Free Radical Research, May 2009; 43(5): 505–513

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healthcare

Non-invasive monitoring of redox status in mice with dextran sodium sulphate-induced colitis

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(Received 12 December 2008; revised 4 March 2009)

Abstract

Change of redox status is associated with colitis induced by dextran sodium sulphate (DSS). This study monitored redox status in DSS-induced colitis in mice using *in vivo* electron spin resonance (ESR) spectroscopy with nitroxyl probes. Colitis was induced in male ICR mice by supplementing their drinking water with 3% DSS for 3, 5 or 7 days. The ESR signal decay rate of carbamoyl-PROXYL administered into the rectum was enhanced by DSS treatment and the enhancement on day 7 was suppressed by membrane-permeable antioxidants, tiron and dimethylsulphoxide and a membrane-impermeable antioxidant, mannitol. The enhancement on day 5 was suppressed by tiron and dimethylsulphoxide, while that on day 3 was inhibited only by tiron. These results suggest that redox change occurs in or around of epithelial cells on day 7, but only intracellularly on day 5, and that redox change such as generation of less reactive radicals occurs only intracellularly on day 3.

Keywords: *Colon, electron spin resonance, nitroxide, Reactive Oxygen Species (ROS), redox status*

Abbreviations: *CD, Crohn's disease; DFO, desferrioxamine; DMSO, dimethylsulphoxide; DSS, dextran sodium sulphate; ESR, electron spin resonance; IBD, inflammatory bowel diseases; iNOS, inducible nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PROXYL, 2, 2, 5, 5-tetramethyl-pyrrolidine-1-oxyl; ROS, reactive oxygen species; TEMPO, 2, 2, 6, 6-tetramethyl-piperidine-1-oxyl; TNBS, trinitrobenzene sulphonic acid; UC, ulcerative colitis*

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are representative chronic inflammatory bowel diseases (IBD) of unknown cause. In patients with UC, inflammation occurs selectively at the rectal colon and expands continuously into the large intestine. On the other hand, in patients with CD, the inflammation occurs discontinuously in the gastrointestinal tract. Patients with UC show the cycles of remission and relapse with symptoms of bloody diarrhoea, weight loss, anaemia and rectal bleeding [1]. Several factors, including intestinal bacterial flora, abnormality in the local immune system of the colonic mucosa and environmental factors in genetically susceptible individuals may participate in the pathogenesis of IBD [2]. Dextran sodium sulphate (DSS) challenge in rodents is widely used as a model to study the pathology of UC, because the DSS-induced colitis, which includes colonic inflammation with ulceration, body weight loss and bloody diarrhoea during the acute phase, resembles human UC [3].

Change of redox status including excessive generation of reactive oxygen species (ROS) is reportedly associated with the pathogenesis of IBD [4–7].

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760902883036

Luminol-dependent chemiluminescence, generated by the stimulation of formyl-methionyl-leucyl-phenylalanine, is enhanced in the polymorphonuclear cells of patients with active UC compared with controls, indicating increased ROS generation [4]. In addition, luminol-dependent and lucigenin-dependent chemiluminescence in colorectal mucosal biopsy specimens increase in active UC [5]. In experimental animals with DSS-induced colitis, an increase in urinary 8-hydroxydeoxyguanosine excretion is strongly associated with increased bowel myeloperoxidase activity, which is an index of neutrophil infiltration and the severity of epithelial injury [6]. In a CD model induced by trinitrobenzene sulphonic acid (TNBS), the pre-treatment with superoxide dismutase or catalase reduces the macroscopic damage score and inhibits the luminol-dependent chemiluminescence 6 days after the induction of colitis [7].

Nitroxyl radicals are valuable tools for non-invasive assessment of the redox status involved in pathological conditions *in vivo*. These agents, being effective antioxidants *in vivo* as well as sensitive imaging probes, have the ability to non-invasively assess redox status in various pathological conditions that are mediated by oxidative stress. The *in vivo* electron spin resonance (ESR)/nitroxyl probe technique has been successfully applied to the examination of redox status *in vivo* under various physiological conditions and experimental diseases [8–26], such as NH_4OH-induced [19], water immersion restraint-induced [24] and indomethacin-induced [26] gastric mucosal lesions. The *in vitro* ESR measurement of three nitroxyl probes with different membrane permeabilities: carboxy-PROXYL, methoxycarbonyl-PROXYL and carbamoyl-PROXYL in liposomes showed that carboxy-PROXYL, methoxycarbonyl-PROXYL and carbamoyl-PROXYL localize to the aquatic phase, the lipidic phase and the interface between the aquatic and lipidic phases, respectively [18]. This ability to localize the imaging probe or antioxidant is an additional advantage of the *in vivo* ESR/nitroxyl probe technique for the non-invasive and real-time evaluation of redox status in living animals.

In this study, redox status in mice with DSS-induced colitis was directly investigated using *in vivo* ESR spectroscopy and nitroxyl probes.

Materials and methods

Chemicals

3-Carbamoyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (carbamoyl-PROXYL), 3-carboxy-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (carboxy-PROXYL) and urethane were purchased from Aldrich Chemical Co. (Milwaukee, WI). Mannitol, DSS, dimethylsulphoxide (DMSO) and Alcian Blue 8GX were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Tiron and catalase were obtained from Sigma Chemical Co. (St. Louis, MO). Desferrioxamine (DFO) was from Novartis Pharma (Tokyo, Japan). All other chemicals were commercially available and of reagent-grade quality.

Animal treatment

Male ICR mice (4 weeks old; 18–22 g body weight) were purchased from Japan SLC Co. (Hamamatsu, Japan) and acclimatized for 1 week before experimentation. Food (MF, Oriental Yeast Co. Tokyo, Japan) and water were provided *ad libitum*. Colonic mucosal lesions were induced by replacing the drinking water with tap water supplemented with 3% DSS (mol wt 5000, 15–20% sulphur content) for 3, 5 or 7 days *ad libitum*. The mice were fasted for 24 h with free access to water before experimentation.

To estimate the effect of antioxidants on colonic mucosal lesion formation, 500 μL of DMSO (0.2 mmol at a time) or mannitol (0.2 mmol at a time) was administered into the rectum once a day during DSS treatment. Antioxidants were dissolved in 0.9% saline.

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

ESR measurement

Carbamoyl- or carboxy-PROXYL (0.089 μmol/g) was administered into the rectum of mice anaesthetized by intramuscular injection of urethane (2 mg/g) and then the ESR spectra were observed *in vivo* in the colonic region with an L-band ESR spectrometer (JES-RE-3X, JEOL, Japan). The microwave power was 1 mW. The microwave frequency was 1.1 GHz. 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 signal decay rate was calculated as described previously [19].

To determine the causes of DSS-induced enhanced signal decay, DMSO (0.2 mmol), tiron (0.2 mmol), mannitol (0.2 mmol), catalase (100 U) or DFO (0.015 mmol) was administered into the rectum together with the spin probe. In each experiment, control mice were treated with distilled water as a vehicle.

Macroscopic and histological examination of colonic mucosal injury

The extent of colonic mucosal injury in mice treated with DSS was investigated macroscopically. The colon was removed and inflated with 10% formaldehyde for 30 min. The colon sample was opened longitudinally and extended by pinching with filter

paper. The sample was then fixed by immersion in 10% formaldehyde for 5 days. The fixed colon sample was washed with distilled water for 5 min, immersed in 3% acetic acid for 5 min and stained with 0.5% Alcian blue dissolved in 3% acetic acid for 10 min. The stained colon was washed with 3% acetic acid until the unbound Alcian blue had been eluted from the sample. The area of the colonic mucosa that stained strongly blue (mm^2 per colon) was determined under a dissecting microscope with a square grid micrometer.

The histologic damage of DSS-treated colonic mucosa was examined by hematoxylin and eosin (H&E) staining. The colons of mice treated with DSS for 3, 5 or 7 days were collected. The specimens were fixed overnight in 10% neutral-buffered formalin and embedded in paraffin. Sections, 5-μm thick, were cut, mounted on glass slides, dried overnight and then stained with H&E.

Statistical analysis

Statistical analyses were carried out using Stat View 5.0. The data were analysed by Student's *t*-test or by ANOVA with the Dunnett's test as a post hoc test. All the results are expressed as the mean ± SD; $p < 0.05$ was considered statistically significant.

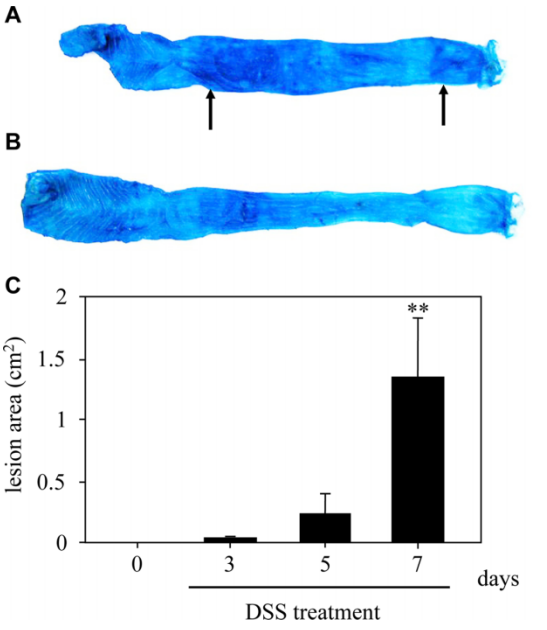

Figure 1. Alcian blue staining of colonic mucosa in mice treated with DSS for 7 days (A) and with tap water (B) and the colonic mucosal lesion area in mice treated with DSS over time (C). Mice were given free access to water containing 3% DSS for 3, 5 or 7 days. The colon was removed, fixed with 10% formalin and stained with Alcian blue. The area of the colonic mucosa that stained strongly blue was evaluated under a dissecting microscope with a square grid micrometer. The strongly blue-stained regions are indicated by arrows in (A). Each value in (C) represents the mean ± SD of 4–6 mice.

Results

The induction of colitis in mice given 3 and 5% DSS solution to drink was assessed. When 3% DSS solution was given, most of the mice developed diarrhoea on day 3 and a slightly bloody stool and anaemia on day 5. On day 7, severe bleeding from the rectum, a decrease in body weight and a shortening of the colon length were observed in almost all of the DSS-treated mice. These symptoms were similar to those seen in patients afflicted with UC [1]. However, when 5% DSS solution was given, some mice died within 7 days (data not shown). Therefore, we used a 3% DSS solution to produce colitis in this study.

Lesions stained with Alcian blue were produced in the rectal colon of the mice treated with DSS for 7 days (Figure 1A, arrows); however, no lesions were observed in the water-treated mice (Figure 1B). Evaluation of the area of the DSS-treated colonic mucosa that stained strongly blue showed that it was small for 5 days of DSS treatment and markedly increased at 7 days of treatment (Figure 1C).

The DSS-treated colonic mucosa was examined by H&E staining to investigate the histologic damage of the colonic mucosal layer. H&E staining of the colonic mucosa on day 3 showed partial disruption of the goblet cells, contraction of the epithelium thickness and lymphocyte infiltration (Figure 2B), while that of the control group showed no damage of the colonic mucosal layer (Figure 2A). On day 5, a marked disruption of goblet cells and crypts and lymphocyte and neutrophil infiltration into the lamina propria were observed (Figure 2C). On day 7, almost all the epithelium, goblet cells and crypts had disappeared and a marked infiltration of inflammatory cells, including lymphocytes and neutrophils, was seen (Figure 2D).

To evaluate the *in vivo* redox status in mice with DSS-induced colitis, a carbamoyl-PROXYL probe (0.089 μmol/g) was injected into the rectum and *in vivo* ESR measurement was carried out at the colonic domain. The typical ESR spectrum of carbamoyl-PROXYL injected into the rectum showed isotropic triplet lines (Figure 3A). The ESR signal intensity of the carbamoyl-PROXYL gradually decreased in the control group. The semi-logarithmic plot was linear for at least 12 min (Figure 3B). The signal decay rate was calculated from the decline of the signal decay curve [19]. In the DSS-treated mice, the signal decay rate on day 7 was much faster than that in the control group (Figure 3B).

To verify that the enhanced signal decay of the carbamoyl-PROXYL probe on day 7 was mediated by change of redox status, the selected antioxidants were administered to the DSS-treated mice together with the spin probe and the decay rates in the colon were determined by *in vivo* ESR spectroscopy. Tiron

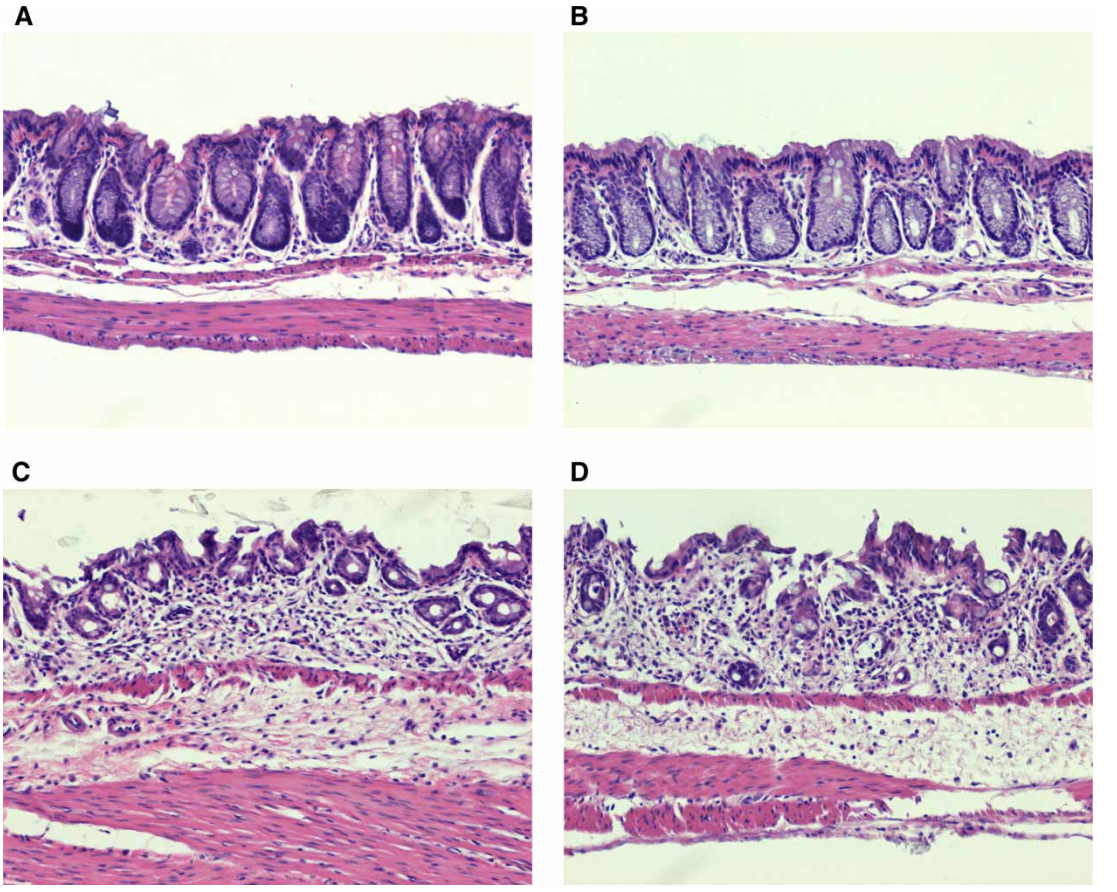

Figure 2. Hematoxylin and eosin (H&E) staining of colon tissues from the control (A), 3-day DSS (B), 5-day DSS (C) and 7-day DSS (D) groups. Colon tissues of mice treated with DSS for 3, 5 or 7 days were collected and slides with 5-μm-thick serial sections of paraffin-embedded tissues were stained with H&E (×50).

(0.2 mmol), which is a membrane-permeable antioxidant, suppressed the enhanced signal decay to the level of the control group (Figure 4A). In addition, DMSO (0.2 mmol), which is another membrane-permeable antioxidant, diminished the enhanced signal decay 50% (Figure 4A). Mannitol (0.2 mmol), which is a membrane-impermeable antioxidant, tended to suppress the enhanced signal decay, but it was not significant (Figure 4A). Catalase and DFO, which is a metal chelator that inhibits the Fenton reaction, also significantly suppressed the enhanced signal decay on day 7 (data not shown). These findings indicate that the enhanced signal decay was due to the change of redox status including the generation of reactive oxygen species such as $O_2{\cdot}^-$, ·OH and secondary radicals derived from these species at the surface of the cell membrane and the epithelium. To investigate the relationship between the change of redox status and the initiation and/or the progression of DSS-induced colitis, the signal decay rates after the shorter periods of DSS treatment were also evaluated. On day 3, the signal decay rate was enhanced by 45%, although this enhancement was not statistically significant (Figure 4B). On day 5, the signal decay rate was enhanced by 70% compared with the control group and was statistically significant (Figure 4B) ($^\star p < 0.05$).

To verify that the enhanced signal decay on days 3 and 5 was mediated by the change of redox status, the specific antioxidants were administered to the DSS-treated mice together with the spin probe, as above for day 7, and the decay rates were determined. On day 5, as on day 7, tiron (0.2 mmol) completely suppressed the enhanced signal decay and DMSO (0.2 mmol) significantly suppressed it, by 87%. However, mannitol (0.2 mmol) had little effect on the enhanced signal decay (Figure 5A). These results suggest that the change of redox status including the generation of highly reactive oxygen species occurs at the surface of the cell membrane and/or the intracellular space of the epithelium and is consistent with the enhanced signal decay observed by *in vivo* ESR. On day 3, the simultaneous administration of tiron with the spin probe suppressed the enhanced signal decay to the level of the control group (Figure 5B). The suppressive effect of tiron on the enhanced signal decay was dose-dependent (0.076 ± 0.022 min^{-1} with 0.05 mmol, 0.073 ± 0.041 min^{-1} with 0.1 mmol and 0.041 ± 0.014 min^{-1} with 0.2 mmol). However, the co-administration of DMSO or mannitol with the spin probe showed no inhibitory effect

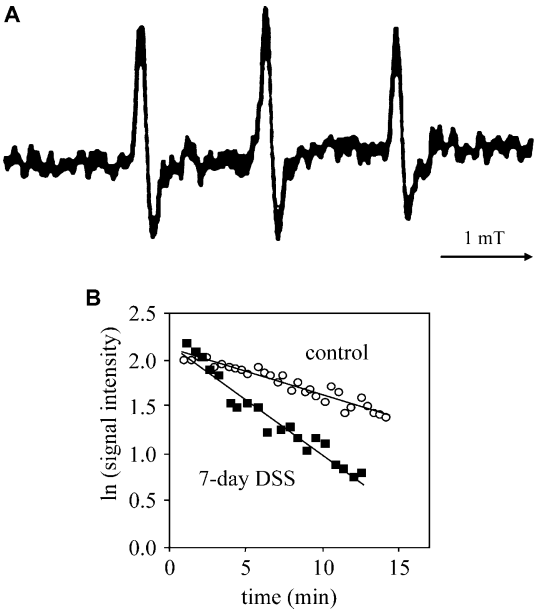

Figure 3. Typical ESR spectrum of carbamoyl-PROXYL in the colonic domain after its administration into the rectum (A) and the signal decay curve of the 7-day DSS group (■) and the control group (○) (B). The carbamoyl-PROXYL solution (0.089 μmol/g) was administered into the rectum of a mouse treated with tap water or with DSS for 7 days and the ESR spectra were measured in the colonic region by L-band ESR spectroscopy. The logarithm of the peak height at the centre magnetic field $h(0)$ was plotted against the time after probe administration and the signal decay rate was calculated from the decline of the signal decay curve, as described previously [19].

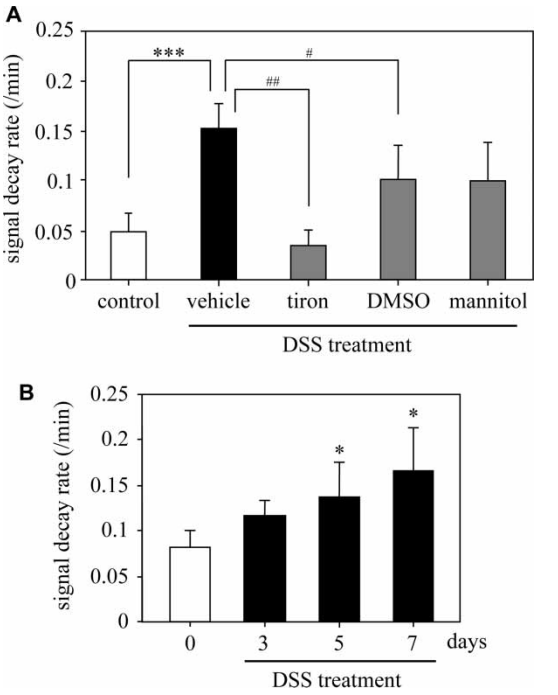

Figure 4. Effect of antioxidants on the enhanced signal decay of carbamoyl-PROXYL on day 7 (A) and the time course of the signal decay rate of carbamoyl-PROXYL on days 3, 5 and 7 in mice with DSS-induced colitis (B). Seven days after the onset of DSS treatment, 500 μL of 400 mmol/L tiron, DMSO or mannitol was mixed with 100 μL of 25 mmol/L carbamoyl-PROXYL and the mixture was then administered into the rectum. No direct chemical reactions were observed between the nitroxyl probe and the antioxidants used. Immediately after administration of the nitroxyl probe, the *in vivo* ESR measurement was performed. Each value represents the mean ± SD of 3–8 mice. $^{\star}p<0.05$, $^{\star\star\star}p<0.005$ as determined by Student's *t*-test when compared with the control group and $^{\#}p<0.05$, $^{\#\#}p<0.01$ as determined by Dunnett's test when compared with the DSS group treated with 0.9% saline as a vehicle.

on day 3 (Figure 5B). These results suggest that the change of redox status including the generation, not of highly reactive oxygen species, but of other, less-reactive species at the surface of the cell membrane and/or in the intracellular space of the epithelium contributed to the enhancement of signal decay on day 3.

A carboxy-PROXYL probe, which is membrane-impermeable, was administered into the rectum of DSS-treated mice and the decay rates were calculated. The signal decay rates on days 3 and 5 were unchanged, while those on day 7 were significantly enhanced, by 70% (Figure 5C) ($^{\star}p<0.05$). These results indicate that the enhanced signal decay of the nitroxyl probes on days 3 and 5 occurred not in the extracellular space of the epithelium but at the surface of the cell membrane and/or in the intracellular space of the epithelium; on day 7, however, it occurred at the surface of the cell membrane and in both the intracellular and extracellular spaces of the epithelium. In addition, the finding that membrane-impermeable protectors also suppressed the enhanced signal decay on day 7 suggests that significant transformation or modification of the membrane integrity may have occurred.

To clarify the relation between the degree of mucosal damage and the signal decay rate, all the paired data of colonic mucosal lesion area that is shown in Figure 1B and the corresponding signal decay rate of carbamoyl-PROXYL that is shown in Figure 4A were described in Figure 6. On day 3, the signal decay rate of carbamoyl-PROXYL is enhanced, but lesion area in colonic mucosa is hardly observed, suggesting that the enhanced signal decay rate of carbamoyl-PROXYL occurs before colonic mucosal lesion formation during DSS treatment.

To investigate the relationship between the changes of redox status *in vivo* evaluated by this technique and lesion formation, it was important to evaluate the suppressive effect of the antioxidants on the DSS-induced lesion formation. Tiron, DMSO and mannitol were administered into the rectum once a day during 7 days of DSS treatment (0.2 mmol per administration) and the formation of colonic mucosal lesions was evaluated. Tiron, which suppressed the enhanced signal decay on days 3, 5 and 7, decreased the area of the colonic lesions to 46% of that in the saline group (Table I). The administration of DMSO, which suppressed the enhanced signal decay on days 5 and 7, also reduced the lesion area to 36% of that in

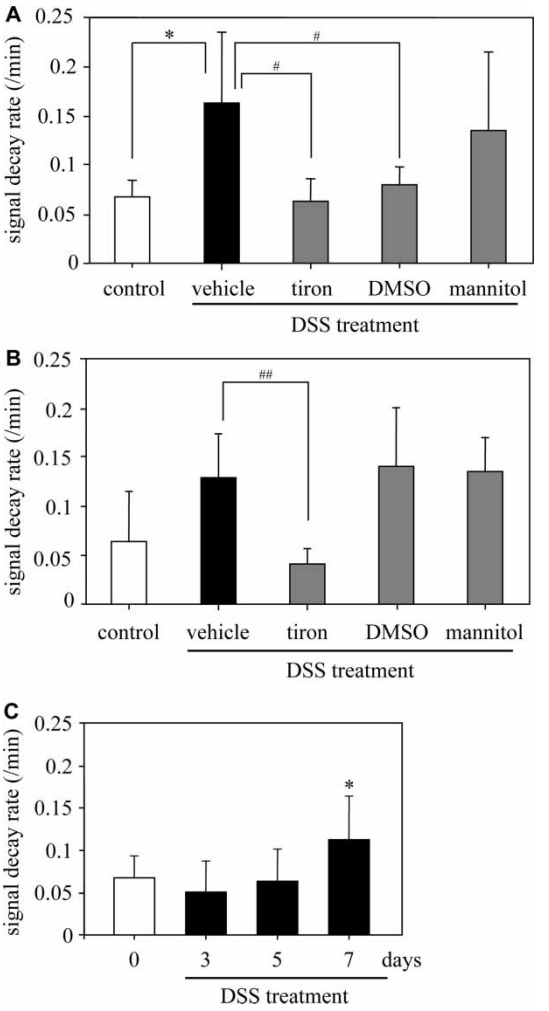

Figure 5. Effect of antioxidants on the enhanced signal decay of carbamoyl-PROXYL on day 5 (A) and on day 3 (B) and the time-course of the signal decay rate of carboxy-PROXYL during DSS treatment (C). In (A) and (B), 5 or 3 days after the onset of DSS treatment, 500 μL of 400 mmol/L tiron, DMSO or mannitol was mixed with 100 μL of 25 mmol/L carbamoyl-PROXYL and the mixture was administered then into the rectum. No direct chemical reactions were observed between the nitroxyl probe and the antioxidants used. Immediately after administration of the nitroxyl probe, the *in vivo* ESR measurement was performed. In (C), carboxy-PROXYL solution (0.089 μmol/g) was administered into the rectum of a mouse treated with DSS for 3, 5 or 7 days and then the *in vivo* ESR measurement was carried out at the colonic domain. Each value represents the mean ± SD of 3–9 mice. $^{\star}p < 0.05$ as determined by Student's *t*-test when compared with the control group and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ as determined by Dunnett's test when compared with the DSS group treated with 0.9% saline as a vehicle.

the saline group (Table I). Mannitol, which suppressed the enhanced signal decay on day 7 only, moderately prevented lesion formation, reducing it to 59% of the lesioned area in the saline group (Table I). These findings suggest that the normalization of redox change in the intracellular space of the epithelium was important for suppressing the DSS-induced formation of colonic mucosal lesions and that the enhanced signal decay observed with the *in vivo* ESR/nitroxyl probe technique reflected the change of redox status contributed to the DSS-induced colitis.

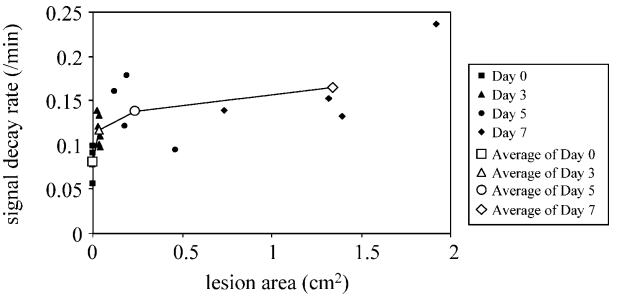

Figure 6. The relation between the colonic mucosal lesion formation and the signal decay rate. Each plot represents the paired data of lesion area and the corresponding signal decay rate of carbamoyl-PROXYL.

Discussion

The changes of redox status *in vivo*, including generation of ROS and free radicals, have been implicated in DSS-induced colitis [4–7] and here we applied the *in vivo* ESR/nitroxyl probe technique, which provides real-time, non-invasive information on the *in vivo* redox status, to the monitoring of redox status in this model. Our results from non-invasive ESR examinations using nitroxyl probes, which are both antioxidants and imaging probes, provide the first *in vivo* evidence that the change of redox status including the generation of highly reactive oxygen species such as $O_2\cdot^-$ and ·OH occurs in the large intestine of mice with DSS-induced colitis and that the degree and the location of redox reactions *in vivo* change with the progression of the colitis. In these experiments, enhanced signal decay of the probe, which is suppressed by antioxidants [14–19,21,22,24,26], was used as an index of free radical generation. Additionally, by using antioxidants with different scavenging capabilities and localizing characteristics, we obtained information about the reactivities and sites of generation as a function of DSS treatment.

Recently, an *in vivo* ESR study using carbamoyl-PROXYL administered into the rectum of mice with colitis induced by TNBS, which is a widely used experimental model of CD, was reported by Togashi et al. [27]. The authors reported that the ESR signal decay of carbamoyl-PROXYL in the TNBS-treated group decreased and then recovered, which paralleled the change in sulphydryl compounds in the injured colonic mucosa. However, we observed the enhanced signal decay of carbamoyl-PROXYL in the DSS-treated group. Both oral administration of DSS and intrarectal administration of TNBS can induce acute and chronic mucosal lesions in colitis, but some pathophysiological mechanisms and immunological processes of DSS-induced colitis (UC-like) are different from those of TNBS-induced colitis

Table I. Effect of tiron (A), DMSO and mannitol (B) on DSS-induced lesion formation.

		DSS (A)		DSS (B)		
	Control	Vehicle	Tiron	Vehicle	DMSO	Mannitol
Lesion area (cm^2)	0 ± 0 (4)	0.45 ± 0.34* (5)	0.21 ± 0.19 (6)	1.02 ± 0.28* (4)	0.37 ± 0.27# (5)	0.6 ± 0.46 (4)

At the same time of day during the 7 days of DSS treatment, 500 μL of tiron (0.2 mmol per dose), DMSO (0.2 mmol per dose) or mannitol (0.2 mmol per dose) was administered into the rectum. Antioxidants were dissolved in 0.9% saline. Seven days after DSS treatment, the area of mucosal lesions was measured under a dissecting microscope with a square grid micrometer. The data of (A) and (B) were from two individual experiments. The mean of lesion area in the vehicle group was varied from 0.45–1.02 through the experiment using this DSS-induced colitis model. Each value represents the mean ± SD of 4–6 mice. *$p < 0.05$, compared with the control group and #$p < 0.05$, compared with the DSS group treated with 0.9% saline as a vehicle. The number in parenthesis indicates the number of trials.

(CD-like). For example, DSS-induced colitis is the result of a change in epithelial barrier function [28], whereas TNBS-induced colitis develops as a delayed-type hypersensitivity reaction to haptenized proteins [29] and some up-regulated or down-regulated gene expressions in DSS-induced colitis are different from those of TNBS-induced colitis [30]. Furthermore, multidrug resistance protein-1, which is one of the active transporters of anti-cancer drugs, widely expressed in the intestinal epithelium and on T lymphocytes [31], has a protective role in cellular defense against oxidative stress responses [32], protects against DSS-induced colitis but has little effect on TNBS-induced colitis [33]. Such differences might be involved in the differences of *in vivo* redox changes in mice with DSS-induced colitis from those in mice with TNBS-induced colitis, leading to the difference of results observed with the *in vivo* ESR/nitroxyl probe technique.

In this study, mucosal lesions on the 7-day DSS group were observed in the medial and distal colon, as shown in Figure 1A. After a single cycle of 4% DSS administration (mol wt 36 000–44 000) to male Sprague-Dawley rats, the colonic mucosa appears oedematous and haemorrhagic erosions are scattered along the entire colon; however, histological observation reveals the typical histological alterations of colitis to be more prevalent on the left side, with minor lesions on the transverse and right side of the colon [34]. Furthermore, toll-like receptor 4 and CD14 mRNA are up-regulated during DSS-induced inflammation in the distal colon and toll-like receptor 2 mRNA is up-regulated in the proximal colon, but the DSS-induced inflammation was localized in the distal colon [35]. Toll-like receptors are transmembrane proteins characterized by an extracellular domain with leucine-rich repeats and an intracellular domain homologous to the IL-1R or Toll/IL-1R (TIR) and activated TIR binds to a homologous domain in the adaptor protein MyD88 then starts a cascade that finally leads to activation of NF-κB, which is one of the most important transcription factors associated with the induction of redox imbalance and production of its related proinflammatory cytokines [36]. These reports reveal the importance of investigating the relationship between the change of redox status and lesion formation in each part of the large intestine, such as distal, medial and proximal colon. This could be achieved by using a free radical imaging system with high spatial and temporal resolution.

To clarify the relation between the degree of mucosal damage and the signal decay rate, all the paired data of colonic mucosal lesion area and the corresponding signal decay rate of carbamoyl-PROXYL were plotted. On day 3, the signal decay rate of carbamoyl-PROXYL is enhanced, but lesion area in the colonic mucosa is hardly observed. Furthermore, no epithelial damage on day 3 was observed with H&E stain. These findings suggest that the enhanced signal decay rate of carbamoyl-PROXYL occurs before the initiation of colonic mucosal macroscopic and histologic damage during DSS treatment.

In our study, the suppressive effect of antioxidants on the enhanced signal decay of carbamoyl-PROXYL differed depending on the kind of antioxidant used and the duration of DSS treatment. On day 7, the enhanced signal decay was suppressed not only by tiron and DMSO but also by mannitol, catalase and DFO. The nitroxyl probes used for the *in vivo* ESR measurement lose their paramagnetism by reacting with $\cdot OH$ [37] and $O_2\cdot^-$ in the presence of an electron donor [38]. Our observation of severe destruction of the epithelium with numerous infiltrating neutrophils and lymphocytes in the 7-day DSS-treated colon suggests that a study using the enhanced signal decay of the membrane-impermeable probe, carboxy-PROXYL, might be feasible, because the infiltrating inflammatory cells had invaded through the surface of the injured intestinal wall.

The enhanced signal decay on day 5 was inhibited by tiron and DMSO. Superoxide and H_2O_2 generated by inflammatory cells might interact with redox-active low-molecular-weight iron complexes to generate highly reactive oxygen species via the Haber-Weiss reaction or the Fenton reaction. In the injured colon, iron capable of catalysing this reaction is potentially abundant, from unabsorbed dietary iron concentrated in the foecal material and catalytic heme iron supplied by the characteristic mucosal bleeding [39]. In mice

treated with DSS for 5 days, the enhancement on day 5 might be due to the generation of highly reactive oxygen species via the Haber-Weiss reaction or the Fenton reaction or to secondary species derived from these reactions. Coincidentally, symptoms of bleeding from the rectum such as a slight bloody stool and anaemia were observed.

DSS treatment caused the enhanced signal decay of carbamoyl-PROXYL and the enhancement on day 3 was suppressed by tiron, but not by DMSO or mannitol. It is difficult to determine whether the generation of ROS contributes to the enhanced signal decay in the colon on day 3 because tiron, which is known as a superoxide scavenger, reacts not only with $O_2{\cdot}^-$ (1×10^7 mol/L·min) but also with ·OH (1×10^9 mol/L·min) [40] and acts as a chelator of metals such as Fe^{3+} or Cu^{2+} [41]. Furthermore, nitroxyl radicals might also react with some reactive nitrogen species, because they are known to protect against DNA damage induced by nitric oxide (NO), nitroxyl anion and peroxynitrite [42]. No decrease in the ESR signal of nitroxyl radicals is observed in the presence of NO [43,44]; however, several studies have shown that NO_2 reacts with hydroxy-TEMPO [45,46]. A high concentration of NO produced by inducible nitric oxide synthase (iNOS) is associated with UC [47] and the level of iNOS-derived NO is correlated with disease activity in UC [48]. Therefore, the enhancement on day 3 might be due to the change of redox status, including the generation of less reactive secondary radicals or NO in the colonic mucosa.

In vivo ESR spectroscopy with nitroxyl probes provides strong evidence for the involvement of change of redox status in DSS-induced colitis. Using scavengers directed to specific cellular compartments and nitroxyl probes with specific localizing ability, we were able to show that the time course of the injury was correlated with the reactivity of the species generated and with the sites of generation.

Acknowledgements

This work was supported by Grants-in-Aid of Research on Priority Areas, for Co-operative Research, for Developmental Scientific Research and for General Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by 21st Century COE program of Kyushu University from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by JSPS Core-to-Core Program.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on iFirst on 8 April 2009.