Oral Nitrite Ameliorates Dextran Sulfate Sodium-induced Acute Experimental Colitis in Mice

Kazuo Ohtake [a], Midori Koga [a], Hiroyuki Uchida [a], Kunihiro Sonoda [b], Junta Ito [a], Masaki Uchida [c], Hideshi Natsume [c] and Jun Kobayashi [a] *

[a] *Division of Pathophysiology, Department of Clinical Dietetics and Human Nutrition, Faculty of Pharmaceutical Science, Josai University, Saitama, Japan*

[b] *Department of Food and Nutritional Environment, College of Human Life and Environment, Kinjo Gakuin University, Nagoya, Japan*

[c] *Division of Pharmaceutics, School of Pharmaceutical Sciences, Faculty of Pharmaceutical Science, Josai University, Saitama, Japan*

* Corresponding author: Jun Kobayashi, M.D. E-mail address: junkoba@josai.ac.jp

Abstract

Inflammatory bowel diseases (IBDs) such as Crohn's disease and ulcerative colitis are chronic inflammatory disorders of the intestinal tract with excessive production of cytokines, adhesion molecules, and reactive oxygen species. Although nitric oxide (NO) is reported to be involved in the onset and progression of IBDs, it remains controversial as to whether NO is toxic or protective in experimental colitis. We investigated the effects of oral nitrite as a NO donor on dextran sulfate sodium (DSS)-induced acute colitis in mice. Mice were fed DSS in their drinking water with or without nitrite for up to 7 days. The severity of colitis was assessed by disease activity index (DAI) observed over the experimental period, as well as by the other parameters, including colon lengths, hematocrit levels, and histological scores at day 7. DSS treatment induced severe colitis by day 7 with exacerbation in DAI and histological scores. We first observed a significant decrease in colonic nitrite levels and increase in colonic TNF-α expression at day 3 after DSS treatment, followed by increased colonic myeloperoxidase (MPO) activity and increased colonic expressions of both inducible NO synthase (iNOS) and heme oxygenase-1 (HO-1) at day 7. Oral nitrite supplementation to colitis mice reversed colonic nitrite levels and TNF-α expression to that of normal control mice at day 3, resulting in the reduction of MPO activity as well as iNOS and HO-1 expressions in colonic tissues with clinical and histological improvements at day 7. These results suggest that oral nitrite inhibits inflammatory process of DSS-induced experimental colitis by supplying nitrite-derived NO instead of impaired colonic NOS activity.

Key words

inflammatory bowel diseases (IBDs), nitric oxide (NO), nitrite, dextran sulfate sodium (DSS), nitric oxide synthase (NOS), endothelial NOS (eNOS), neuronal NOS (nNOS), inducible NOS (iNOS)

Introduction

Inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis, are chronic inflammatory disorders of the intestinal tract with excessive production of cytokines, adhesion molecules, and reactive oxygen species (ROS) [1]. The etiology of IBDs is believed to involve inappropriate host responses to the complex commensal microbial flora in the gut resulting from mucosal barrier dysfunction, such as an abnormal leaky mucus layer, altered tight junction protein expression for distribution, and increased epithelial apoptosis [2].

In order to study etiology of IBDs, mouse experimental colitis induced by oral administration of dextran sulfate sodium (DSS) is widely used as a standardized colitis model [3]. Histologically, colitis induced by DSS is characterized by infiltration of inflammatory cells into the lamina propria, accompanied by lymphoid hyperplasia, focal crypt damage, and epithelial ulceration [4]. DSS has a direct toxic effect on the epithelium and destroys the mucosal barrier, allowing bacteria to come into contact with lamina propria cells [5], resulting in activation of intestinal macrophages and secreting proinflammatory cytokines such as TNF-α [6]. These innate immune dysfunctions mediated by intestinal macrophage induce excessive production of superoxide, leading to further inflammatory process and endothelial dysfunction as a result of ability of superoxide to inactivate

endogenous NO [7].

There is growing experimental evidence that nitric oxide (NO), enzymatically generated by three isoforms of nitric oxide synthase (NOS) (endothelial NOS: eNOS, neuronal NOS: nNOS, and inducible NOS: iNOS), plays an important role in the intestinal barrier and immune functions. Impaired NO production in intestine, therefore, might have a causative effect on the progression and sequelae of IBDs. Although controversy has continued as to whether the in vivo effects of iNOS-induced NO are beneficial or detrimental on the experimental colitis [8], the majority of studies using selective inhibitors of iNOS and iNOS-deficient mice have shown improvement in experimental colitis, suggesting a possible involvement of inflammatory iNOS in the progression of IBDs. On the other hand, constitutively produced NO by eNOS and nNOS appears to be a homeostatic regulator of numerous essential functions of the gastrointestinal mucosa, such as maintenance of adequate perfusion, and regulation of microvascular and epithelial permeability. The protective actions of constitutive NOS also include reduction of leukocyte adherence, inhibition of macrophage activation, and inhibition of Th1 type cytokines by inactivation of cytokine processing. These evidences suggest protective effects of NO produced by constitutive NOS on the pathogenic processes of acute colitis following barrier dysfunction.

Thus, while the association of NOS-mediated NO dynamics with pathophysiology of IBDs has been investigated in detail, exogenous NO donors are attempted to be applied to experimental colitis models as a therapeutic intervention [9,10]. Salas et al. showed that subcutaneous administration of NO donor modulates DSS-treated colitis in mice, suggesting that the beneficial

effect of NO is related to a reduction in leukocyte recruitment and subsequent proinflammatory cytokine production by inhibiting endothelial adhesion molecule expression in the early phase of this animal model [9].

Recently, an alternative pathway fundamentally different from enzymatic NOS system for NO generation was discovered in which nitrite and nitrate are reduced in vivo to form NO, exerting physiological functions in the gastrointestinal tract and cardiovascular system [11,12]. Moreover, nitrate and nitrite are thought to have therapeutic application for the diseased states where oxygen availability is reduced or NOS activity is decreased [13-15].

Based on these recent observations, we hypothesized that oral nitrite supplementation could be effective on reducing inflammatory processes in DSS-treated colitis mice, which mimics IBDs in that it induces inflammation and macrophage activation accompanied with disrupted epithelial barrier integrity [16]. Since DSS-treated colitis by 5 to 10 days in mice is considered to be an acute colitis model [17], we investigated how constitutive NOS and iNOS are implicated in the onset and progression of DSS-treated mice colitis, and also investigated the effect of oral nitrite on clinical and histological improvement following inflammatory responses including colonic TNF-a, iNOS and HO-1 expressions especially focusing on the role of NO availability in the early phase of this model.

Materials and methods

Animals

Specific pathogen-free male ICR (CD-1) mice, 7 wk old, weighing 30-35 g, from Kiwa Laboratory Animals (Wakayama, Japan) were allowed food (CE-2, CLEA Japan) and reverse osmosis (RO) water *ad libitum*, and were kept on a 12/12 h light/dark cycle with at least 7 days of local vivarium acclimatization before experimental use. All the protocols were approved by the Institutional Animal Care and Use Committee at the University of Josai Life Science Center and were consistent with the Guide for the Care and Use of Laboratory Animals published by the NIH.

Experimental procedures

Experimental colitis was induced in ICR mice [9,18] by administrating 2.5-3.0% DSS (molecular weight 50 kDa, ICN Biomedicals Inc., Costa Mesa, California) in RO water *ad libtum* over the experimental period. Sodium nitrite ($NaNO_2$) was also administered orally in RO water at 25 mmol/l *ad libtum*. As shown in Figure 1, mice were randomly divided into four groups: 1) non-treated group, 2) $NaNO_2$-treated group, 3) DSS-treated group, and 4) DSS+$NaNO_2$-treated group. DSS-treated groups were given 2.5% DSS. The first administration of $NaNO_2$ was given one day before the administration of DSS. Based on our preliminary study suggesting that DSS-treated mice drink 15% more water than DSS+$NaNO_2$-treated mice over the experimental period, DSS+$NaNO_2$-treated mice were, therefore, given 3.0% DSS to provide the same total dose of DSS between them, as evidenced in Table 1. Drinking water containing DSS and/or $NaNO_2$ was freshly prepared and replaced every evening. Fluid intake was recorded daily throughout the duration of the study.

Animal body weight, presence of blood in excreta, and stool consistency were recorded daily, then assessed as disease activity index (DAI) in accordance with the method described by Murthy et al. [19]. In brief, each score in DAI was determined as the extent of body weight loss (0, < 1%; 1, 1-5%; 2, 6-10%; 3, 11-15%; 4, >15%), stool consistency (0, normal; 2, loose; 4, diarrhea), and stool haemoccult positivity or gross bleeding (0, normal color; 2, reddish color; 4, bloody stool) and combined and divided by 3 for each mouse.

In order to examine unfavorable effects of oral nitrite on blood pressure, systolic blood pressure (SBP) as well as heart rate (HR) was measured at day 3 and 7 using a noninvasive computerized tail-cuff method (BP98A; Softron, Tokyo, Japan).

At day 3 or 7, the mice were sacrificed under pentobarbital anesthesia (60 mg/kg), and blood samples (about 0.5 ml) were collected via the abdominal aorta into plastic tubes containing sodium EDTA. Hematocrit (Ht) and total hemoglobin (Hb) were analyzed using an automatic cell counter (Celltac MEK-5208, Nihon Kohden, Japan). The analysis of methemoglobin (Met Hb) in blood was performed using manual spectrophotometric assays based on the observation that Met Hb with absorption peak of 635 nm at pH 6.6 disappears after the conversion of Met Hb to cyan-Met Hb by neutralized cyanide [20]. Plasma samples were prepared by centrifugation at 12,000 g for 5 min at 4 ºC and stored at -80 ºC until use. The colon was excised and washed with ice-cold PBS and its length was measured after exclusion of the cecum. The colon samples were snap-frozen in liquid nitrogen and stored at -80 ºC until use, or fixed in 10% neutral buffered formalin solution.

Histological analysis

The colon was fixed in 10% neutral buffered formalin solution and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (HE) for light microscopic observation. Eight randomly selected fields (magnified 100 times) in each section were graded and averaged according to the method described by Cooper et. al. [21], the severity of mucosal injury was graded as follows: grade 0, normal colonic mucosa; grade 1, loss of 1/3 of the crypts; grade 2, loss of 2/3 of the crypts; grade 3, the lamina propria is covered with a single layer of epithelium and mild inflammatory cell infiltration is present; grade 4, erosions and marked inflammatory cell infiltration are present. Digital images were obtained from a high-resolution digital camera system (Penguin 150CL, Pixera, Los Gatos, CA, USA) linked to a microscope (BX41, Olympus, Tokyo, Japan) and desktop computer (Pentium 4, 2.0 GHz).

Nitrite and nitrate concentrations in colon and plasma

Nitrite concentration in the colon and the plasma was measured using a dedicated HPLC system (ENO-20; EiCom, Kyoto, Japan) [22]. This method is based on the separation of nitrite and nitrate by ion chromatography, followed by on-line reduction of nitrate to nitrite, postcolumn derivatization with Griess reagent, and detection at 540 nm. Proteins in each sample were removed by centrifugation at 10,000 g for 5 min following methanol precipitation (colon : methanol = 1 : 2 weight/volume, plasma : methanol = 1 : 1 volume/volume, 4 ºC).

Myeloperoxidase (MPO) activity

MPO activities in the colonic tissues were measured according to the technique described by Bradley et al. [23]. The results are expressed as MPO units/g wet tissue; 1 unit of MPO activity was defined as that degrading 1 μmol hydrogen peroxide/min at 25 ºC.

Western blot analysis

The colonic samples obtained from the mice were homogenized in 20 volumes of ice-cold lysis buffer; 25 mM HEPES/NaOH; pH 7.4, 150 mM NaCl, 4 mM EDTA, 25 mM NaF,1 mM Na_2VO_4, 0.2% (w/v) SDS and 1% (v/v) Nonidet P-40 with Complete Protease Inhibitor (Roche Diagnostics, Indianapolis, IN). The tissue homogenates were centrifuged at 12,000 g for 10 min at 4 ºC, and the supernatants were retained. Colonic mucosal lysates typically contained 8 mg protein per ml on the basis of the BCA protein assay. Protein lysates (60 μg protein/lane) were resolved by SDS-PAGE, electroblotted to PVDF membranes, and immunoblotted with selected antibodies against TNF-α (goat pAb, Santa Cruz Biotechnology, CA, USA), HO-1 (rabbit pAb, Stressgen Bioreagents, Hamburg, Germany), iNOS (rabbit pAb, BD Bioscience, CA, USA), and β-actin (mouse mAb, Santa Cruz Biotechnology, CA, USA). Bound antibody was visualized using the ECL chemiluminescence detection system (SuperSignal West Dura Extended Duration Substrate, Pierce) with HRP-conjugated secondary antibodies (Pierce). The band intensity was quantified using a Bio Imaging System, with Gene Snap and Gene Tools software (Syngene Bio Imaging, Cambridge, USA). Control of protein loading and transfer was conducted by detection of the β-actin levels.

Statistical analysis

All values are expressed as means ± SE. Data were analyzed by one- or two-way ($NaNO_2$ intake ×DSS intake) ANOVA, and then differences among means were analyzed using the Tukey-Kramer multiple comparison test. A level of $P < 0.05$ was considered significant.

Results

Characteristics of DSS-treated colitis

As described in the experimental procedures in the methods, DSS-treated mice drink 15% more water than DSS+$NaNO_2$-treated mice. Therefore, DSS+$NaNO_2$-treated mice were given 3.0% DSS to provide the same total dose of DSS between them. We calculated the average intake of DSS in DSS-treated and DSS+$NaNO_2$-treated mice (245.6 ± 28.4 mg/mouse/day and 248.6 ± 11.1 mg/mouse/day, respectively) resulting in no significant difference in DSS intake between the two groups (Table 1). No difference of average intake of nitrite was observed between $NaNO_2$-treated mice and DSS+$NaNO_2$-treated mice (6.4 ± 0.1 and 7.2 ± 0.3 mg/mouse/day, respectively) either (Table 1).

Consistent with previous report [18,24,25], mice administered 2.5-3.0% DSS developed severe colitis and suffered weight loss. Bloody stools started at day 4 and continued to the end of the experimental period. DAI consisting of parameters of body weight loss, stool consistency, and

bloody feces significantly started increasing from day 4 to day 7 in DSS-treated mice, whereas the DAI was decreased by oral $NaNO_2$ supplementation in $NaNO_2$+DSS-treated mice, compared with DSS-treated mice (Figure 2). $NaNO_2$ supplementation alone had no impact on DAI both in non-treated and $NaNO_2$-treated mice over the 7 day experimental period (Figure 2). DSS treatment also resulted in the reduction of Ht levels due to continued bloody stools, as well as colon length in DSS-treated mice at day 7, all of which were consequently restored by oral $NaNO_2$ supplementation (Figure 3).

Effects of oral $NaNO_2$ on blood pressure, heart rate, and Met Hb levels

In order to investigate unfavorable side effects of oral $NaNO_2$, blood pressure, heart rate and Met Hb were measured. Irrespective of with or without DSS treatment, the dose of oral $NaNO_2$ used in the present study had no effect on the systolic blood pressure and heart rate both at day 3 and 7 (non-treated versus $NaNO_2$-treated, DSS-treated versus $NaNO_2$+DSS-treated). Consistent with previous report [26], DSS significantly reduced systolic blood pressure irrespective of with or without $NaNO_2$ treatment (non-treated versus DSS-treated, $NaNO_2$-treated versus $NaNO_2$+DSS-treated) (Table 2). Oral $NaNO_2$ had no impact on Hb levels between non-treated and $NaNO_2$-treated mice, while DSS decreased Hb levels (non-treated versus DSS-treated) and improved with oral $NaNO_2$ (DSS-treated versus $NaNO_2$+DSS-treated), which is similar result to that of Ht levels (A in Figure 3). Thus, despite DSS-induced deleterious effects probably due to gross bloody stool and circulating volume loss resulting from poor intake and frequent diarrhea,

oral $NaNO_2$ had no clinical unfavorable effects except for more increased Met Hb formation at day 7 with $NaNO_2$ than without $NaNO_2$ (non-treated versus $NaNO_2$-treated, and DSS-treated versus $NaNO_2$+DSS-treated, respectively) (Table 3).

Histological findings of colitis

Despite normal colonic histological structures in non-treated mice and $NaNO_2$-treated mice at day 7, the colonic histology observed in DSS-treated mice at day 7 was characterized by multifocal dropouts of entire crypts in all parts of the colon, as well as a marked infiltration of inflammatory cells into the mucosa (C versus A and B in Figure 4). The histological exacerbations induced by DSS treatment at day 7 were significantly improved by additional oral $NaNO_2$ supplementation (D in Figure 4), which was definitely manifested by histological scores in Figure 4 (E).

MPO activity in colon

Induction of colitis by DSS represented a remarkable increase in MPO activity in colonic tissue at day 7, whereas additional $NaNO_2$ supplementation to DSS-treated mice significantly reduced MPO activity (Figure 5).

Nitrite and nitrate levels in colon and plasma

Because we believe that a protective effect of NO produced by constitutive NOS occurs before clinical and histological exacerbations are actualized, nitrite levels in plasma and colonic tissue,

which reflect acute changes in regional eNOS activity [27], were measured at day 3. In spite of no significant changes in plasma nitrite levels between non-treated mice and DSS-treated mice at day 3 (C in Figure 6), colonic nitrite levels were significantly decreased by DSS treatment at day 3 due to a decreased activity of the constitutive form of NOS, whereas this decrease in colonic nitrite levels recovered to the control levels of non-treated mice with oral $NaNO_2$ supplementation at day 3 (A in Figure 6). Although there are tendencies towards an increasing concentration of plasma nitrite levels due to oral $NaNO_2$ supplementation at day 7, no significant difference in plasma nitrite levels was found between the four groups (G in Figure 6). Different from plasma nitrite levels, colonic nitrite levels in DSS-treated and DSS+$NaNO_2$-treated mice are directly influenced by iNOS-derived nitrite and oral $NaNO_2$ supplementation, respectively (E in Figure 6).

With respect to nitrate levels in colon and plasma, colonic and plasma nitrate appear to be accumulating due to oxidation process from unstable nitrite to stable nitrate. At day 3, DSS treatment significantly reduced nitrate levels in colon and plasma (between $NaNO_2$-treated group and DSS+$NaNO_2$-treated group, B and D in Figure 6), while no significant reduction of colonic and plasma nitrate levels were observed at day 7 (between $NaNO_2$-treated group and DSS+$NaNO_2$-treated group, F and H in Figure 6), suggesting possible background activities of constitutive NOS (at day 3) and iNOS (at day 7).

Effects of oral $NaNO_2$ on colonic TNF-α, iNOS and HO-1 expressions at day 3 and day 7

We observed the impaired constitutive NOS activity already existing at day 3 in the colitis mice

(A in Figure 6), and also found an increased expression of colonic TNF-α, while there was no significant difference between the colonic iNOS and HO-1 expressions between non-treated control and DSS-treated mice (A, C, E, G in Figure 7). However, at day 7, significantly increased expressions of these proteins were observed after DSS treatment (B, D, F, H in Figure 7) in colitis mice, accompanied with deterioration of clinical (Figure 2) and other parameters (Figure 3, 4, 5), which was consequently almost reversed to non-treated normal control levels by oral $NaNO_2$ supplementation.

Discussion

Constitutive NO production by eNOS prevents the accumulation of platelets and adherent leukocytes, thus creating a nonthrombogenic environment in the vasculature. In the intestinal tract, small amounts of NO produced by constitutive forms of NOS (eNOS and nNOS) are thought to be physiologic and protective by regulating mucosal integrity, especially in response to noxious stimuli. Because acute DSS-induced colitis represents primarily a barrier disruption model which results in the activation of cells of the innate immune system [6], impaired constitutive NOS activities in the colon might be closely related to the progression of this experimental colitis.

Recent studies regarding the effects of eNOS on experimental colitis demonstrated that the colonic mucosal mRNA levels of eNOS were decreased after DSS administration in mice [28], and DSS treatment increased the disease activity in eNOS-deficient colitis mice [29], suggesting that

eNOS plays an important role in limiting intestinal injury during experimental colitis by preventing leukocyte recruitment and subsequent proinflammatory cytokine production, such as IL-12 and IFN-γ [30,31]. nNOS is also another source of constitutively released NO in the intestine, mediating neuronal signal transmission and regulating gut motility in rat and guinea pig [30,31]. Constitutive nNOS also has the ability to suppress iNOS expression in the intestine via down-regulation of NF-κβ. Pharmacological inhibition of nNOS causes IκBα degradation, leading to up-regulation of NF-κβ in rat [32]. This transcription factor, NF-κβ, plays an important role in inflammation by regulating the transcription of proinflammatory cytokines, adhesion molecules, and proinflammatory enzymes such as iNOS [32]. Therefore, reduced NO production by either eNOS or nNOS in the injured intestine could appear responsible for triggering acute colitis and subsequent chronic inflammation in the colon.

In our colitis model, we first observed a significant decrease in colonic nitrite levels at day 3 (A in Figure 6), presumably caused by the reduction of endogenously produced NO by constitutive NOS. At day 7, however, the colonic nitrite levels significantly increased above the control levels due to the prominent NO production by iNOS (E in Figure 6), which is evidenced in our current study by showing the increase in MPO activity (Figure 5) and iNOS expressions in the colon (B and H in Figure 7). Oral $NaNO_2$ supplementation to colitis mice reversed colonic nitrite levels to those of non-treated control mice at day 3 (A in Figure 6), subsequently resulting in clinical and histological improvements at day 7.

Concerning plasma levels of nitrite and nitrate, despite the significant difference of colonic nitrite

levels between non-treated control and DSS-treated colitis mice (A in Figure 6), we observed no difference in plasma nitrite levels between them (C in Figure 6). Because, when oxygen availability is reduced or NOS activity is decreased, tissue nitrite is more susceptible to modification than plasma nitrite which has a function as a reservoir for tissues to maintain steady-state nitrite levels. Therefore, nitrite level starts decrease first at tissues, reflecting NO bioavailability much more in tissues than plasma. On the other hand, plasma nitrite levels are easily affected and fluctuated by oral nitrite intake. Because of a rapid turnover of unstable nitrite in plasma, unless nitrite is required in tissues, excessive nitrite easily undergoes oxidation to form stable nitrate in plasma [33], which is evidenced in Figure 6 (D and H).

Despite the contribution of eNOS and nNOS in preventing the development of colitis, large amounts of NO produced by iNOS are likely to be pro-inflammatory and injurious [34]. Selective pharmacological inhibition of iNOS reduces colonic inflammation and tissue injury by 7 days of DSS treatment [34,35]. Mice genetically deficient in iNOS revealed an attenuated colonic inflammatory response to DSS treatment, suggesting that iNOS-induced NO production is directly responsible for the subsequent development of experimental colitis in mice [36].

Although the potential of either pro-inflammatory or anti-inflammatory effects of NO in IBDs is still controversial [8], Kissa et al. demonstrated that pretreatment of trinitrobenzene sulphonic acid (TNBS)-induced colitis rat with L-NAME, a nonselective NOS inhibitor, exacerbated the subsequent colonic damage by suppressing eNOS activity, whereas delayed administration of L-NAME had a beneficial action on colonic injury and inflammation by suppressing iNOS activity,

suggesting the time-dependent actions of NOS activity might be involved in the development of these experimental colitis [37].

Abnormal interaction between intestinal bacteria and macrophages following barrier dysfunction due to impaired NOS system activates innate immune cells and induces ROS and proinflammatory cytokines such as TNF-α, which is well known inducer of NO generation in intestinal macrophages as well as epithelial cells [6]. Neutralizing anti-TNF antibodies have been shown to lead to an improvement of the colitis score [38,39]. In the current study, a significant increase in the colonic TNF-α expression was observed in colitis mice at day 3 before the appearance of HO-1 and iNOS expression in the colon, and oral $NaNO_2$ supplementation to colitis mice reduced colonic TNF-α expression with the reduction of HO-1 and iNOS expression as well as clinical and histological improvements at day 7, possibly suggesting TNF-α a key factor for the subsequent ROS-mediated inflammatory process. HO is the rate-limiting enzyme in the catabolism of heme, followed by production of biliverdin, free iron, and carbon monoxide. Among three mammalian HO isozymes identified (HO-1, 2, and 3), HO-1 is a stress-responsive protein induced by various oxidative agents [40,41]. Recent studies suggested that the induction of HO-1 expression plays a protective role in intestinal damage models induced by TNBS or DSS, indicating that activation of HO-1 may act as an endogenous defensive mechanism to reduce inflammation and tissue injury in the intestinal tracts. In the present study, impaired constitutive NOS activity already existed at day 3, while colonic HO-1 expression was not remarkable. However, it significantly increased at day 7 with deterioration of clinical and biological parameters, which was reversed by the treatment with oral

$NaNO_2$. This result suggests that intestinal NO deficit precedes oxidative stress, supporting the idea that the preexisting NO, irrespective of being endogenously produced or exogenously supplemented, could play an important role in preventing and limiting oxidative injury following DSS treatment.

There are several NOS-independent mechanisms of NO formation in biological tissues [42]. One of the mechanisms generating NO in the body is the reduction of nitrite in the gastric lumen and colon where acidic and anaerobic conditions are responsible for NO formation, respectively. Besides the NOS-dependent pathway, this NOS-independent pathway provides an important alternative source supplying NO to lesions where NOS activity is impaired [33]. Ourselves and others previously showed that oral nitrite elicits its beneficial effects on experimental diseased states such as hypertension and diabetic kidney [22,43] through the bioactive conversion of nitrite to NO in the stomach and peripheral tissues by non-enzymatic reductions. The recent growing body of evidence regarding the beneficial effects of oral or dietary nitrite on physiologic and pathological conditions has been well demonstrated.

In the present study, from the standpoint of therapeutic intervention for acute experimental colitis, we used high dose of oral nitrite (25 mM ≈ 1.73 g/L) far from what can be achieved through dietary consumption. We therefore evaluated unfavorable side effects on blood pressure and Met Hb formation after oral $NaNO_2$ intake, revealing no effect on the blood pressure (Table 2), but a significantly increased Met Hb formation found to be within a permitted limit [44] and not too toxic to continue the study. Because recent reports use much lower nitrite, as well as nitrate, which is physiologically relevant to dietary sources [13,14,45], lower doses of oral nitrite will be required to

attempt for a possible application to therapeutic and prophylactic use in future.

Another issue to be considered is carcinogenic property of nitrite and nitrate. Certain nitrosamines formed in the stomach by reaction between nitrite and naturally occurring secondary amines in food are known to be experimentally carcinogenic in rodents [46]. Although a causal relationship between nitrite and nitrate exposure and human cancer has not been unequivocally demonstrated [47], there is limitation of this study with respect to carcinogenicity of oral nitrite in that we deal with acute phase of experimental colitis. Whereas, data regarding carcinogenicity of oral or dietary nitrite and nitrate should be accumulated using as lower dose of nitrite and nitrate as possible.

In summary, the results of the current study demonstrate that oral nitrite significantly ameliorate DSS-treated experimental colitis by supplying nitrite-derived NO instead of impaired colonic NOS activity, consequently inhibiting inflammatory process of DSS-treated experimental colitis

Acknowledgments

The authors would like to thank Yoshinori Kubo, Masafumi Saito, Yukari Takahashi, and Sonoko Harada for their technical assistance.

References

[1] M.I. Torres, A. Rios, Current view of the immunopathogenesis in inflammatory bowel disease and its implications for therapy, World J. Gastroenterol. 14 (2008) 1972-1980.

[2] A.H. Gitter, K. Bendfeldt, J.D. Schulzke, M. Fromm, Leaks in the epithelial barrier caused by spontaneous and TNF-alpha-induced single-cell apoptosis, FASEB. J. 14 (2000) 1749-1753.

[3] I. Okayasu, S. Hatakeyama, M. Yamada, T. Ohkusa, Y. Inagaki, R. Nakaya, A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice, Gastroenterology 98 (1990) 694-702.

[4] A. Venkatraman, B.S. Ramakrishna, A.B. Pulimoos, S. Patra, S. Murthy, Increased permeability in dextran sulphate colitis in rats: time course of development and effect of butyrate, Scand. J. Gastroenterol. 35 (2000) 1053-1059.

[5] S. Kitajima, S. Takuma, M. Morimoto, Histological analysis of murine colitis induced by dextran sulfate sodium of different molecular weights, Exp. Anim. 49 (2000) 9-15.

[6] C. Bauer, F. Loher, M. Dauer, C. Mayer, H.A. Lehr, M. Schonharting, R. Hallwachs, S. Endres, A. Eigler, The ICE inhibitor pralnacasan prevents DSS-induced colitis in C57BL/6 mice and suppresses IP-10 mRNA but not TNF-α mRNA expression, Dig. Dis. Sci. 52 (2007) 1642-1652.

[7] M. Mori, K.Y. Stokes, T. Vowinkel, N. Watanabe, J.W. Elrod, N.R. Harris, D.J. Lefer, T. Hibi, D. N. Granger, Colonic blood flow responses in experimental colitis: time course and underlying

mechanisms, Am. J. Physiol., 289 (2005) G1024-G1029.

[8] R.K. Cross, K.T. Wilson, Nitric oxide in inflammatory bowel disease, Inflamm. Bowel. Dis. 9 (2003) 179-189.

[9] A. Salas, M. Gironella, A. Salas, A. Soriano, M. Sans, J. Iovanna, J.M. Pique, J. Panes, Nitric oxide supplementation ameliorates dextran sulfate sodium-induced colitis in mice, Lab. Invest. 82 (2002) 597-607.

[10] S. Fiorucci, E. Antonelli, E. Distrutti, P.D. Soldato, R.J. Flower, M.J.P. Clark, A. Morelli, M. Perretti, L.J. Ignarro, NCX-1015, a nitric-oxide derivative of prednisolone, enhances regulatory T cells in the lamina propria and protects against 2,4,6-trinitrobenzenesulfonic acid-induced colitis in mice, Proc. Natl. Acad. Sci. USA 99 (2002) 15770-15775.

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

[12] J.O. Lundberg, E. Weitzberg, NO generation from inorganic nitrate and nitrite: role in physiology, nutrition and therapeutics, Arch. Pharm. Res. 32 (2009) 1119-1126.

[13] N.S. Bryan, J.W. Calvert, J.W. Elrod, S. Gundewar, S.Y. Ji, D.J. Lefer, Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury, Proc. Natl. Acad. Sci. U S A 104 (2007) 19144-19149.

[14] K.Y. Stokes, T.R. Dugas, Y. Tang, H. Garg, E. Guidry, N. S. Bryan, Dietary nitrite prevents hypercholesterolemic microvascular inflammation and reverses endothelial dysfunction, Am. J. Physiol. 296 (2009) H1281 - H1288.

[15] N.J.H. Raat, A.C. Noguchi, V.B. Liu, N. Raghavachari, D. Liu, X. Xu, S. Shiva, P.J. Munson, M.T. Gladwin, Dietary nitrate and nitrite modulate blood and organ nitrite and the cellular ischemic stress response, Free Radic. Biol. Med. 47 (2009) 510-517.

[16] M.A. McGuckin, R. Eri, L.A. Simms, T.H.J. Florin, G.R. Smith, Intestinal barrier dysfunction in inflammatory bowel diseases, Inflamm. Bowel Dis. 15 (2009) 100-113.

[17] T. Hibi, H. Ogata, A. Sakuraba, Animal models of inflammatory bowel disease, J. Gastoroenterol. 37 (2002) 409-417.

[18] K. Yasukawa, R. Miyakawa, T. Yao, M. Tsuneyoshi, H. Utsumi, Non-invasive monitoring of redox status in mice with dextran sodium sulphate-induced colitis, Free Radic. Res. 43 (2009) 505-513.

[19] S.N. Murthy, H.S. Cooper, H. Shim, R.S. Shah, S.A. Ibrahim, D.J. Sedergran, Treatment of dextran sulfate sodium-induced murine colitis by intracolonic cyclosporine Dig. Dis. Sci. 38 (1993) 1722-1734.

[20] T. Leahy, R. Smith, Notes on methemoglobin determination, Clin. Chem. 6 (1960) 148-152.

[21] H.S. Cooper, S.N. Murthy, R.S. Shah, D.J. Sedergran, Clinicopathologic study of dextran sulfate sodium experimental murine colitis, Lab. Invest. 69 (1993) 238-249.

[22] K. Ohtake, Y. Ishiyama, H. Uchida, E. Muraki, J. Kobayashi, Dietary nitrite inhibits early glomerular injury in streptozotocin-induced diabetic nephropathy in rats, Nitric Oxide 17 (2007)

[23] P.P. Bradley, D.A. Priebat, R.D. Christensen, G. Rothstein, Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker, J. Invest. Dermatol. 78

(1982) 206-209.

[24] P.L. Beck, R. Xavier, J. Wong, I. Ezedi, H. Mashimo, A. Mizoguchi, E. Mizoguchi, A.K. Bhan, D.K. Podolsky, Paradoxical roles of different nitric oxide synthase isoforms in colonic injury, Am. J. Physiol. Gastrointest. Liver Physiol. 286 (2004) G137-147.

[25] K. Tanaka, T. Namba, Y. Arai, M. Fujimoto, H. Adachi, G. Sobue, K. Takeuchi, A. Nakai, T. Mizushima, Genetic evidence for a protective role for heat shock factor 1 and heat shock protein 70 against colitis, J. Biol. Chem. 282 (2007) 23240-23252.

[26] I.M. Garrelds, J.P. Heiligers, M.E. Van Meeteren, D.J. Duncker, P.R. Saxena, M.A. Meijssen, F.J. Zijlstra, Intestinal blood flow in murine colitis induced with dextran sulfate sodium, Dig. Dis. Sci. 47 (2002) 2231-2236.

[27] T. Lauer, M. Preik, T. Rassaf, B.E. Strauer, A. Deussen, M. Feelisch, M. Kelm, Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action, Proc. Natl. Acad. Sci. USA 98 (2001) 12814-12819.

[28] Y. Naito, K. Katada, T. Takagi, H. Tsuboi, Y. Isozaki, O. Handa, S. Kokura, N. Yoshida, H. Ichikawa, T. Yoshikawa, Rosuvastatin, a new HMG-CoA reductase inhibitor, reduces the colonic inflammatory response in dextran sulfate sodium-induced colitis in mice, Int. J. Mol. Med. 17 (2006) 997-1004.

[29] M. Sasaki, S. Bharwani, P. Jordan, J.W. Elrod, M.B. Grisham, T.H. Jackson, D.J. Lefer, J.S. Alexander, Increased disease activity in eNOS-deficient mice in experimental colitis, Free Radic. Biol. Med. 35 (2003) 1679-1687.

[30] P. Holzer, I.T. Lippe, A.L. Tabrizi, L. Lenard Jr, L. Bartho, Dual excitatory and inhibitory effect of nitric oxide on peristalsis in the guinea pig intestine, J. Pharmacol. Exp. Ther. 280 (1997) 154-161.

[31] K. Nichols, W. Staines, A. Krantis, Nitric oxide synthase distribution in the rat intestine: a histochemical analysis, Gastroenterology 105 (1993) 1651-1661.

[32] X.W. Qu, H. Wang, I.G. De Plaen, R.A. Rozenfeld, W. Hsueh, Neuronal nitric oxide synthase (NOS) regulates the expression of inducible NOS in rat small intestine via modulation of nuclear factor kappa B, FASEB. J. 15 (2001) 439-446.

[33] N.A. Bryan, B.O. Fernandez, S.M. Bauer, M.F. Garcia-Saura, A.B. Milsom, T. Rassaf, R.E. Maloney, A. Bharti, J. Rodriguez, M. Feelisch, Nitrite is a signaling molecule and regulator of gene expression in mammalian tissue, Nat. Chem. Biol. 1 (2005) 290-297.

[34] C.F. Krieglstein, W.H. Cerwinka, F.S. Laroux, J.W. Salter, J.M. Russell, G. Schuermann, M.B. Grisham, C.R. Ross, D.N. Granger, Regulation of murine intestinal inflammation by reactive metabolites of oxygen and nitrogen: divergent roles of superoxide and nitric oxide, J. Exp. Med. 194: 1207-1218, 2001.

[35] F. Obermeier, G. Kojouharoff, W. Hans, J. Scholmerich, V. Gross, W. Falk, Interferon-gamma (IFN-gamma)-and tumor necrosis factor (TNF)-induced nitric oxide as toxic effector molecule in chronic dextran sulphate sodium (DSS)-induced colitis in mice, Clin. Exp. Immunol. 116 (1999) 238-245.

[36] C.F. Krieglstein, C. Anthoni, W.H. Cerwinka, K.Y. Stokes, J.M. Russell, M.B. Grisham, D.N.

Granger, Role of blood- and tissue-associated inducible nitric-oxide synthase in colonic inflammation, Am. J. Pathol. 170 (2007) 490-496.

[37] J. Kissa, D. Lamarquea, J.C. Delchiera, B.J.R. Whittleb, Time-dependent actions of nitric oxide synthase inhibition on colonic inflammation induced by trinitrobenzene sulphonic acid in rats, Eur. J. Pharmacol. 336 (1997) 219-224.

[38] D.H. Present, P. Rutgeerts, S. Targan, S.B. Hanauer, L. Mayer, R.A. van Hogezand, D.K. Podolsky, B.E. Sands, T. Braakman, K.L. DeWoody, T.F. Schaible, S.J. van Deventer, Infliximab for the treatment of fistulas in patients with Crohn's disease, Crohn's disease cA2 study group, N. Engl. J. Med. 337 (1997) 1029-1035.

[39] S.R. Targan, S.B. Hanauer, S.J. van Deventer, L. Mayer, D.H. Present, T. Braakman, K.L. DeWoody, T.F. Schaible, P.J. Rutgeerts, A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease, N. Engl. J. Med. 340 (1999) 1398-1405.

[40] Y. Naito, T. Takagi, T. Yoshikawa, Heme oxygenase-1: a new therapeutic target for inflammatory bowel disease, Aliment. Pharmacol. Ther. 20 (2004) S177-S184.

[41] Y. Erbil, M. Giris, S.D. Abbasoglu, U. Barbaros, B.T. Yanik, A. Necefli, V. Olgac, G.A. Toker, Effect of heme oxygenase-1 induction by octreotide on TNBS-induced colitis, J. Gastroenterol. Hepatol. 22 (2007) 1852-1858.

[42] J.L. Zweier, P. Wang, A. Samouilov, P. Kuppusamy, Enzyme independent formation of nitric oxide in biological tissues, Nat. Med. 1 (1995) 804-809.

[43] K. Nakanishi, Y. Kubo, K. Ohtake, H. Uchida, F. Kotake, H. Natsume, J. Kobayashi, Effect of

mitochondrial Aldehyde dehydrogenase on pharmacokinetic profile of nitrite in plasma and organs, Nitric Oxide 20 (2009) S38.

[44] L. Fewtrell, Drinking-water nitrate, methemoglobinemia and global burden of disease: a discussion, Environ. Health Persp. 112 (2004) 1371-1374.

[45] Y. Kanematsu, K. Yamaguchi, H. Ohnishi, Y. Motobayashi, K. Ishizawa, Y. Izawa, K. Kawazoe, S. Kondo, S. Kagami, S. Tomita, K. Tsuchiya, T. Tamaki, Dietary doses of nitrite restore the circulating nitric oxide level and improve renal injury in L-NAME-induced hypertensive rats, Am. J .Physiol. Renal Physiol. 295 (2008) F1457-F1462.

[46] P.N. Magee, J.M. Barnes, The production of primary hepatic malignant tumours in the rat by feeding dimethylnitrosoamine, Br. J. Cancer 10 (1956) 114-122.

[47] H.J.M. van Grinsven, M.H. Ward, N. Benjamin, T.M. de Kok, Does the evidence about health risks associated with nitrate injection warrant an increase of the nitrate standard for drinking water?, Environ. Health 5 (2006) 26-30.

Legends

Table 1 Averaged amounts of supplemented $NaNO_2$ and DSS.

There was no difference in total amounts of supplemented $NaNO_2$ between $NaNO_2$-treated and $NaNO_2$+DSS-treated groups, and no difference in total amounts of supplemented DSS between the DSS-treated and $NaNO_2$+DSS-treated groups. Data represent mean (n=8-10/group) ± S.E.

Table 2 Effect of $NaNO_2$ on blood pressure and heart rate

The dose of oral $NaNO_2$ used in the present study had no effect on the systolic blood pressure (SBP) and heart rate (HR) both at day 3 and 7 (non-treated versus $NaNO_2$-treated, DSS-treated versus $NaNO_2$+DSS-treated). DSS significantly reduced systolic blood pressure irrespective of with or without $NaNO_2$ treatment (non-treated versus DSS-treated, $NaNO_2$-treated versus $NaNO_2$+DSS-treated). These data were analyzed with two-way ANOVA ($NaNO_2$ intake and DSS intake as main effects). Data represent mean (n=8-10/group) ± S.E. NS ; no significance.

Table 3 Effect of $NaNO_2$ on Methemoglobin level

Oral $NaNO_2$ had no impact on Hb levels between non-treated and $NaNO_2$-treated mice, while DSS significantly decreased Hb levels (non-treated versus DSS-treated) and improved with oral

$NaNO_2$ (DSS-treated versus $NaNO_2$+DSS-treated). Methemoglobine (Met Hb) formation at day 7 was significantly more induced in non-treated and DSS-treated mice with $NaNO_2$ than without $NaNO_2$. These data were analyzed with two-way ANOVA ($NaNO_2$ intake and DSS intake as main effects). Data represent mean (n=8-10/group) ± S.E. $^{a}P<0.05$ versus non-treated group. $^{b}P<0.05$ versus $NaNO_2$-treated group. $^{c}P<0.05$ versus DSS-treated group. NS; no significance.

Figure 1

Experimental protocol for DSS-treated colitis in mice. Experimental colitis was induced in ICR mice by administrating 2.5-3.0% DSS in drinking water *ad libtum* over the experimental period. Sodium nitrite ($NaNO_2$) was administered orally in drinking water at 25 mmol/l *ad libtum* over the experimental period. In group 4, the first administration of $NaNO_2$ was given 1 day before the administration of DSS. ‡; Based on our preliminary study, suggesting that DSS-treated mice drink 15% more water than DSS+$NaNO_2$-treated mice over the experimental period. Therefore, group 4 mice were given 3.0% DSS in drinking water.

Figure 2

Effects of DSS and/or $NaNO_2$ supplementation on the disease active index (DAI) over the experimental time-course. DSS induced a significant increase in DAI of DSS-treated mice at day 4.

Treatment of DSS-treated mice with $NaNO_2$ significantly attenuated the development of the colonic inflammatory process from day 6 up to the end of the experimental period. Data represent mean (n=8-10/group) ± S.E. $^{a}P<0.05$ versus non-treated group. $^{b}P<0.05$ versus $NaNO_2$-treated group. $^{c}P<0.05$ versus DSS-treated group.

Figure 3

Effects of DSS and/or $NaNO_2$ supplementation on hematocrit (Ht), and colon length in the DSS model of mouse colitis. Treatment with $NaNO_2$ significantly attenuated the decrease both in Ht (A) and in colon length at day 7 (B and C). Data represent mean (n=8/group) ± S.E. $^{a}P<0.05$ versus non-treated group. $^{b}P<0.05$ versus $NaNO_2$-treated group. $^{c}P<0.05$ versus DSS-treated group.

Figure 4

Histological findings of colitis in mice. Excised colons at day 7 were stained with hematoxylin and eosin (original magnification ×100): non-treated group (A), $NaNO_2$-treated group (B), DSS-treated group (C), DSS+$NaNO_2$-treated group (D). Closed arrow shows the crypt loss, and open arrow shows inflammatory cell infiltration. The histological scores [21] for eight independent sections were determined (E). DSS-treated mice showed a significant increase in the histological scores compared with non-treated mice. Treatment of mice with DSS and $NaNO_2$ significantly

reduced the histological scores compared with DSS-treated mice. Data represent mean (n=8/group) ± S.E. [a]$P<0.05$ versus non-treated group. [b]$P<0.05$ versus $NaNO_2$-treated group. [c]$P<0.05$ versus DSS-treated group.

Figure 5

Effects of DSS and/or $NaNO_2$ supplementation on colonic MPO activity at day 7. DSS-treated mice showed a significant increase in the colonic MPO activity compared with non-treated mice. Treatment of mice with DSS and $NaNO_2$ significantly reduced the colonic MPO activity compared with DSS-treated mice. Data represent mean (n=8/group) ± S.E. [a]$P<0.05$ versus non-treated group. [b]$P<0.05$ versus $NaNO_2$-treated group. [c]$P<0.05$ versus DSS-treated group.

Figure 6

Nitrite and nitrate levels in the colon and plasma of each of the four groups at day 3 (A, B, C, D) and day 7 (E, F, G, H). At day 3, DSS-treated mice exhibited a reduced colonic nitrite level compared with non-treated and $NaNO_2$-treated mice, whereas this decrease in colonic nitrite levels recovered to the control levels of non-treated mice with $NaNO_2$ supplementation (A). At day 7, DSS-treated mice exhibited an increased colonic nitrite level compared with non-treated mice. Additional $NaNO_2$ supplementation to DSS-treated mice further increased colonic nitrite level

compared with DSS-treated mice (E). No difference in plasma nitrite levels was observed between the four groups at day 3 and day 7 (C and G). At day 3, DSS treatment significantly reduced nitrate levels in colon and plasma (B and D), while no significant reduction of colonic and plasma nitrate levels were found at day 7 (F and H).Data represent mean (n=8-10/group) ± S.E. Data represent mean (n=8/group) ± S.E. [a]$P<0.05$ versus non-treated group. [b]$P<0.05$ versus $NaNO_2$-treated group. [c]$P<0.05$ versus DSS-treated group.

Figure 7

Effects of DSS and/or $NaNO_2$ supplementation on colonic TNF-α, HO-1, and iNOS expressions at day 3 and day 7. Colonic TNF-α, HO-1 and iNOS expressions at day 3 in Western blot (A), and at day 7 in Western blot (B). Densitometric quantification of colonic TNF-α, HO-1 and iNOS at day 3 (C, E, and G, respectively), and at day 7 (D, F, and H, respectively). At day 3, a significant increase in the colonic TNF-α expression was observed in colitis mice, which was reversed to control level by $NaNO_2$ supplementation (A, C). On the other hand, there was no significant difference in the colonic iNOS and HO-1 expressions between non-treated control and DSS-treated mice (A, E, G). At day 7, significant increases in all of these proteins were observed in colitis mice, which were almost reversed to control levels by oral $NaNO_2$ supplementation (B, D, F, H). Data represent β-actin standardized mean (n=8/group) ± S.E. [a]$P<0.05$ versus non-treated group. [b]$P<0.05$ versus $NaNO_2$-treated group. [c]$P<0.05$ versus DSS-treated group.

Figure 1

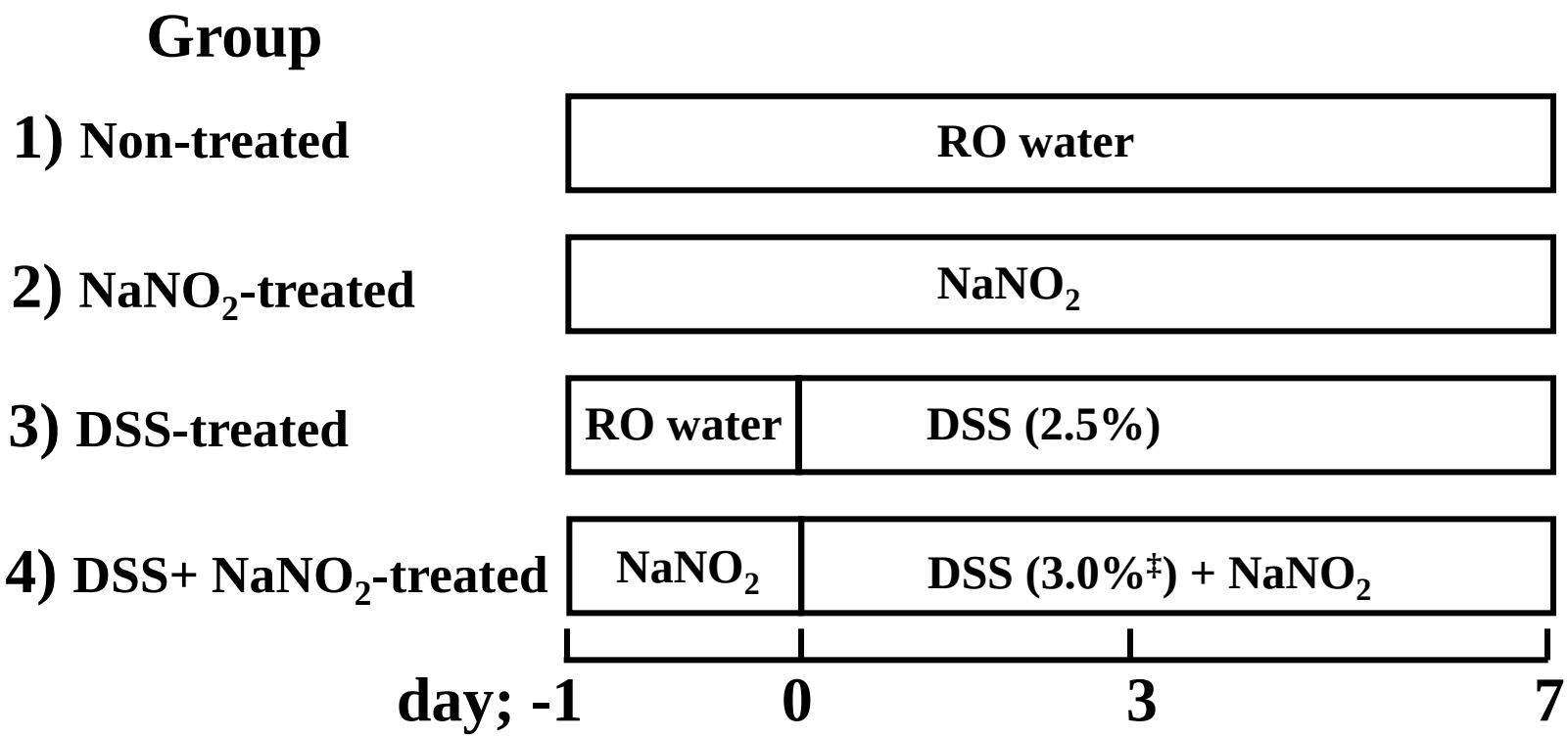
Group
1) Non-treated
RO water
2) NaNO2-treated
NaNO2
3) DSS-treated
RO water
DSS (2.5%)
4) DSS+ NaNO2-treated
NaNO2
DSS (3.0%‡) + NaNO2
day; -1
0
3
7

Figure 2

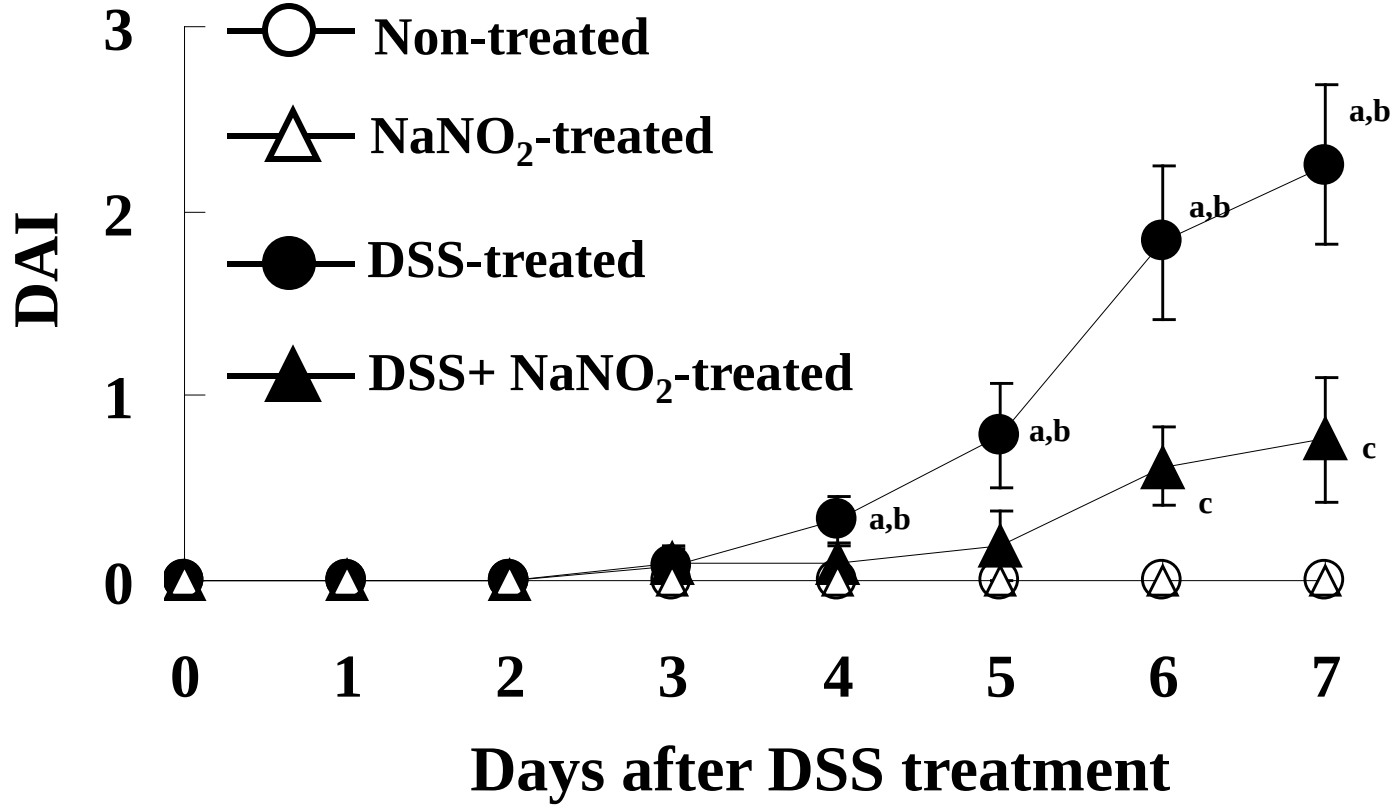
Non-treated
NaNO2-treated
DSS-treated
DSS+ NaNO2-treated
DAI
3
2
1
0
0
1
2
3
4
5
6
7
Days after DSS treatment
a,b
a,b
a,b
c
c

Figure 3

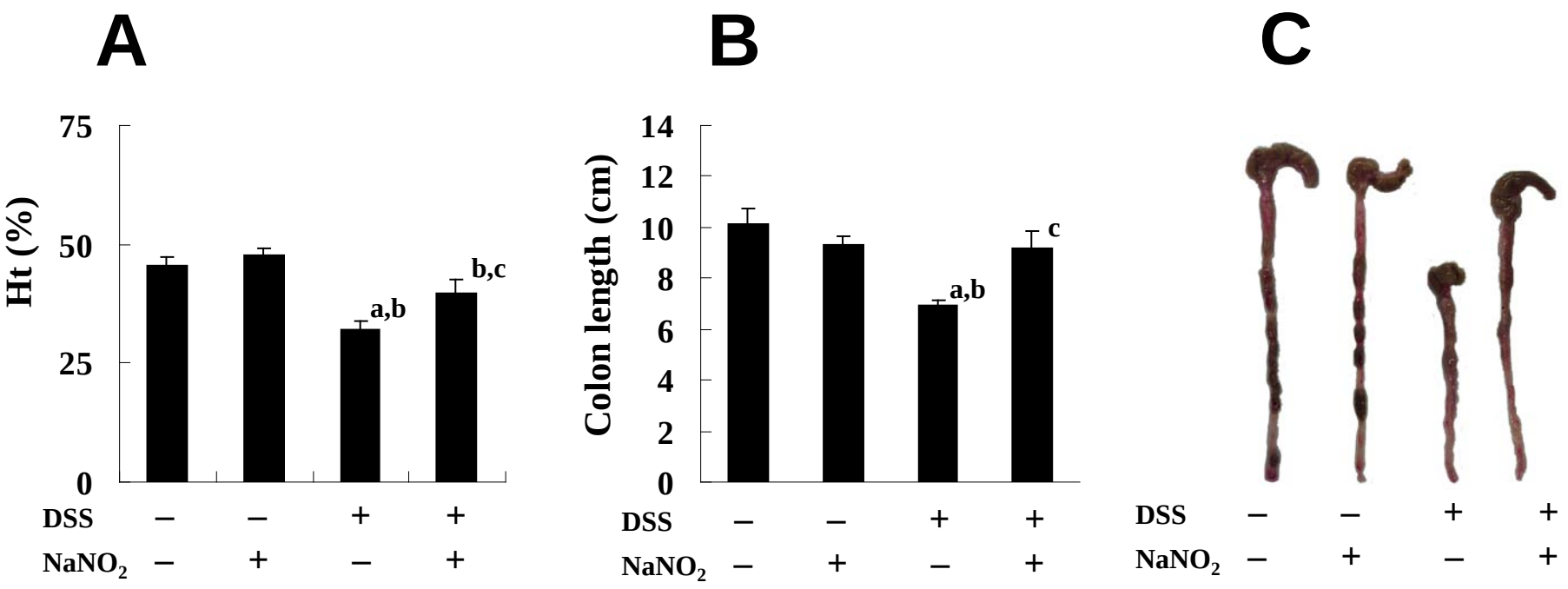
A
Ht (%)
75
50
25
0
a,b
b,c
DSS – – + +
NaNO2 – + – +
B
Colon length (cm)
14
12
10
8
6
4
2
0
a,b
c
DSS – – + +
NaNO2 – + – +
C
DSS – – + +
NaNO2 – + – +

Figure 4

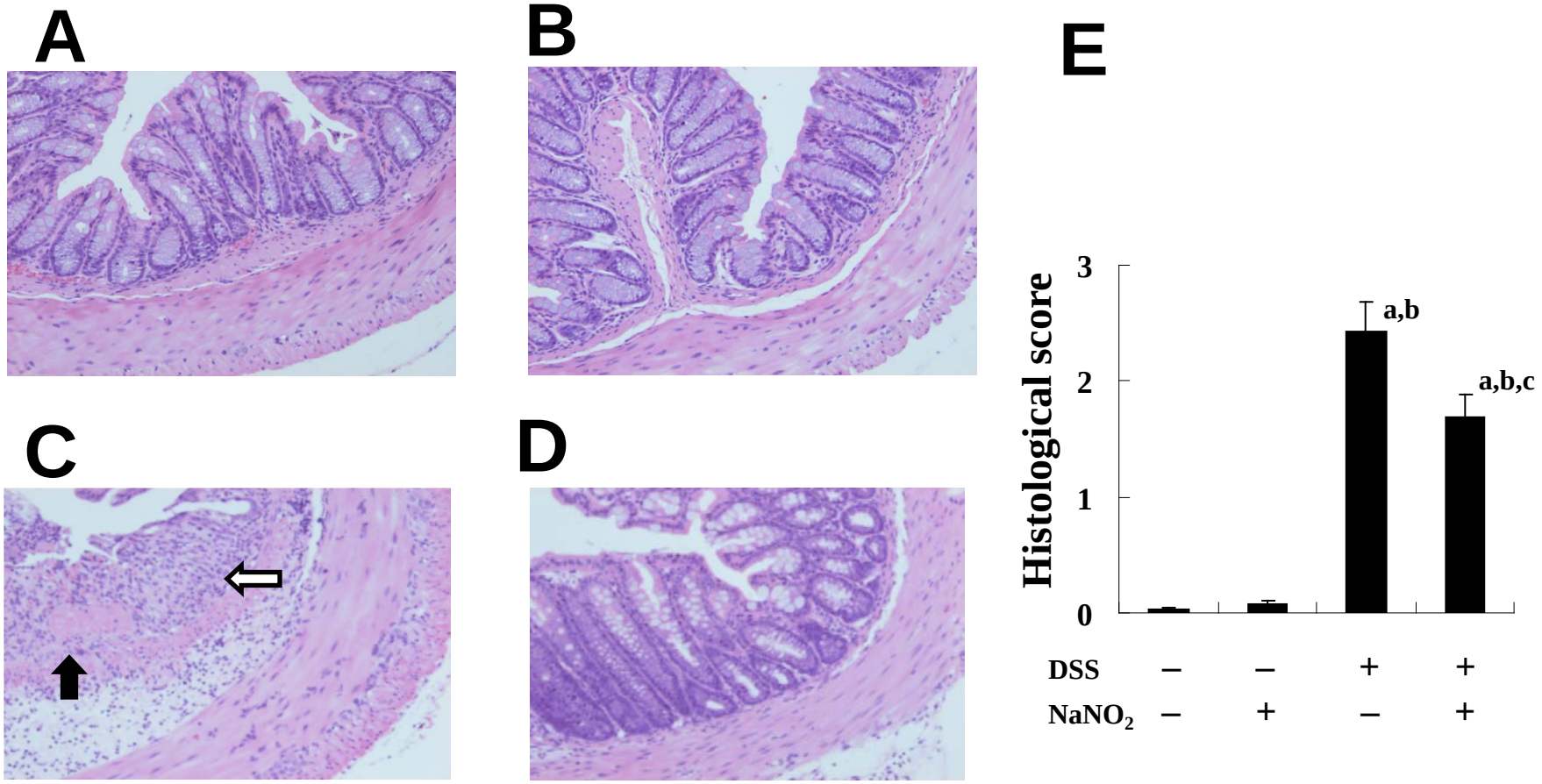

Figure 5

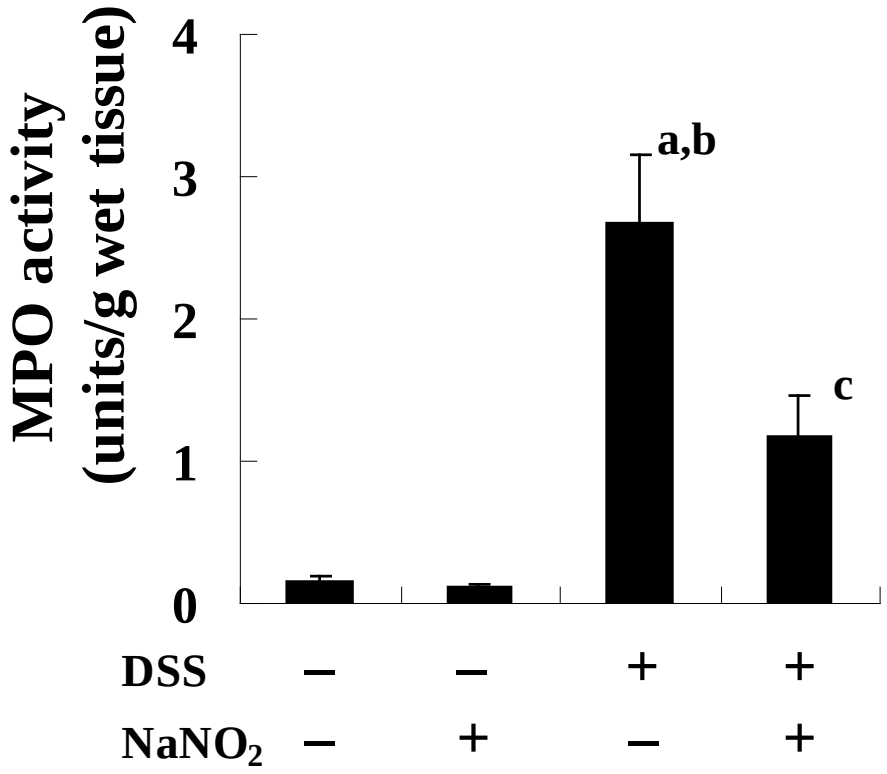

Figure 6

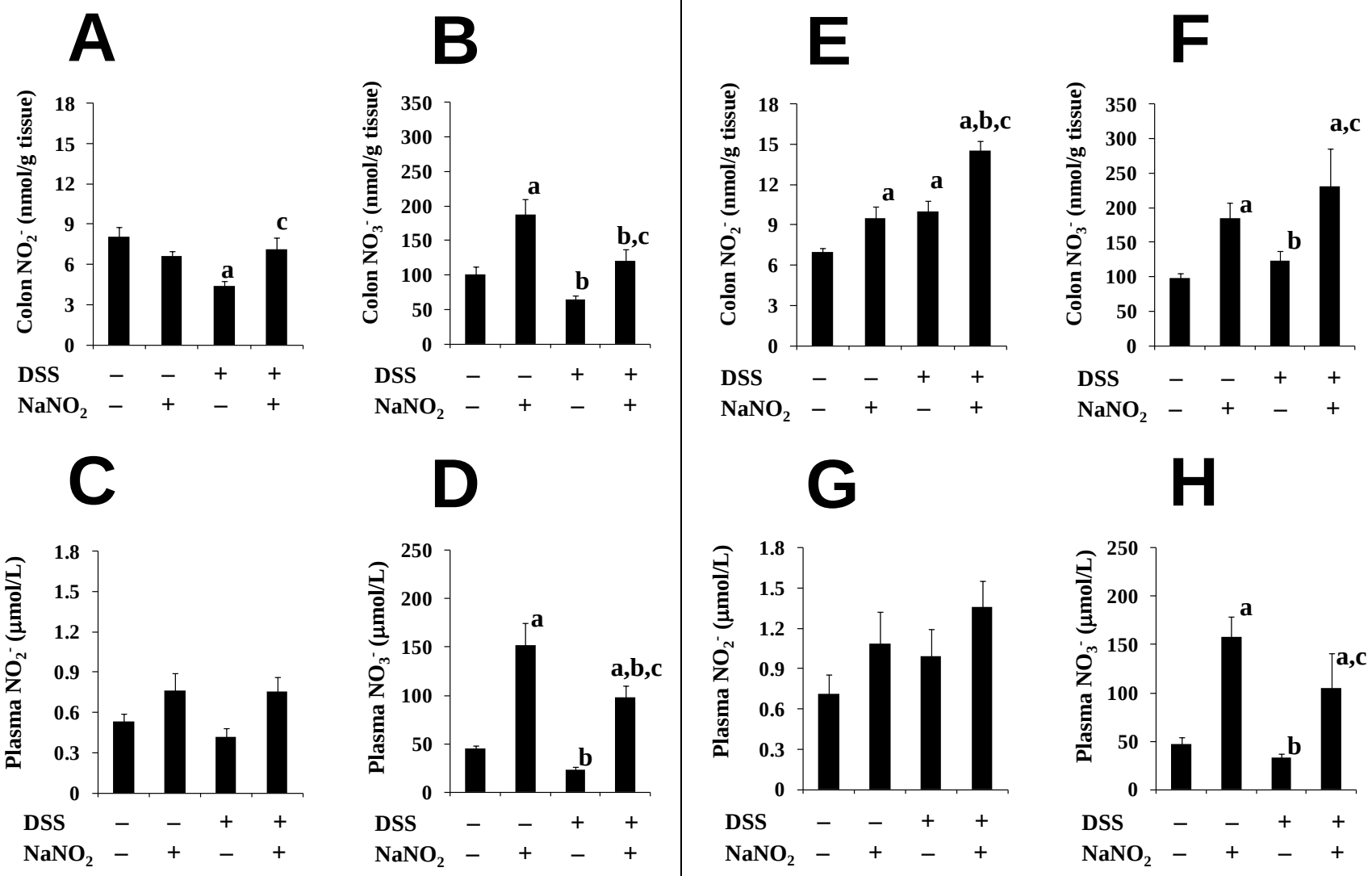
A
Colon NO_2^- (nmol/g tissue)
18
15
12
9
6
3
0
a
c
DSS – – + +
$NaNO_2$ – + – +
B
Colon NO_3^- (nmol/g tissue)
350
300
250
200
150
100
50
0
a
b
b,c
DSS – – + +
$NaNO_2$ – + – +
E
Colon NO_2^- (nmol/g tissue)
18
15
12
9
6
3
0
a
a
a,b,c
DSS – – + +
$NaNO_2$ – + – +
F
Colon NO_3^- (nmol/g tissue)
350
300
250
200
150
100
50
0
a
b
a,c
DSS – – + +
$NaNO_2$ – + – +
C
Plasma NO_2^- (µmol/L)
1.8
1.5
1.2
0.9
0.6
0.3
0
DSS – – + +
$NaNO_2$ – + – +
D
Plasma NO_3^- (µmol/L)
250
200
150
100
50
0
a
b
a,b,c
DSS – – + +
$NaNO_2$ – + – +
G
Plasma NO_2^- (µmol/L)
1.8
1.5
1.2
0.9
0.6
0.3
0
DSS – – + +
$NaNO_2$ – + – +
H
Plasma NO_3^- (µmol/L)
250
200
150
100
50
0
a
b
a,c
DSS – – + +
$NaNO_2$ – + – +

Figure 7

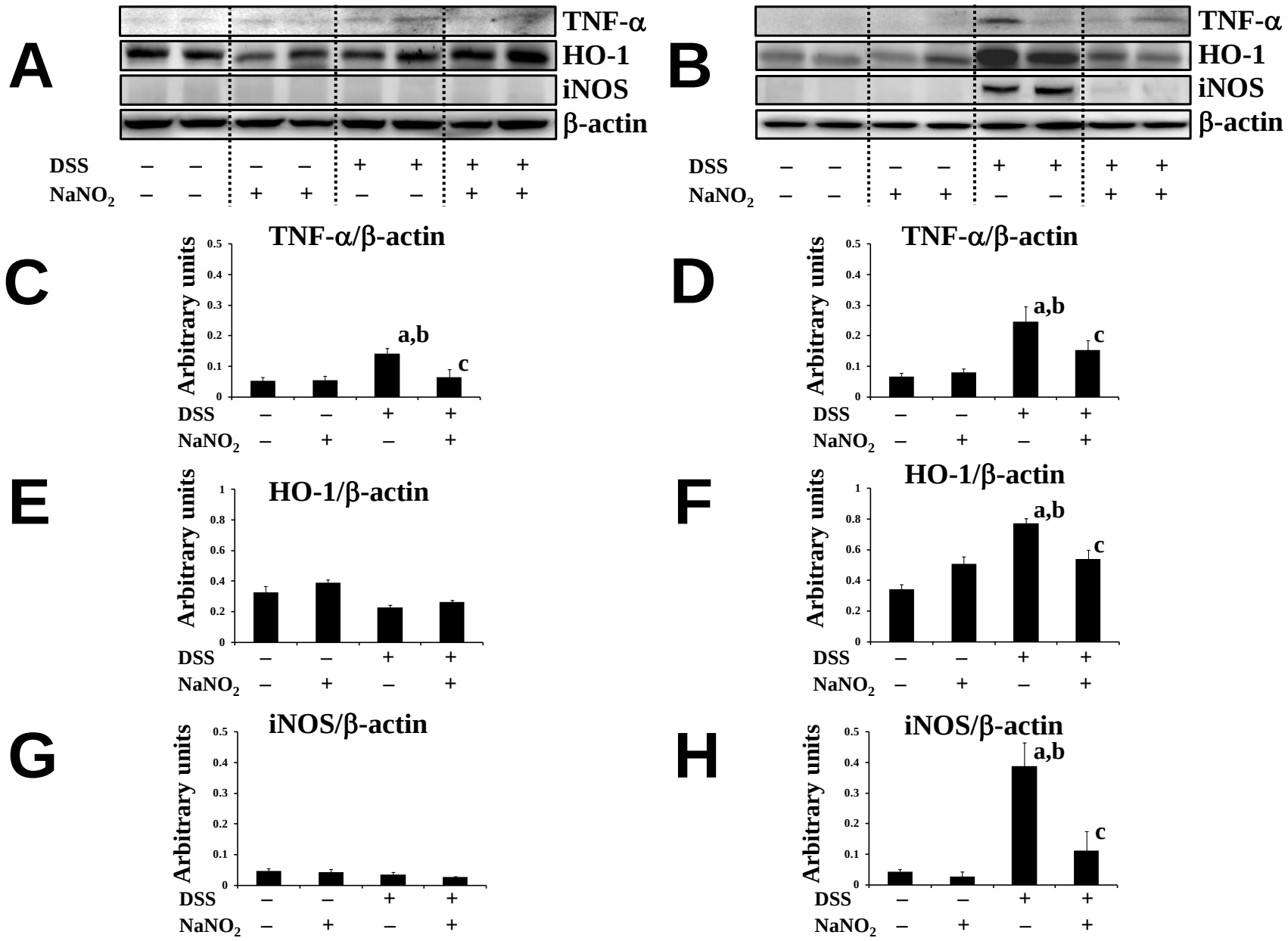
A
TNF-α
HO-1
iNOS
β-actin
DSS – – – – + + + +
NaNO2 – – + + – – + +
B
TNF-α
HO-1
iNOS
β-actin
DSS – – – – + + + +
NaNO2 – – + + – – + +
C
TNF-α/β-actin
Arbitrary units
0 0.1 0.2 0.3 0.4 0.5
a,b
c
DSS – – + +
NaNO2 – + – +
D
TNF-α/β-actin
Arbitrary units
0 0.1 0.2 0.3 0.4 0.5
a,b
c
DSS – – + +
NaNO2 – + – +
E
HO-1/β-actin
Arbitrary units
0 0.2 0.4 0.6 0.8 1
DSS – – + +
NaNO2 – + – +
F
HO-1/β-actin
Arbitrary units
0 0.2 0.4 0.6 0.8 1
a,b
c
DSS – – + +
NaNO2 – + – +
G
iNOS/β-actin
Arbitrary units
0 0.1 0.2 0.3 0.4 0.5
DSS – – + +
NaNO2 – + – +
H
iNOS/β-actin
Arbitrary units
0 0.1 0.2 0.3 0.4 0.5
a,b
c
DSS – – + +
NaNO2 – + – +

Table 1 Averaged amounts of supplemented $NaNO_2$ and DSS

Group	Non-treated	$NaNO_2$-treated	DSS-treated	$NaNO_2$+DSS-treated
$NaNO_2$ intake (mg/mouse/day)	-	6.4 ± 0.1	-	7.2 ± 0.3
DSS intake (mg/mouse/day)	-	-	245.6 ± 28.4	248.6 ± 11.1

There was no difference in total amounts of supplemented $NaNO_2$ between $NaNO_2$-treated and $NaNO_2$+DSS-treated groups, and no difference in total amounts of supplemented DSS between the DSS-treated and $NaNO_2$+DSS-treated groups. Data represent mean (n=8-10/group) ± S.E.

Table 2 Effect of $NaNO_2$ on blood pressure and heart rate

Group	Non-treated	$NaNO_2$-treated	DSS-treated	$NaNO_2$+DSS-treated	Two-way ANOVA		
					Main effects		Interaction
					$NaNO_2$ intake (N)	DSS intake (D)	N × D
At day 3							
SBP (mmHg)	98.1 ± 6.0	99.4 ± 4.3	79.1 ± 6.3	78.9 ± 6.9	NS	P = 0.0003	NS
HR (beats/min)	498.0 ± 23.4	496.1 ± 27.0	466.0 ± 27.4	524.8 ± 24.5	NS	NS	NS
At day 7							
SBP (mmHg)	98.3 ± 6.0	97.4 ± 5.6	82.3 ± 6.6	85.3 ± 7.0	NS	P = 0.02	NS
HR (beats/min)	479.5 ± 15.0	478.8 ± 41.4	437.9 ± 24.1	493.1 ± 11.6	NS	NS	NS

The dose of oral $NaNO_2$ used in the present study had no effect on the systolic blood pressure (SBP) and heart rate (HR) both at day 3 and 7 (non-treated versus $NaNO_2$-treated, DSS-treated versus $NaNO_2$+DSS-treated). DSS significantly reduced systolic blood pressure irrespective of with or without $NaNO_2$ treatment (non-treated versus DSS-treated, $NaNO_2$-treated versus $NaNO_2$+DSS-treated). These data were analyzed with two-way ANOVA ($NaNO_2$ intake and DSS intake as main effects). Data represent mean (n=8-10/group) ± S.E.
NS ; no significance.

Table 3 Effect of $NaNO_2$ on Methemoglobin level

Group	Non-treated	$NaNO_2$-treated	DSS-treated	$NaNO_2$+DSS-treated	Two-way ANOVA		
					Main effects		Interaction
					$NaNO_2$ intake (N)	DSS intake (D)	N × D
At day 7							
Hb (g/dL)	15.9 ± 0.5	15.8 ± 0.7	9.0 ± 0.3[a,b]	13.7 ± 0.6[c]	P = 0.0001	P = 0.001	P = 0.0007
Met Hb (g/dL)	0.2 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	P = 0.0001	NS	NS
Met Hb (%)	1.3 ± 0.2	2.3 ± 0.3	1.4 ± 0.2	2.4 ± 0.5	P = 0.002	NS	NS

Oral $NaNO_2$ had no impact on Hb levels between non-treated and $NaNO_2$-treated mice, while DSS significantly decreased Hb levels (non-treated versus DSS-treated) and improved with oral $NaNO_2$ (DSS-treated versus $NaNO_2$+DSS-treated). Methemoglobine (Met Hb) formation at day 7 was significantly more induced in non-treated and DSS-treated mice with $NaNO_2$ than without $NaNO_2$. These data were analyzed with two-way ANOVA ($NaNO_2$ intake and DSS intake as main effects). Data represent mean (n=8-10/group) ± S.E. [a]P<0.05 versus non-treated group. [b]P<0.05 versus $NaNO_2$-treated group. [c]P<0.05 versus DSS-treated group. NS; no significance.