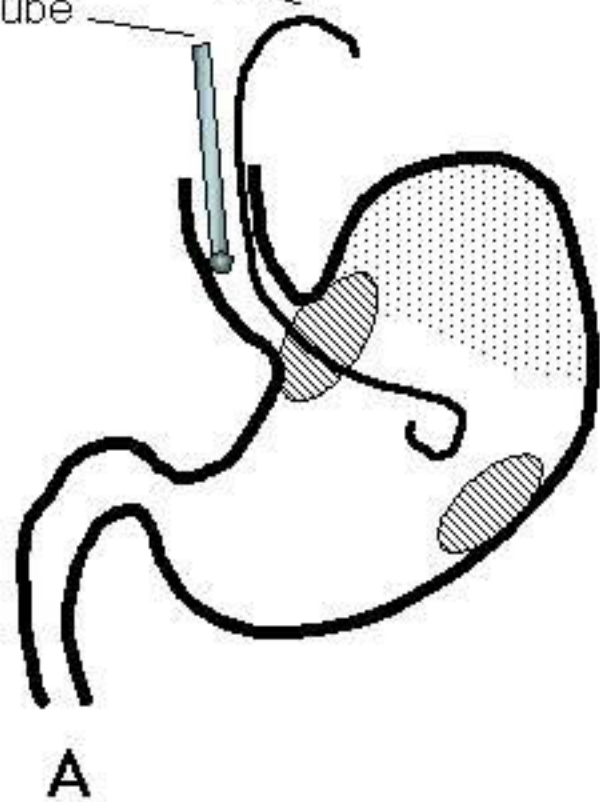
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Figure 1

Polyethylene tube

Metallic tube



Electrode

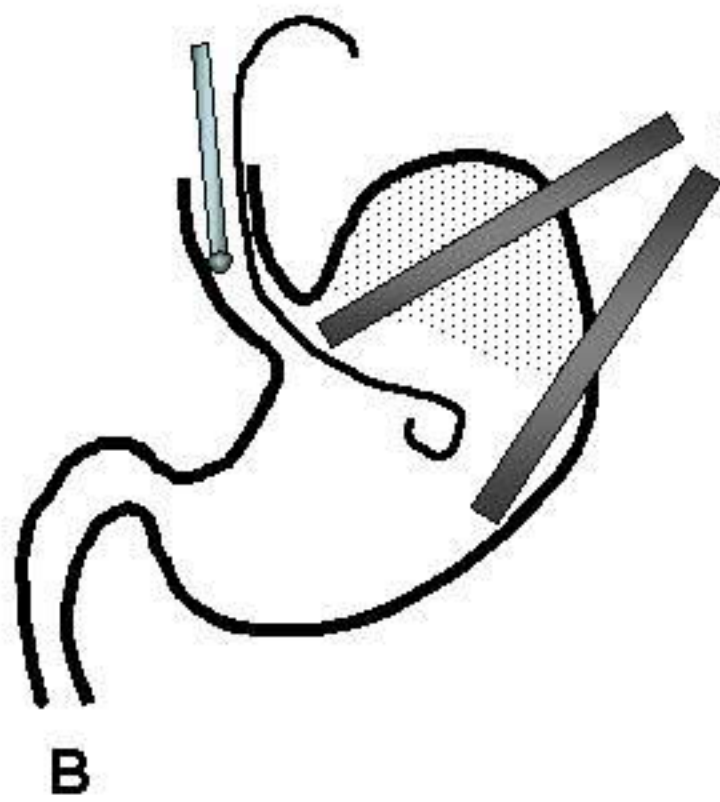


Figure 2

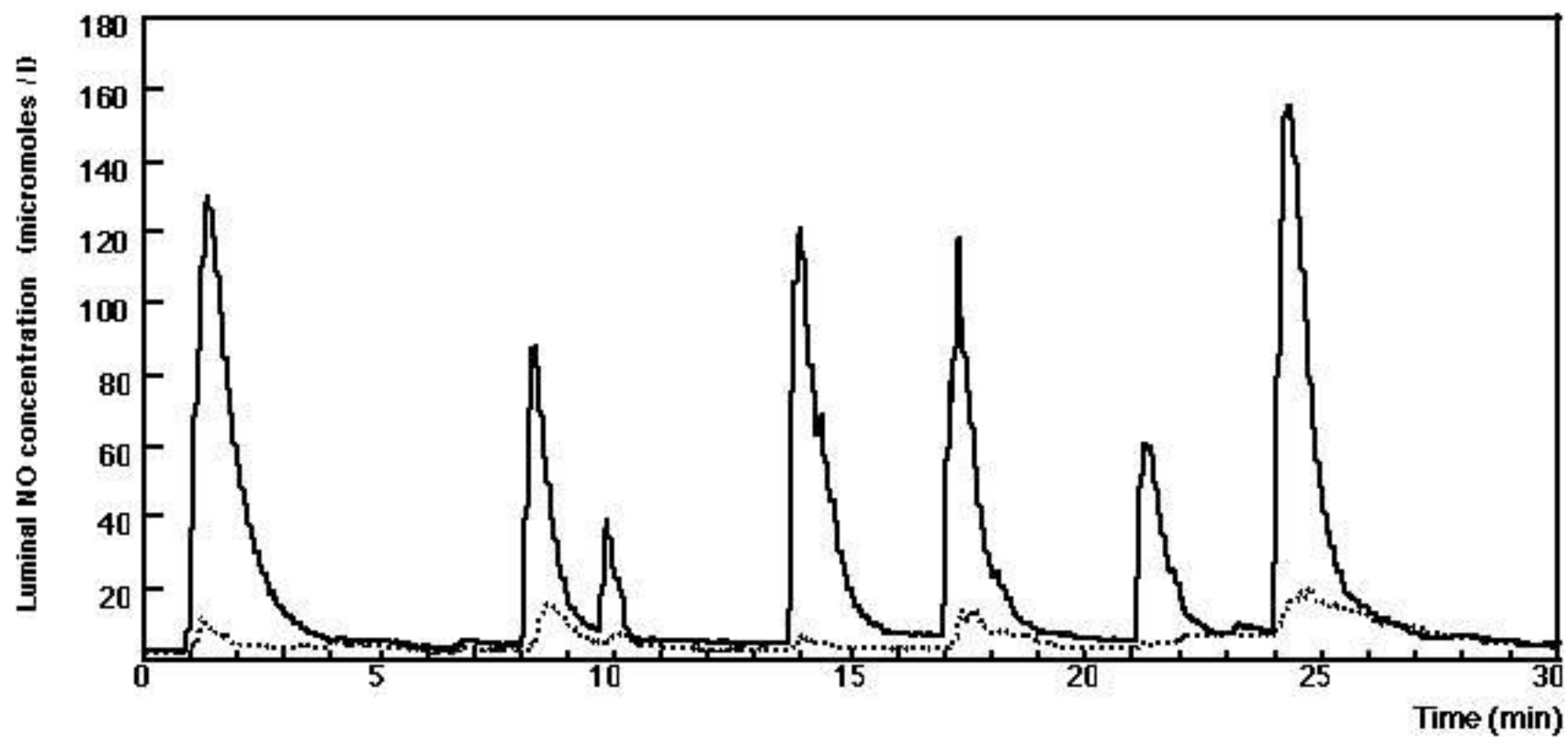


Figure 3

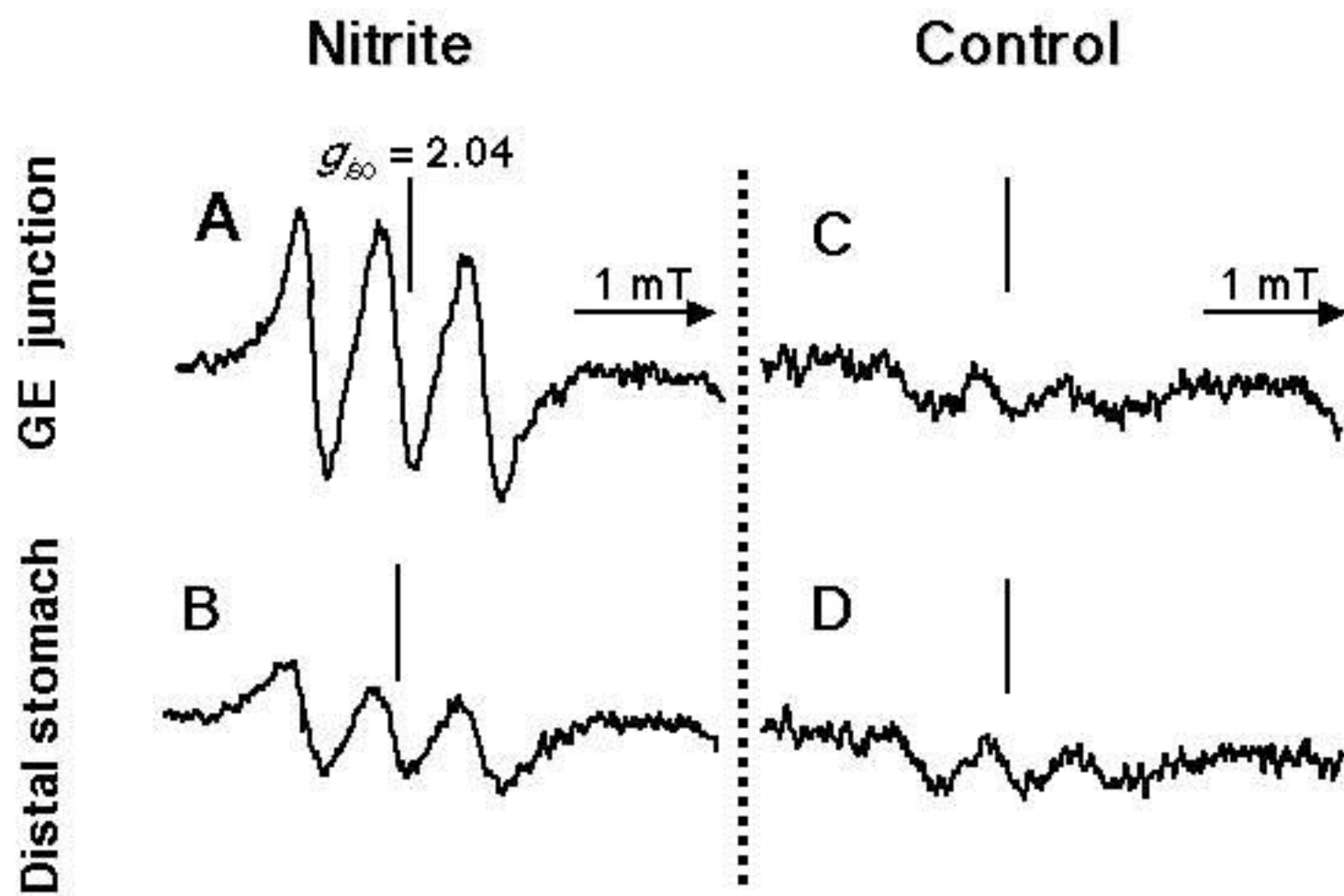


Figure 4

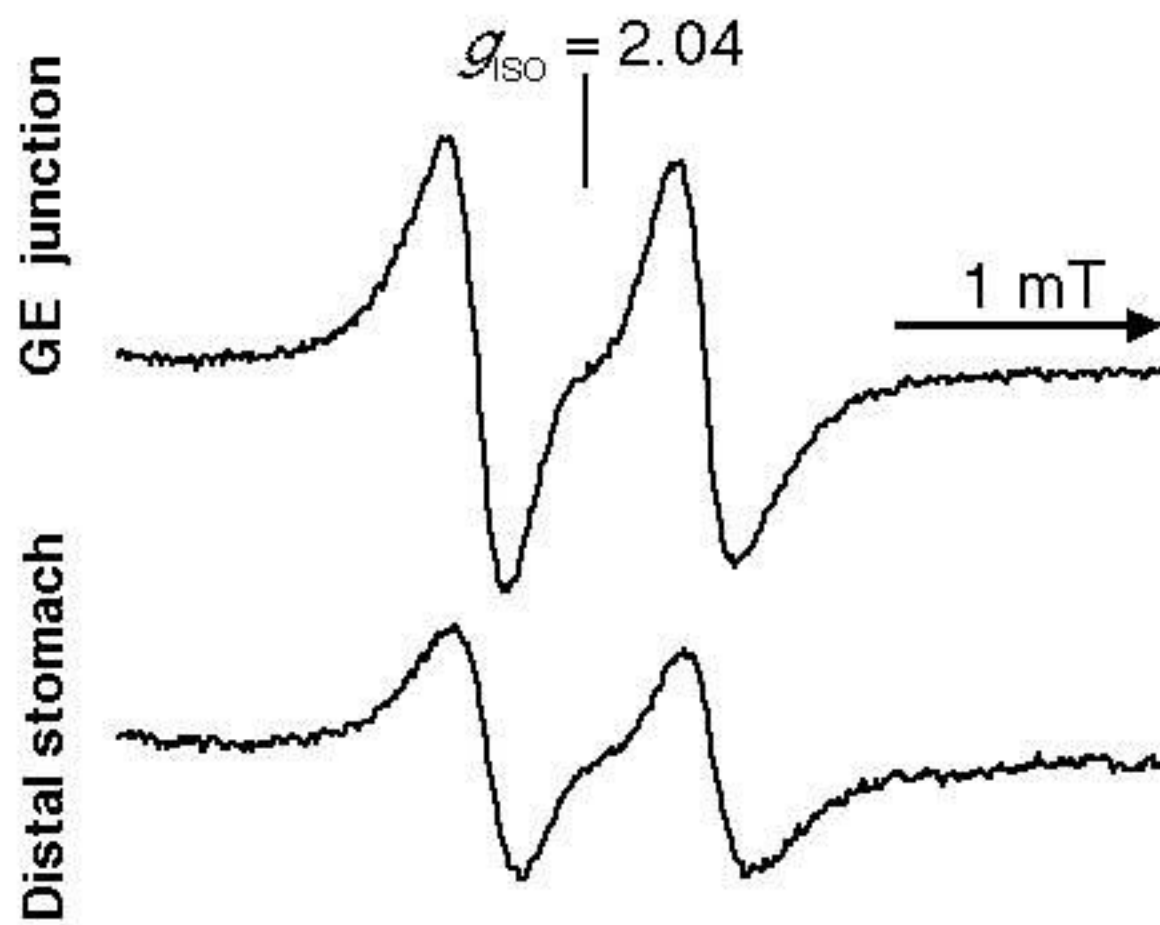




Figure 5

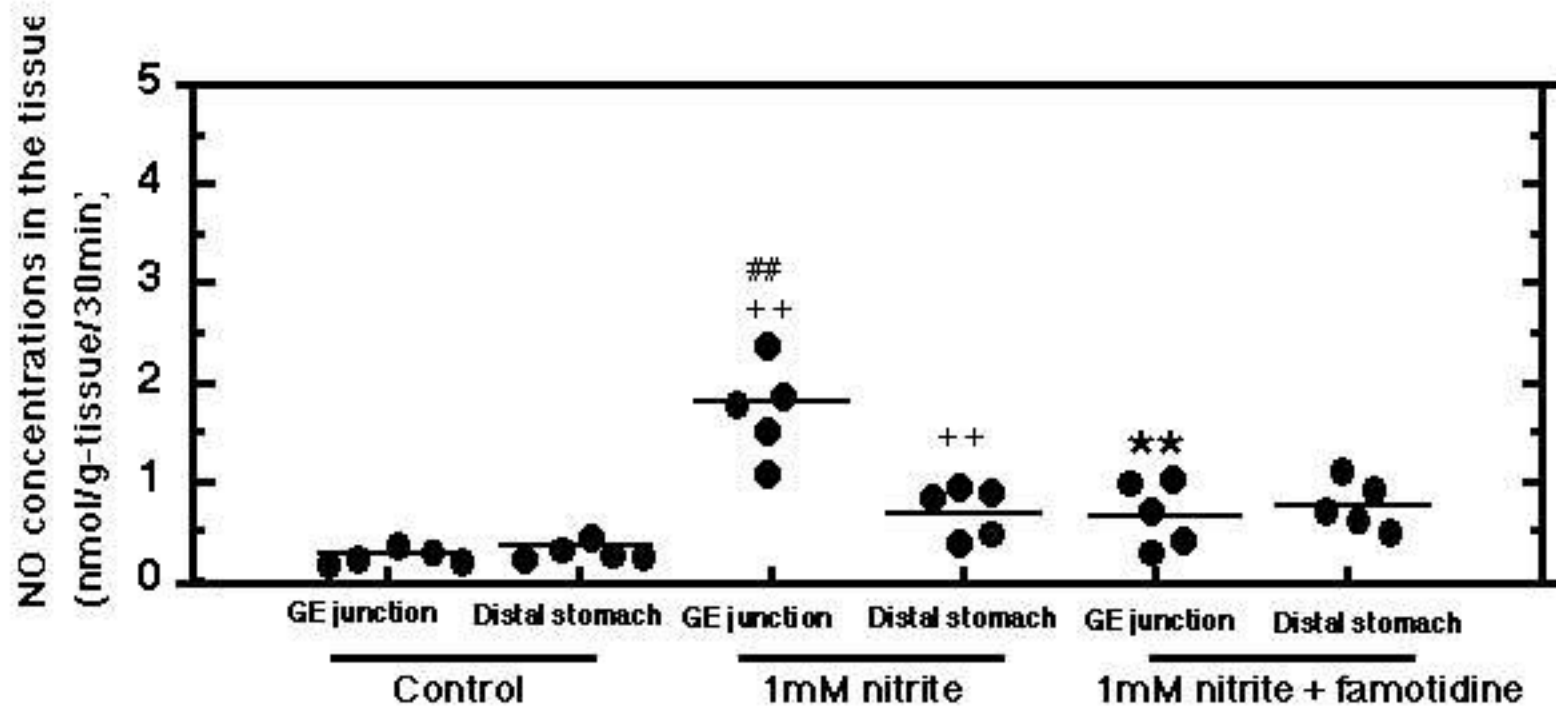


Figure 6

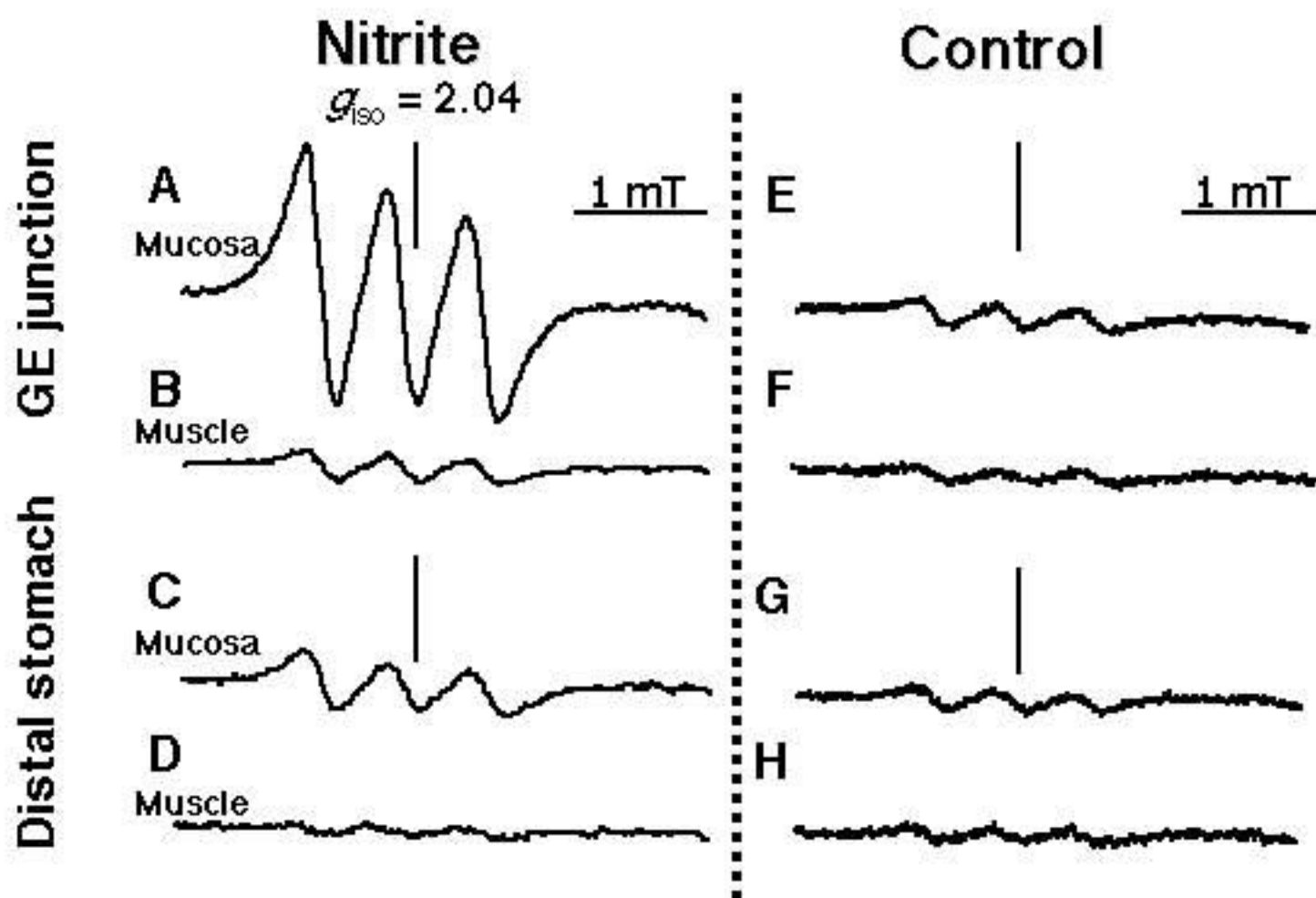


Figure 7

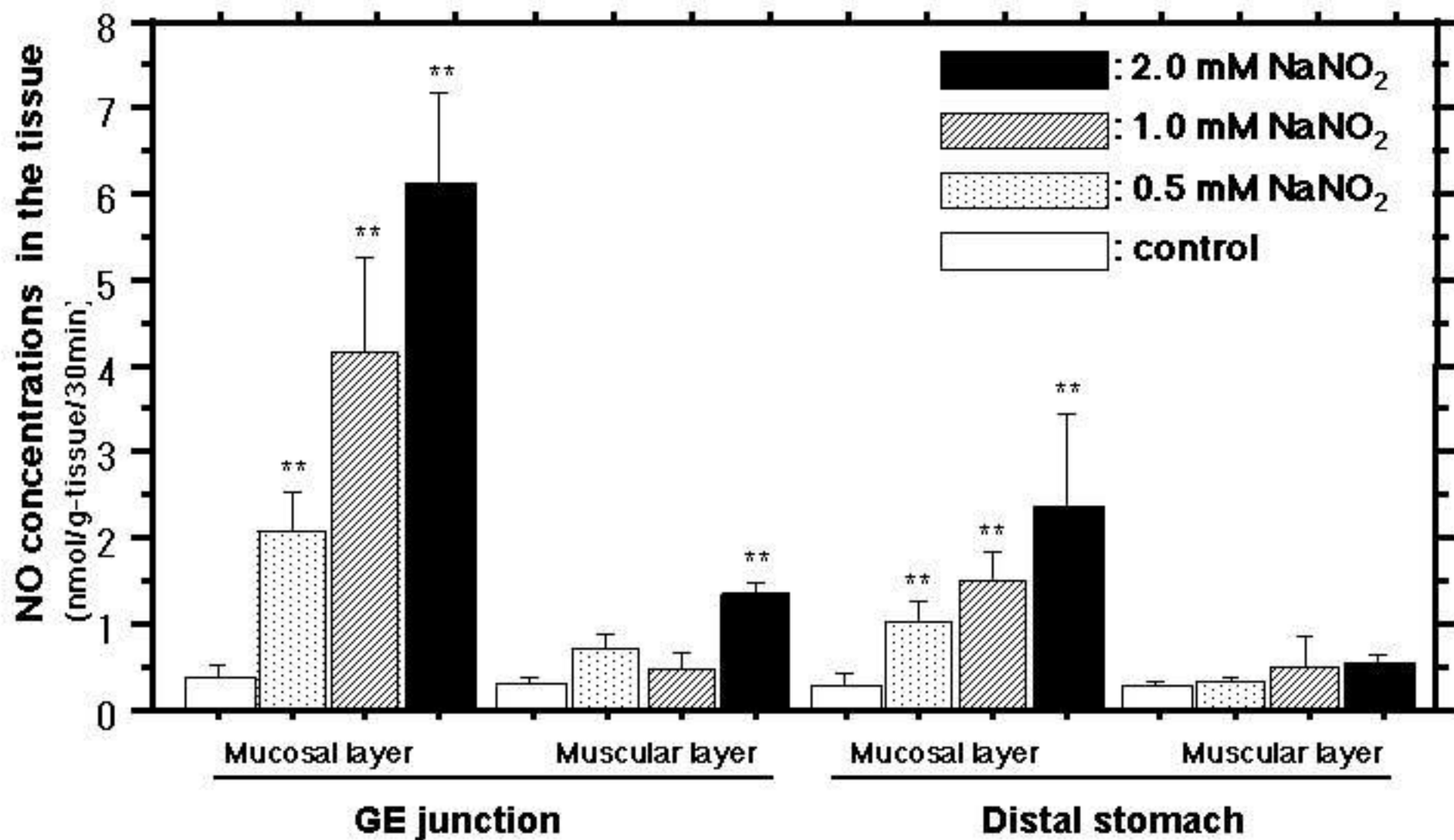
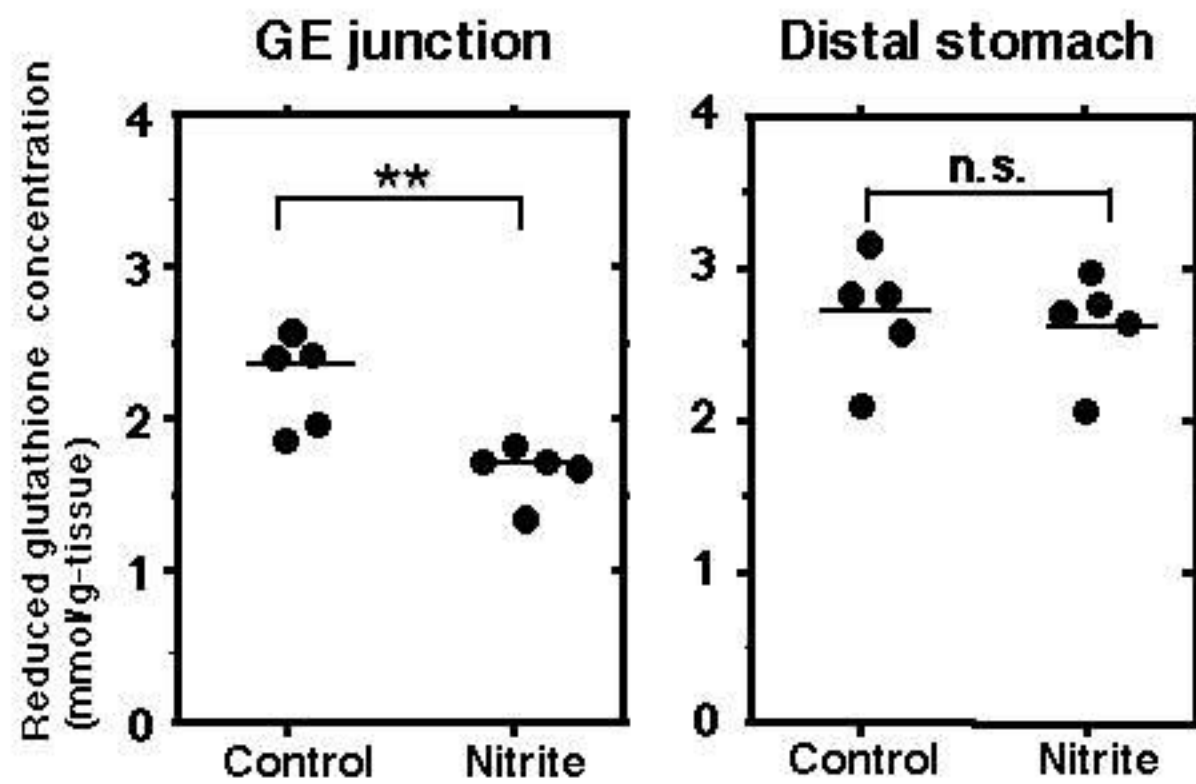


Figure 8





## Diffusion of cytotoxic concentrations of nitric oxide generated luminally at the gastro-oesophageal junction of rats

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