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Non-invasive Analysis of Reactive Oxygen Species Generated in NH_4OH-induced Gastric Lesions of Rats using a 300 MHz *In Vivo* ESR Technique

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Free radicals are reportedly involved in mucosal injury, including NH_4OH-induced gastric lesions, but the kind, location and origin of radical generation have yet to be clarified. We developed the non-invasive measurement of reactive oxygen species (ROS) in stomach, and applied to mucosal injury. NH_4OH-induced gastric lesions were prepared in rats, which were then given a nitroxyl probe intragastrically or intravenously, and the spectra of the gastric region were obtained by *in vivo* 300 MHz electron spin resonance (ESR) spectroscopy. The spectral change of the nitroxyl probe administered intragastrically was significantly enhanced 30 min after NH_4OH administration, but no change occurred when the probe was given by intravenous injection. The enhanced change was confirmed to be due to •OH generation, because it was completely suppressed by mannitol, catalase and desferrioxamine (DFO), and was not observed in neutropenic rats. NH_4OH-induced neutrophil infiltration of the gastric mucosa was suppressed by intravenous injection of superoxide dismutase (SOD) or catalase, or by administration of allopurinol. The present study provided the direct evidence in NH_4OH-treated living rats that •OH produced from $O_2^{\bullet -}$ derived from neutrophils caused gastric lesion formation, while $O_2^{\bullet -}$ or H_2O_2 derived from the xanthine oxidase system in endothelial cells was involved in neutrophil infiltration.

Keywords: Hydroxyl radical; Free radicals; Oxidative injury; ESR; *Helicobacter pylori*

Abbreviations: ANS, anti neutrophil serum; DFO, desferrioxamine; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ESR, electron spin resonance; H&E, hematoxylin and eosin; MPO, myeloperoxidase; NS, normal serum; NSAIDs, non-steroidal anti inflammatory drugs; PROXYL, 2,2,5,5-tetramethylpyrrolidine-1-oxyl; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid

INTRODUCTION

Recently, it has been proven that reactive oxygen species (ROS) are closely involved with various diseases. In diseases involving gastric mucosal injury, ROS are thought to either cause the injury directly or enhance its formation. The generation of ROS is inferred indirectly by detecting $O_2^{\bullet -}$ by chemiluminescence or increases in thiobarbituric acid (TBA)-reactive substances, and these methods can be used to study the results of generating ROS.

Neutrophils, macrophages and endothelial cells are reported to be a source of free radicals in gastric mucosal lesions.[1] For example, neutrophil infiltration is observed in gastric ulcers induced by non-steroidal anti inflammatory drugs (NSAIDs), ethanol, stress, or ischemia-reperfusion and ROS are thought to enhance the neutrophil infiltration.[2–5] Gastric mucosal lesion formation is suppressed by the administration of allopurinol, which inhibits xanthine oxidase in endothelial cells. Xanthine oxidase is activated by a decrease in blood flow, which can be transiently induced by NSAIDs.[6,7] In addition, generated ROS decreases the amount of antioxidants,[8,9] which may limit the extent of gastric mucosal damage. It is not certain, however, which cells are the major source of ROS, or how ROS contribute to gastric lesions.

In 1983, Warren *et al.* succeeded in isolating and cultivating *Helicobacter pylori*.[10] Invading neutrophils in the gastric mucosa,[11] increased lipid

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peroxidation, and changes in antioxidant activity[12] are all reported in *H. pylori* colonized Mongolian gerbils or *H. pylori*-infected patients, suggesting that free radical reactions are also involved in *H. pylori* related disease. On the other hand, *H. pylori* has a very high urease activity, and 4-fold higher NH_4OH is generated from urea in *H. pylori*-infected patients.[13] The NH_4OH concentration is thought to be even higher in the submucosal layer. Evidence from *in vitro*, *in situ* and *ex vivo* experiments suggests the involvement of •OH,[14] monochloramine,[15] apoptosis[16] and mitochondrial damage[17] in gastric lesion formation by NH_4OH. Unrelated mechanisms to the ROS formation, such as the back diffusion of H^+[18] may also be involved in the gastric mucosal lesions. There are still many uncertainties on how ROS are related to the origin and progress of gastric mucosal lesions induced by NH_4OH. It is of the utmost importance to clarify *in vivo* when, where, what and how ROS are produced, and how they cause and contribute to the progression of mucosal injury. The establishment of a non-invasive method for direct, quantitative observation of ROS is, therefore, desirable and critically eager for investigating these oxidative injuries.

We have examined *in vivo* free radical reactions in various physiological conditions and experimental diseases using *in vivo* electron spin resonance (ESR) and a nitroxyl probe.[19–22] We have used this method to analyze free radical reactions and ROS generation in experimental diseases, including hyperoxia,[19] streptozotocin-induced diabetes[21] and iron-overload.[22] Other groups have applied this technique to cancers,[23] X-ray irradiation[24] and ischemia-reperfusion-induced heart injury.[25] The role of ROS generation can be clarified by using a blood–brain barrier permeable probe[26] or imaging techniques.[27]

Until now, most *in vivo* ESR measurements have been limited to mice or the heads of rats, because less skin depth of microwave penetration. To improve the skin depth of microwave penetration, lower frequent ESR spectroscopy than 300 MHz has been developed by Decorps and Fric[28] and Halpern *et al.*[29] Recently, we[21] and the other group[30] also developed an *in vivo* 300 MHz ESR spectrometer, and we succeeded in the observation that vitamin E and insulin suppressed the free radical reactions in the upper abdomen of streptozotocin-induced diabetic rats.[21] If the *in vivo* 300 MHz ESR spectrometer is applicable to gastric injury, it can be determined whether free radical reactions occur in the stomach or in blood vessels by comparing the signal changes of a nitroxyl probe administered intragastrically and intravenously.

In this study, the non-invasive measurement of ROS was developed using the *in vivo* 300 MHz ESR spectrometer and the kind and origin of ROS generation were directly analyzed in rats with NH_4OH-induced gastric lesions. The mechanism of ROS generation, the area of the gastric lesions, and the roles of vascular permeability and neutrophil infiltration were investigated using anti-neutrophil antibody and antioxidants.

MATERIALS AND METHODS

Induction of Gastric Mucosal Damage

Male Sprague–Dawley rats (age 5 weeks, weight 120–150 g) were purchased from Seac Yoshitomi Co. (Fukuoka, Japan), and were acclimatized for 1 week before experiments. Diet (MF, Oriental Yeast Co. Tokyo, Japan) and water were provided *ad libitum*. The animals were fasted for 24 h, but allowed free access to water until 1 h before the experiment. Gastric mucosal lesions were induced by the intragastric administration of an NH_4OH solution (5 ml/kg) under anesthesia brought about by the intramuscular injection of urethane (2 g/kg). Control rats were administered saline instead of NH_4OH.

To measure the area of the mucosal lesions, the stomach was removed, inflated by injecting 10 ml of 1% formaldehyde, immersed in water and opened along the greater curvature. The area of lesions (mm^2 per glandular stomach) was measured under a dissecting microscope with a square grid micrometer.

To confirm neutrophil infiltration, the damaged region ($0.5 \times 2\,cm^2$) of glandular stomach was collected and fixed overnight in 10% neutral-buffered formalin. Sections with thickness of 5 μm were cut on a cryostat (Coldtome, Sakura Seiki Co., Tokyo, Japan) at −5°, taken up on glass slides, dried overnight at 4° and then counterstained with hematoxylin and eosin (H&E) stain.

All procedures and animal care were approved by the Committee on Ethics of Animal Experiments, Graduate School of Pharmaceutical Sciences, Kyushu university, and were conducted according to the Guidelines for Animal Experiments of Graduate School of Pharmaceutical Sciences, Kyushu university.

Drug Treatment

3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL) and urethane were purchased from Aldrich Chemical Co. (Milwaukee,WI). Cu/Zn-superoxide dismutase (SOD) from bovine erythrocytes, catalase, mannitol and allopurinol were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The *o*-Dianisidine-(3,3′-dimethoxybenzidine)-dichloride and desferrioxamine (DFO) were obtained from Sigma Chemical Co. (St. Louis, MO)

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and Ciba-Geigy (Japan), respectively. Rabbit normal serum (NS) and rabbit anti neutrophil serum (ANS, >75% agglutinability against rat neutrophils at 1:20 dilution) were from Inter-cell Technologies, Inc. All other chemicals were obtained either from Sigma Chemical Co. or Wako Pure Chemical Industries, Ltd.

Mannitol (0.5 mmol/rat), carbamoyl-PROXYL (0.1 mmol/rat), SOD (3000 U/rat) or catalase (3000 U/rat) dissolved in distilled water was intragastrically or intravenously administered immediately before the lesions were induced. DFO (10 mg/rat) in saline was administered intravenously 5 min before the lesions were induced, because the intragastric administration is reported to be not affective.[31] Allopurinol (5 mg/rat) suspended in distilled water was given orally every day for 7 days and then 2 h before the lesions were induced. The rats intraperitoneally received 500 μl of 4-fold diluted ANS 24 h before the lesions were induced. Control rats were given NS instead of ANS. In ANS-treated rats, the number of neutrophils counted using Turk's stain decreased to 25% of the level in control rats.

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Assay of MPO Activity

Gastric mucosal myeloperoxidase (MPO) activity was determined as an index of neutrophil accumulation. The corpus mucosa was scraped off using two glass slides and homogenized with 0.1 M phosphate buffer (pH 6.2) in a Teflon Potter Elvehjem homogenizer. The samples were sonicated (10 s × 3), subjected to three freeze-thaw cycles, and centrifuged (4000 rpm, 10 min) at 4°C. The supernatant (0.4 ml) was reacted with 0.2 ml of 0.15% H_2O_2 in the presence of 3.9 mM *o*-dianisidine (0.2 ml). After 15 min, 0.2 ml of 1% NaN_3 was added, and the MPO activity was assayed at 460 nm.

Determination of Microvascular Permeability

The microvascular permeability was evaluated by measuring the amount of Evan's blue dye extravasated in the mucosa. Twenty minutes after the intravenous injection of Evan's blue (5 ml/kg of 1%w/v), the rat was treated with heparin, and the intravessel contents were removed by transcardiac perfusion with saline. Evan's blue in the gastric mucosa was extracted by incubating with formamide for 72 h, and then measured at 620 nm. The amount of dye was expressed as μg/g wet tissue.

In Vivo ESR Measurement

Anesthetized rats were given 1.5 ml of 3 mM carbamoyl-PROXYL intragastrically or 1 ml/kg of 200 mM carbamoyl-PROXYL intravenously, and then the ESR spectrum was observed *in vivo* in the gastric region with a 300 MHz-ESR spectrometer (JES-CM-3L, JEOL, Japan). The microwave power was 1.19 mW. The amplitude of the 100 kHz field modulation was 0.1 mT. The external magnetic field was swept at a scan rate of 1.25 mT/min. The calculation of the signal decay rate is described in the "Results" section.

To evaluate the effect of antioxidants on the signal decay rate, mannitol or catalase was administered with the spin probe. DFO was injected intravenously immediately before the spin probe was administered. The catalase was completely inactivated by boiling it for 6 h (data not shown). In each experiment, NH_4OH-treated control rats were treated with the vehicle instead of the antioxidant or iron chelator.

In Vitro ESR Measurement of •OH Generation and its Relation to the Enhanced Signal Decay

•OH was generated using Fenton reaction by mixing $FeSO_4$ (2 μM) with hydrogen peroxide (28.8 μM) in 0.01 N HCl (pH 2.0). The amount of •OH was estimated from the signal intensity of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO)—OH adduct, and the contribution of •OH to the signal decay of nitroxyl radical was confirmed by adding carbamoyl-PROXYL (10 μM) to the Fenton solution. The relation of nitroxyl signal decay with •OH generated was semi-quantitatively determined with the inhibitory effect of nitroxyl signal decay by various amount of DMPO (1, 10, 50, 100 μM, 1, 100 mM, or 1 M). ESR spectrum was observed with a X-band ESR spectrometer (JES-RE 1X, JEOL, Japan). The center of magnetic field was 338.3 mT and the microwave power was 3 mW. The amplitude of the 100 kHz field modulation was 0.1 mT. The external magnetic field was swept at a scan rate of 5 mT/min.

Statistical Analysis

Statistical analyses were carried out using Stat View 5.0. The data were analyzed by Student's *t* test, by one way analysis of variance (ANOVA) with Dunnett test or Tukey–Kramer test as a post hoc test, or by two way ANOVA with Tukey–Kramer test. All the results are expressed as the mean ± S.E.

RESULTS

Effect of NH_4OH on the Gastric Mucosa

Under urethane anesthesia, the intragastric administration of NH_4OH induced gastric lesions on the glandular stomach (shown in an insert of Fig. 1). The area of the lesions was maximal 30 min after NH_4OH

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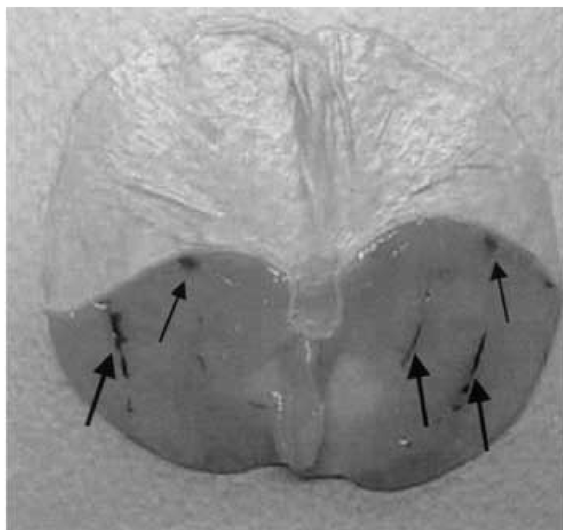

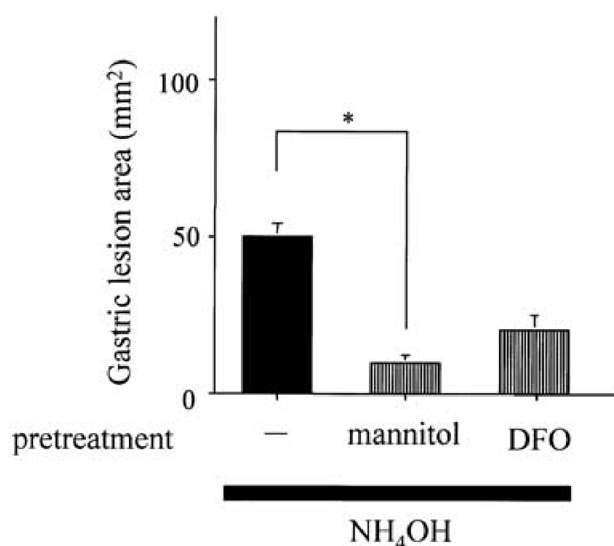

FIGURE 1 Effects of mannitol and DFO on NH_4OH-induced gastric lesions. The picture shown in the insert was taken 30 min after intragastric administration of 900 mM NH_4OH–water (5 ml/kg wt.) in an anesthetized rat. Note that the lesions located on the glandular stomach are linear (↑) or round (↑). Mannitol and DFO were administered intragastrically and intravenously, respectively, 5 min before inducing lesions by the intragastric administration of 900 mM NH_4OH. The area of gastric lesions was measured 30 min after induction. Values represent the mean ± S.E. (bar) of 5 rats. *$p < 0.05$ as determined by Dunnett test when compared with vehicle-treated group.

treatment, and then decreased gradually until 240 min afterwards, probably due to the restitution of epithelial cells. Histological investigation by H&E stain demonstrated that almost of epithelial cells were disrupted and neutrophils were infiltrated into gastric mucosa of NH_4OH-treated rats, while any damage was not observed in the mucosa of saline-treated rats (Fig. 2).

Pretreatment with mannitol, which is an •OH scavenger, significantly suppressed the gastric lesion area by 20% of that in control group (Fig. 1). The pretreatment with DFO, which is a Fenton-like reaction inhibitor, inhibited the lesion formation up to 41% of that in control group. These results indicate that •OH radicals generated from H_2O_2 were the direct cause of the lesions.

In Vivo ESR Study of NH_4OH-induced Gastric Lesions in Rats

Using an *in vivo* ESR technique, free radical reactions in the stomach and blood vessels were evaluated by the intragastric and intravenous injection of nitroxyl spin probe, respectively. Figure 3A and B demonstrate typical ESR spectra in the gastric region of living rats after intragastric and intravenous injection of carbamoyl-PROXYL, respectively.

The carbamoyl-PROXYL spectra after intragastric injection decreased gradually in the rat's stomach. The very slow decay in control animals indicated that spin probe remained in the stomach. The semi logarithmic plot was linear for at least 20 min, indicating that the signal decay obeys first order kinetics (Fig. 3A). In NH_4OH-treated stomachs, the signal decay rate calculated from the slope of this

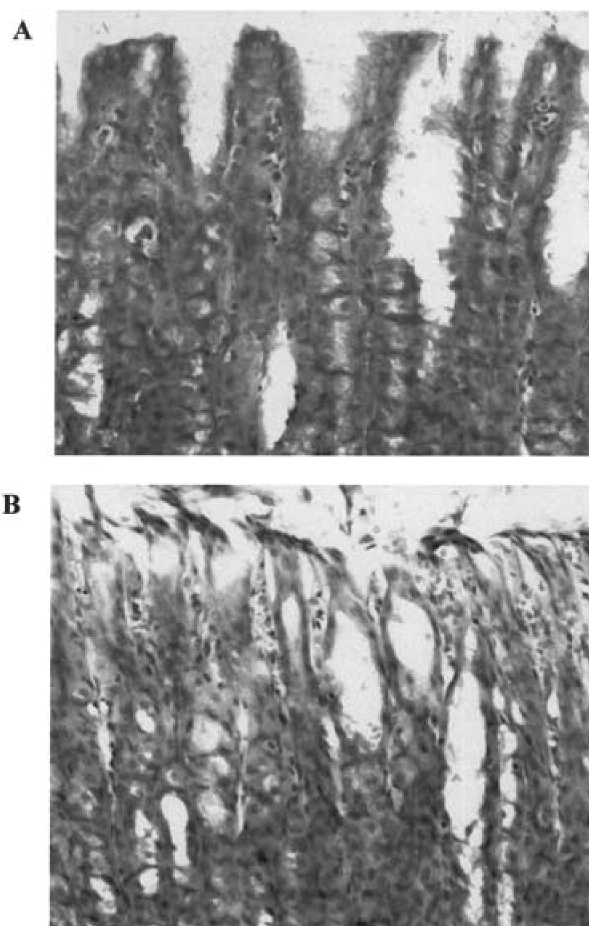

FIGURE 2 Hematoxylin and eosin (H&E) staining of stomachs from (A) saline-treated rats and (B) NH_4OH-treated rats. Thirty minutes after NH_4OH treatment, the stomach was removed, and slides with 5 μm thick serial sections of wax-embedded tissues were stained with H&E stain. (magnification = ×400).

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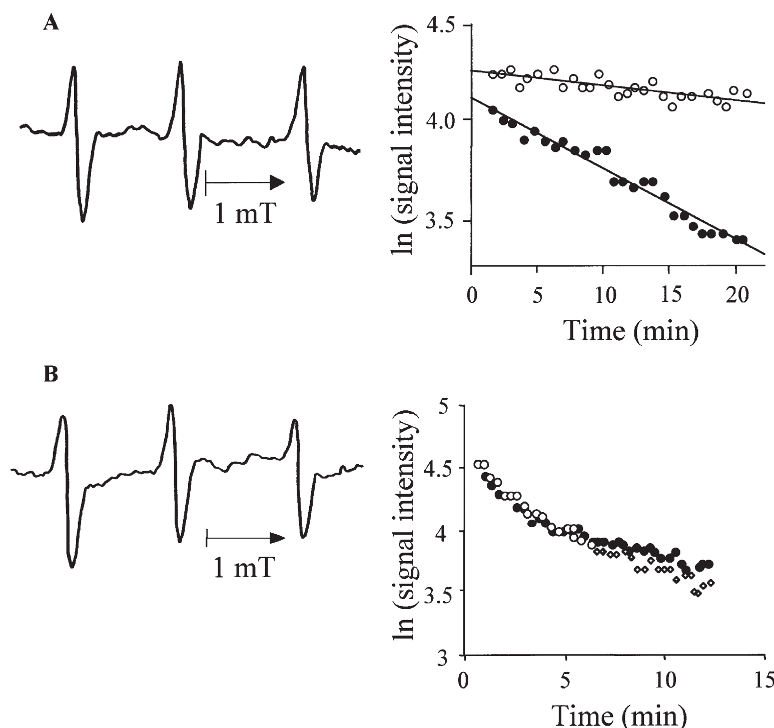

FIGURE 3 Typical ESR spectra and signal decay curves for carbamoyl-PROXYL in the gastric region after (A) intragastric (3 mM, 1.5 ml) and (B) intravenous (200 mM, 1 μl/g wt.) injection of carbamoyl-PROXYL. The arrow indicates the direction and amplitude (1.0 mT) of the external magnetic field. Thirty minutes after treatment with saline (○) or 900 mM NH_4OH (•) under anesthesia, the carbamoyl-PROXYL ESR spectra were measured in the gastric region with a 300 MHz ESR spectrometer. The logarithm of peak height $h(+1)$ was plotted against time after administration.

curve was much faster than that in saline-treated stomachs.

On the other hand, the carbamoyl-PROXYL signal decay after intravenous injection did not obey first order kinetics, and no difference was observed between control and NH_4OH-treated rats (Fig. 3B).

The intragastric carbamoyl-PROXYL signal decay was enhanced 30 min after NH_4OH treatment. Then, the enhanced signal decay decreased gradually until 240 min after treatment (Fig. 4). The time-course of the signal decay was quite similar to those seen in the studies of gastric lesion area, microvascular permeability and neutrophil infiltration. The enhanced

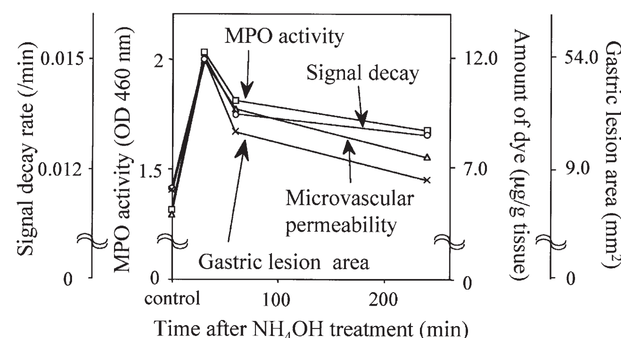

FIGURE 4 Time course of the signal decay rate, lesion area, MPO activity, and amount of extravasated Evan's blue in rats treated with 900 mM NH_4OH. The carbamoyl-PROXYL signal decay rate in the stomach was determined from the decay curve shown in Fig. 2 at 30, 60 and 240 min after the intragastric administration of 900 mM NH_4OH under anesthesia, and compared with the lesion area, MPO activity, and amount of extravasated Evan's blue. Values represent the mean ± S.E. (bar) of 6 rats.

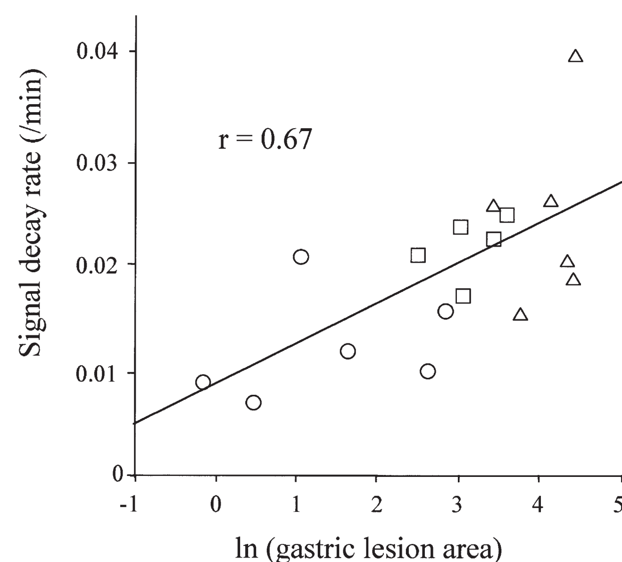

FIGURE 5 Correlation between signal decay and the area of gastric lesions. Under anesthesia, 500, 700 or 900 mM NH_4OH was administered intragastrically, and the signal decay rate and lesion area were determined in individual rats. The symbols (○), (□) and (Δ) indicate the results of treating rats with 500, 700 and 900 mM NH_4OH, respectively.

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TABLE I Effect of antioxidants and an iron chelator on the enhanced signal decay

	Signal decay rate (/min)		
Control	0.007 ± 0.006	0.008 ± 0.005	0.006 ± 0.002
NH_4OH-treated (vehicle)	0.026 ± 0.015*	0.027 ± 0.012**	0.023 ± 0.007**
Mannitol			
(0.05 mmol/rat)	0.017 ± 0.005		
(0.5 mmol/rat)	0.005 ± 0.005***		
Catalase			
(10 U/rat)		0.014 ± 0.009	
(100 U/rat)		0.012 ± 0.004***	
DFO			
(5 mg/rat)			0.014 ± 0.006
(50 mg/rat)			0.009 ± 0.005***

Gastric lesions were induced by the intragastric administration of 900 mM NH_4OH. After 30 min, 1.5 ml of 3 mM carbamoyl-PROXYL was administered intragastrically and the ESR spectrum was observed in the gastric region. To estimate the effect of an antioxidant on the enhanced signal decay, either mannitol or catalase was administered with the spin probe solution. DFO was injected intravenously immediately before administering the spin probe. NH_4OH-treated (vehicle) groups were treated with the corresponding vehicle instead of the antioxidant. Values represent the mean ± S.E. of 5–6 rats. $^*p < 0.05$, $^{**}p < 0.01$ as determined by Student's *t* test when compared with control group, and $^{***}p < 0.05$ as determined by Dunnett test when compared with NH_4OH-treated (vehicle) group.

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signal decay acted in an NH_4OH concentration-dependent manner and correlated well with gastric lesion formation in individual rats 30 min after NH_4OH treatment (Fig. 5).

To identify the radical species contributing to the enhanced signal decay, either mannitol or catalase was administered intragastrically together with the spin probe. Mannitol and catalase suppressed the enhanced signal decay in a dose dependent manner, although inactivated catalase did not (Table I). Intravenous injection of DFO 5 min before the administration of spin probe suppressed the enhanced signal decay (Table I). These findings indicate that the enhanced signal decay is caused by •OH generated from H_2O_2 in a Fenton-like reaction.

As described above, •OH is a direct cause of gastric lesion formation. As shown in Fig. 6, pretreatment with carbamoyl-PROXYL suppresses NH_4OH-induced gastric lesion formation. Therefore, the enhanced signal decay confirms the involvement of •OH in lesion formation and hence reflects •OH generation.

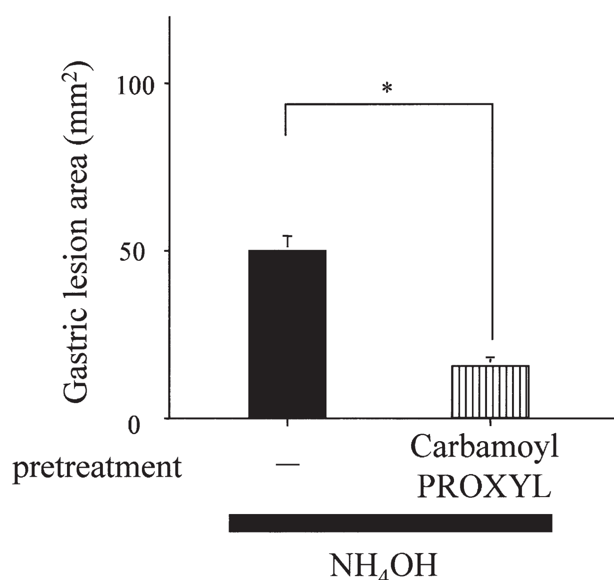

FIGURE 6 Inhibitory effect of carbamoyl-PROXYL on gastric lesion formation. Carbamoyl-PROXYL was administered intragastrically immediately before the administration of 900 mM NH_4OH. The area of gastric lesions was measured 30 min after NH_4OH treatment. Values represent the mean ± S.E. (bar) of 5 rats. $^*p < 0.05$ as determined by Student's *t* test when compared with vehicle-treated group.

In Vitro ESR Measurement of •OH Generation and its Relation to the Enhanced Signal Decay

To confirm the relation between *in vivo* decay of carbamoyl-PROXYL and •OH generation in stomach, •OH was generated using Fenton reaction by mixing $FeSO_4$ with hydrogen peroxide in 0.01 N HCl (pH 2.0). As shown in Fig. 7C with dark arrows, typical ESR signal of DMPO-OH was observed, confirming •OH generation in the solution. In the absence of DMPO, the *in vitro* carbamoyl-PROXYL signal was decreased with time after mixing, and the decay rate constant was 0.026/min (Fig. 7A). The presence of DMPO distinctly suppressed the *in vitro* signal decay. The rate constant was 0.002/min at 1 mM DMPO, and DMPO-OH signal was hardly observed (Fig. 7B). At 1 M DMPO, the *in vitro* signal decay was not occurred (Fig. 7C). The *in vitro* signal decay was suppressed as DMPO increased from 10 μM to 1 mM (data not shown), indicating that the minimum amount of •OH observable using *in vivo* ESR spectrometer and carbamoyl-PROXYL is semi-quantitatively estimated to be that of •OH trapped by 10 μM DMPO. The enhancement of *in vivo* signal decay in NH_4OH-treated stomach was within the range from 0.014 /min to 0.020 /min as shown in Fig. 3 and Table I. These facts suggested that the amount of •OH generated in stomach of

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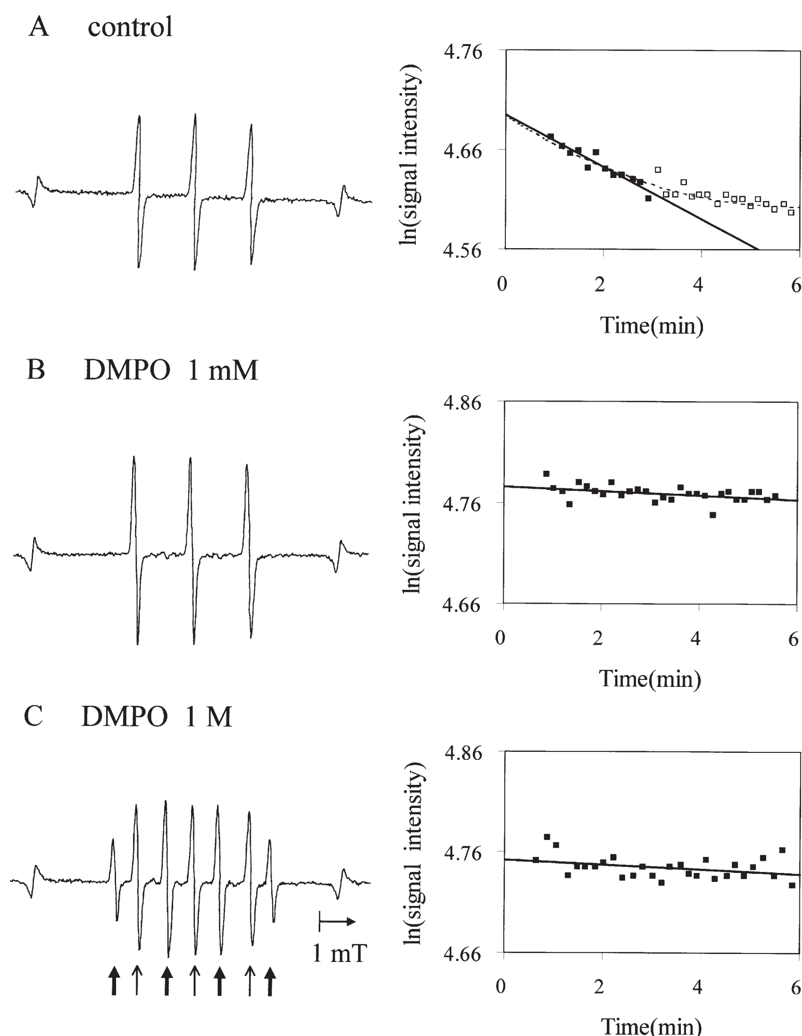

FIGURE 7 Typical ESR spectra (left panel) and the *in vitro* decay curve of carbamoyl-PROXYL signal (right panel) in the mixture of Fenton reagents, carbamoyl-PROXYL and various amount of DMPO (A; 0 mM, B; 1 mM and C; 1 M). Arrows, ↑ and ↑, in (C) indicate the triplet carbamoyl-PROXYL signal and the quartet DMPO-OH signal, respectively. ·OH was generated using Fenton reaction by mixing $FeSO_4$ (2 μM) with hydrogen peroxide (28.8 μM) in 0.01 N HCl (pH 2.0). The amount of ·OH was estimated from the signal intensity of DMPO-OH adduct, and the contribution of ·OH to the signal decay of nitroxyl radical was confirmed by adding carbamoyl-PROXYL (10 μM) to the Fenton solution. The relation of nitroxyl signal decay with ·OH generated was semi-quantitatively determined with the inhibitory effect of nitroxyl signal decay by various amount of DMPO (1, 10, 50, 100 μM, 1, 100 mM, or 1 M).

NH_4OH-treated rat is semi-quantitatively estimated to be lower than that of •OH trapped by 1 mM DMPO.

Contribution of Neutrophils and Endothelial Cells to Free Radical Reactions

When neutrophils, an important source of free radicals, were depleted by the intraperitoneal administration of ANS, the enhanced signal decay was diminished and gastric lesion formation tended to be suppressed (Fig. 8), suggesting the involvement of neutrophil in •OH generation in stomach.

In order to examine whether ROS are involved in neutrophil infiltration, the effect of antioxidants on neutrophil infiltration was measured. The intravenous injection of SOD or catalase suppressed neutrophil infiltration, while DFO, mannitol and carbamoyl-PROXYL did not (Table II). The intragastric administration of the antioxidants did not influence on the neutrophil infiltration. The oral administration of catalase to control rats did not inhibit the MPO activity (data not shown) in the present experiments. All facts suggest that O_2^- and H_2O_2 in blood vessels is involved in neutrophil infiltration.

The involvement of xanthine oxidase, which exists mainly locally in endothelial cells, was confirmed by using allopurinol, which is a xanthine oxidase inhibitor (Table III). Allopurinol suppressed neutrophil infiltration to the control level, and also suppressed both the enhanced signal decay and gastric lesion formation, indicating that endothelial cells contribute to •OH generation in stomach and

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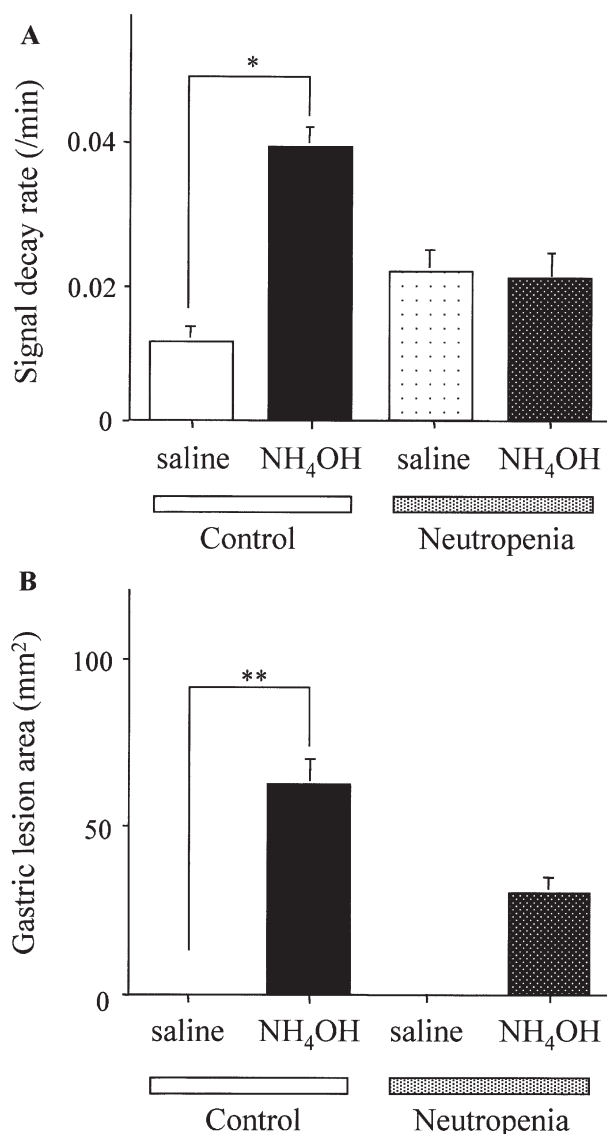

FIGURE 8 Effect of ANS pretreatment on signal decay (A) and gastric lesion area (B). NS (normal) or ANS (neutropenia) were injected intraperitoneally 24 h before lesion induction, and the signal decay rate and gastric lesion area were measured 30 min after induction. Each value represents the mean ± S.E. of 5–6 rats. Stastical analyses were carried out using two-way analysis of variance, followed by Tukey–Kramer test as a *post hoc* test. $*p < 0.05$, $**p < 0.01$ compared with control (vehicle) group.

hence lesion formation through enhanced neutrophil infiltration.

DISCUSSION

This non-invasive study using *in vivo* 300 MHz ESR spectrometry and NH_4OH-induced gastric lesion model of *H. pylori*-related disease provided the first direct evidence that •OH produced in the stomach from $O_2^{\bullet -}$ derived from neutrophils caused gastric lesion formation, while $O_2^{\bullet -}$ or H_2O_2 generated by

TABLE II Effect of antioxidants on neutrophil infiltration

	OD 460 nm	
(a) intravenous injection		
Control (vehicle)	0.88 ± 0.06	1.11 ± 0.06
NH_4OH (vehicle)	1.84 ± 0.07***	2.12 ± 0.09***
+SOD	1.21 ± 0.03+	
+catalase	1.34 ± 0.07+	
+DFO		2.15 ± 0.16
+mannitol		1.96 ± 0.05
+carbamoyl-PROXYL		2.23 ± 0.05
(b) intragastric administration		
Control	1.04 ± 0.07	1.28 ± 0.02
NH_4OH (vehicle)	1.92 ± 0.05***	2.13 ± 0.06***
+SOD	1.87 ± 0.14	
+catalase	1.80 ± 0.08	
+mannitol		2.00 ± 0.08
+carbamoyl-PROXYL		2.13 ± 0.06

Antioxidants were injected intravenously (a) or intragastrically (b) 5 min before lesion induction. Gastric lesions were induced by the intragastric administration of 900 mM NH_4OH. Values represent the mean ± S.E. of 4–10 rats. *** $p < 0.005$ as determined by Student's *t* test when compared with the control (vehicle) group and $^+p < 0.05$ as determined by Dunnett test when compared with the NH_4OH (vehicle) group.

endothelial cells was involved in neutrophil infiltration.

The nitroxyl probe is reported to react with •OH or carbon radicals or with $O_2^{\bullet -}$ in the presence of thiol, causing loss of the paramagnetism of the probe.[29] The loss of paramagnetism is observed as enhanced signal decay in experimental diseases such as hyperoxia,[19] ischemia-reperfusion,[19] liver damage induced by CCl_4,[20] streptozotocin-induced diabetes,[21] iron-overload[22] and X-irradiation.[24] This enhancement is suppressed by antioxidants such as catalase and SOD.[20–22,24] These facts indicate that the combination of our *in vivo* ESR technique with a nitroxyl probe is a very effective way of analyzing ROS generation in disease.

It was confirmed that the gastric lesions on the glandular stomach induced by NH_4OH were caused directly by •OH generated from H_2O_2 via a Fenton-like reaction, because lesion formation was suppressed by pretreatment with an •OH scavenger or a substance that blocked Fenton-like reactions.

TABLE III Effect of allopurinol on neutrophil infiltration, gastric lesion formation and the enhanced signal decay

	OD 460 nm	Lesion area (mm^2)	Signal decay (/min)
control (vehicle)	1.19 ± 0.11	0	0.012 ± 0.003
+ allopurinol	1.14 ± 0.04	0.2 ± 0.1	0.011 ± 0.002
NH_4OH (vehicle)	2.19 ± 0.05*	33.6 ± 2.1*	0.035 ± 0.003
+allopurinol	1.28 ± 0.29**	10.5 ± 1.5**	0.013 ± 0.002

Rats were given allopurinol 5 mg/day for 7 days with a final dose 2 h before the experiment (total dose: 40 mg/rat). Gastric lesions were induced by the intragastric administration of 900 mM NH_4OH and MPO activity, gastric lesion area or signal decay rate was assessed 30 min after lesion induction. Values represent the mean ±S.E. of 3–6 rats. Stastical analyses were carried out using two-way analysis of variance, followed by Tukey–Kramer test. $*p < 0.01$ compared with control (vehicle) group, and $**p < 0.01$ compared with NH_4OH (vehicle) group.

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The *in vivo* •OH formation from H_2O_2 through a Fenton-like reaction was confirmed by the enhanced ESR signal decay of carbamoyl-PROXYL after intragastric administration in the NH_4OH-treated group and by suppression of the enhanced signal decay with the simultaneous administration of mannitol, catalase, or DFO. These results agree well with previous *in vitro* observations that the paramagnetism of the nitroxyl radical was diminished very quickly by direct reaction with •OH ($3-4 \times 10^{11}$/M per min).[32]

If the enhanced signal decay really reflects •OH generation, the nitroxyl probe should be a competitive inhibitor in NH_4OH-induced mucosal injury. We knew that the nitroxyl probe reacted with •OH in NH_4OH-induced gastric lesions, because lesion formation was almost completely suppressed by pretreatment with carbamoyl-PROXYL. The protective effect of the nitroxyl probe concurred with previous reports in HCl-, ethanol- and NSAID-induced gastric lesions.[33]

Enhanced signal decay was not observed in neutropenic rats, indicating that •OH, which enhances signal decay, is derived mainly from neutrophils that have infiltrated the gastric mucosa. These results strongly demonstrate that this *in vivo* ESR method with a nitroxyl probe can monitor radical reactions in the stomach. Using this method, we succeeded, for the first time, in providing direct evidence of •OH generation from neutrophils in the stomach of rats with NH_4OH-induced gastric mucosal lesions. It is noteworthy that the signal decay did not change when carbamoyl-PROXYL was injected intravenously. This made it clear that in stomachs with NH_4OH-induced lesions •OH was generated on the luminal side of the gastric mucosa, and not on the vascular side.

Neutrophil infiltration occurs through a complicated process involving the expression of adhesion molecules on endothelial cells or neutrophils, and ROS are involved in this process *in vivo*[2–4] and *in vitro*.[5] In our study, neutrophil infiltration in the gastric mucosa was almost completely suppressed by the intravenous injection of SOD or catalase, while the intragastric administration of these antioxidants had no effect. Pretreatment with allopurinol, an inhibitor of xanthine oxidase found in endothelial cells, almost completely inhibited both neutrophil infiltration and the enhanced signal decay. These results indicate that $O_2^{\bullet -}$ or H_2O_2 produced by endothelial cells indirectly induces the •OH generation and lesion formation by controlling neutrophil infiltration. On the other hand, DFO, mannitol and carbamoyl-PROXYL treatment did not affect neutrophil infiltration. Similar results have also been reported in the intestinal ischemia model[3] and dermal vasculitis.[4] •OH is probably not involved in neutrophil infiltration *in vivo*.

It remains unclear why ROS from the xanthine oxidase system do not affect the signal decay of intravenous carbamoyl-PROXYL. It is probably a result of difference in the amount of ROS production. *In vitro* experiments showed that neutrophils produce approximately 3.5×10^5 nmol of $O_2^{\bullet -}$/min 10^6 cells after activation with PMA,[34] opsonized zymozan[34] or arachidonic acid,[35] while about 2×10^2 nmol/min/10^6 cells is produced in lung endothelial cells.[36] ROS from endothelial cells might be detectable, if the sensitivity of *in vivo* ESR spectroscopy were improved 10^3 times.

The present study provides, for the first time, non-invasive method to determine the kind and origin of ROS generation in gastric mucosal injury of living arts, and direct evidence that •OH which caused gastric lesion formation was produced through Fenton-like reaction from $O_2^{\bullet -}$ derived from neutrophils in the stomach in a NH_4OH-induced gastric lesion rats, while $O_2^{\bullet -}$ or H_2O_2 derived from the xanthine oxidase system in endothelial cells was involved in neutrophil infiltration.

Quite recently, we determined the generation of ROS from mitochondrial electron transport complex I in the failing myocardium,[37] and also the protection of cardiac myocytes against oxidative injury by free radical scavenger.[38] The present non-invasive method can be utilized widely for animal disease model to investigate the mechanism of oxidative injuries and the *in vivo* effect of antioxidant drugs, even though sample size is limited within 80 mm o.d. It may have large potential as a new diagnostic system for various oxidative injuries in human, if much wide-range resonator is improved.

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