Review Article

Role of Sulforaphane in Protection of Gastrointestinal Tract against *H.pylori*- and NSAID-Induced Oxidative Stress

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Abstract: Sulforaphane (SFN), a phytochemical found in abundance in broccoli sprouts, potently induces a variety of antioxidant enzymes, and thereby protects cells from injury induced by various kinds of oxidative stresses. It has been suggested that both *H. pylori* infection and intake of non-steroidal anti-inflammatory drugs (NSAIDs) induce chronic oxidative stress in gastrointestinal (GI) mucosa, thereby causing mucosal injury in the GI tract. Therefore, it would be a reasonable assumption that SFN protects GI mucosa against oxidative injury induced by *H. pylori* or NSAIDs. In this review, we report our recent data on the effects of SFN on *H. pylori*-induced gastric mucosal inflammation and NSAID-induced small intestinal injury. Our data from the *H. pylori* infection study clearly demonstrated that SFN inhibited *H. pylori* viability both *in vitro* and *in vivo*, and mitigated *H. pylori*-induced gastritis in mice and humans. Similarly, our study on NSAID-induced small intestinal injury showed that SFN not only mitigated aspirin-induced injury of small intestinal epithelial cells *in vitro*, but also ameliorated indomethacin-induced small intestinal injury in mice *in vivo*. These data strongly suggest that SFN contributes to the protection of GI mucosa against oxidative injury induced by *H. pylori* or NSAIDs.

Keywords: Sulforaphane, *Helicobacter pylori,* stomach, aspirin, indomethacin, small intestine

Introduction

Sulforaphane (SFN), an abundant antioxidant phytochemical found in broccoli sprouts (BS), potently induces a variety of antioxidant enzymes, which protect cells and organs against various kinds of oxidative stresses [1]. Recent studies have shown the antibacterial activity of SFN against *Helicobacter pylori (H. pylori) in vitro* [2]. We have recently shown that SFN induces antioxidant enzymes in the gastro-intestinal (GI) tract of mice, and protects GI mucosa against injuries induced by *H. pylori* and NSAIDs [3-5]. Our studies show that SFN not only enhances the antioxidant activity of GI mucosa, but also demonstrates antibacterial activity against *H. pylori* in gastric mucosa and anaerobic bacteria in the small intestine of mice [5]. We have conducted clinical trials with *H. pylori*-infected human subjects who consume BS, and have shown that SFN clearly inhibits *H. pylori* activity and mitigates *H. pylori*-induced gastritis [4]. This review introduces our recent data on the protective effects of SFN, which demonstrate that SFN prevented *H. pylori*- and NSAID-induced GI mucosal injury.

Role of SFN in Protection of the Cells Against Oxidative Stress

It has been suggested that environmental factors, especially dietary factors, are more important in the development of GI cancer than genetic factors [6,7]. Recent studies have clearly associated the development of gastric cancer not only with *H. pylori* infection [8,9], but also with the intake of a high salt diet [10,11]. In contrast, it has also been reported that daily intake of fruits and vegetables decreased the risk of developing GI cancers [6,12]. However, the mechanisms by which intake of fruits and vegetables decreased the risk of GI cancers were not clarified until recently. It is naturally important to identify the protective substances from the various phytochemicals in fruits and vegetables. Furthermore, it is necessary to clarify the mechanisms by which each substance protects the GI tract against oxidative injury. Finally, the effects of such substances on human health should be examined through well-designed clinical trials.

SFN has been studied extensively in the past two decades, and several studies have suggested the possibility that SFN may contribute to cancer chemoprotection [13]. SFN, a member of the isothiocyanate (ITC) family, is abundant in cruciferous vegetables, in particular broccoli sprouts (BS) [13,14]. Previous studies have shown that the unique molecular functional group -N=C=S is common to ITCs, which accounts for the pungency of these vegetables [15] (Fig 1). Different ITCs have been reported in a variety of foods. For example, Wasabi and mustard oils are rich in allyl ITCs [15]. Phenethyl ITC and 4-(methylthio)-3-butenyl ITC are found abundantly in Daikon and Cresson, respectively [16]. As previously stated, BS are rich in SFN. SFN potently induces various antioxidant (or phase 2) enzymes, such as glutathione S-transferase (GST), heme oxygenase-1 (HO-1), and NAD(P)H: quinone oxidoreductase 1 (NQO1), via Nf-E2 related factor 2-Kelch-like ECH-associated protein 1 (Nrf2-Keap1)-dependent pathways (17), thereby enhancing the antioxidant activity of the cells in the GI tract [4,5] (Fig 2).

In raw BS, SFN exists in the biologically inactive form of sulforaphane glucosinolates (SGS) [14]. The transformation of SGS to SFN occurs during the chewing process, where SGS is subjected to the action of myrosinase, also a component of BS [14]. SGS is also transformed to SFN in the intestinal lumen, by myrosinase of the intestinal microflora [14]. As previously stated, SFN upregulates phase 2 enzymes via Nrf2-Keap1-dependent mechanisms [17]. Under basal conditions, Nrf2 proteins are located in the cytoplasm and are biologically inactive as Nrf2 is bound to the Keap1 protein. Following exposure of the cells to oxidative stresses or SFN, Nrf2 proteins become dissociated from Keap1 and translocate into the nucleus. After entering the nucleus, Nrf2 protein binds to the antioxidant response element, and upregulates expression of a variety of xenobiotic and antioxidant enzymes [18-20] (Fig 2). It has been shown that induction of the phase 2 enzymes by SFN lasts almost for 72 h [17]. In addition to activation of the Nrf2-Keap1 pathway within cells, SFN has also been shown to inhibit *H. pylori* viability *in vitro*; these effects were demonstrated in both the clarithromycin-sensitive and resistant strains [2]. Furthermore, other studies have shown that SFN decreases colonization in mice stomach *ex vivo* [21], and in a small number of human cases [22].

Based on these results, we aimed to determine if oral intake of SFN contributed to the protection of GI mucosa against GI diseases induced by oxidative stresses, such as those induced by *H. pylori* and NSAIDs.

Roles of SFN in Protection Against *H. pylori*-Induced Gastric Mucosal Inflammation

H. pylori infection induces chronic oxidative stress in gastric mucosa, which eventually induces gastric cancer. However, SFN has been shown to mitigate various kinds of oxidative stresses. In this study, we aimed to determine if SFN inhibited *H. pylori* activity *in vitro*, and if SFN mitigated *H. pylori*-induced oxidative injury in gastric mucosa in mice and humans *in vivo.*

1. Effect of SFN on Urease Activity and *H. pylori* Viability *in vitro*

In this series of experiments, we examined whether SFN showed direct antibacterial activity against *H. pylori in vitro* [23]. The *H. pylori* strain Sydney Strain-1 (SS-1) was used in this study. The viability of *H. pylori* was determined by evaluating the number of colony forming units (CFU) after incubation of the *H. pylori* in the absence or presence of various concentrations of SFN for 3 h. The urease activity of *H. pylori* was assessed by measurement of the concentration of ammonia released into the medium during incubation with 5 mM urea for 1 h. The effects of SFN on urease activity and viability of *H. pylori* were examined at ambient pH 7.4 *in vitro*. At doses from 1-100 μg/mL, SFN dose-dependently decreased urease activity and viability of *H. pylori* (Fig 3). These results suggest that SFN shows antibacterial activity against *H. pylori in vitro.*

2. Effect of SFN on *H. pylori* Colonization and Gastric Mucosal Inflammation in *H. pylori*-Infected Mice *in vivo*

Based on the *in vitro* experimental data, we conducted the next series of studies to determine if SFN inhibited colonization of *H. pylori* and mitigated inflammation in *H. pylori*-infected gastric mucosa in mice *in vivo*.

Gastric mucosal infections with *H. pylori* Sydney Strain-1 were established in 6-week-old female C57BL/6 mice of both wild-type (Nrf2+/+) and Nrf2 knockout (Nrf2-/-) strains by inoculation with 5×10^7 CFU of *H. pylori* [24]. The *H. pylori*-infected mice were fed a high-salt diet (7.5% NaCl) for 2 months in order to exacerbate inflammation in gastric corpus mucosa [11]. The wild-type and Nrf2-/- mice were fed the homogenized BS (+BS), or no BS (−BS). Approximately 3 μmol/mouse/day of SGS was administered to the +BS group. Eight weeks later, all mice were sacrificed. Gastric mucosal preparations were fixed with formalin, stained with hematoxylin and eosin, and examined using light microscopy. The degree of gastric mucosal inflammation was measured using the updated Sydney system [25]. Activities of the gastric mucosal phase 2 detoxification enzymes, NQO1 and GST, were measured by ELISA. *H. pylori* colonization of mice gastric mucosa was assessed as described in our previous report [4].

1) Effect of BS Treatment on Gastric Mucosal Inflammation

Morphological examination by light microscopy of the gastric mucosae in *H. pylori*-infected Nrf2+/+ mice showed massive infiltration of inflammatory cells in the mice not receiving BS, while the mice fed BS showed less inflammation (Fig 4).

Following the administration of BS, activation of the antioxidant enzymes NQO1 and GST increased significantly in Nrf2+/+, but not Nrf2-/- mice, as expected (26). In agreement with these findings, inflammation of the gastric corpus mucosa in *H. pylori*-infected mice was substantially attenuated by treatment with BS in Nrf2+/+, but not in Nrf2-/- mice (Fig 4, Fig 5).

2) Effect of BS Treatment on H. pylori Colonization

BS treatment induced an almost 2-log reduction in *H. pylori* colonization in wild-type mice but not in Nrf2-/- mice (Fig. 5), thus confirming the integral role of the SFN-induced Nrf2 activation in protection against *H. pylori*-induced gastric inflammation.

3. Effect of Daily Intake of SFN-Rich BS on *H. pylori* Infection in Human Subjects

Based on the *in vivo* experimental data from *H. pylori*-infected mice, we conducted the next experiments to determine if daily intake of SFN-rich BS mitigated *H. pylori*-induced gastritis in human subjects.

Fifty *H. pylori*-positive volunteers, whose endoscopy showed no abnormalities other than gastritis, were randomized to either the BS group (n=25) or the alfalfa sprouts (AS) group (n=25). In this study, AS were used as the placebo, since they do not contain SGS or other isothiocyanates (Fig 6). Subjects were instructed to consume 70 g/day of SGS-rich 3-day-old BS (Broccoli Super Sprout®, Murakami Pharm Ltd, Japan) for 8 weeks; these sprouts were validated to have an SGS content of approximately 6 μmol/g [27, 28]. Subjects in the AS (placebo) group were instructed to consume an equivalent amount of AS for 8 weeks. All participants were required to attend the hospital for collection of blood and stool samples at 0, 4, 8, and 16 weeks (eight weeks after the completion of the intervention); the dates corresponded to study days 0, 28, 56, and 112, respectively. Stool samples were analyzed for *H. pylori* stool antigen (HpSA) using an HpSA-ELISA kit from Meridian Bioscience, Inc., as previously described [29]. Serum pepsinogens I and II (PGI and PGII) were measured in blood samples collected from volunteers at these same time points, and the PGI/PGII ratio was computed [30, 31]. All test results were compared using a Student's *t*-test. Error bars on all figures represent ±1 SD from the mean.

The study protocol (outlined in Fig. 6) randomized 50 subjects to daily consumption of either 70 g of BS or a placebo (AS). The mean age of subjects at randomization was 54.5 years. There were more female (n=28) than male (n=19) subjects, but there was no difference in pre-intervention *H. pylori* infection and inflammation status for the two groups (Table 1). Two subjects in the AS group dropped out during the intervention period owing to acute viral infection. Thus, the data obtained from 25 subjects in the BS group and 23 in the AS group were analyzed.

1) Effects of BS/AS Treatment on Serum PGI and PGII

During the intervention period, significant reductions in both PGI and PGII ($P < 0.05$) compared with baseline levels were only observed in the BS group, and these returned to baseline values 2 months after the intervention. The ratio of PGI to PGII, used as a more robust indicator of changes in gastric inflammation (31), increased significantly ($P < 0.05$) during the intervention in the BS group (Fig. 7).

2) Effects of BS/AS Treatment on HpSA

The HpSA levels measured in the BS intervention arm were significantly lower ($P < 0.05$) during the intervention than at baseline, and returned to baseline levels ($P < 0.05$) at 16 weeks (2 months after intervention). The placebo group receiving AS showed no significant change in HpSA (Fig.8). Of the 25 subjects in the BS treatment group, eight subjects had HpSA values below the cutoff (0.100) at the end of the 8-week BS treatment period. In six of these subjects, the HpSA values became positive again at 8 weeks after cessation of BS consumption, and the HpSA values of the remaining two subjects became positive again, 6 months after intervention, thus indicating that BS treatment reduced *H. pylori* colonization but did not result in complete eradication.

4. Discussion

This study was designed to determine whether regular dietary consumption of BS, which is rich in the SFN precursor SGS, inhibited *H. pylori* colonization and attenuated inflammation in the gastric mucosa of mice and humans.

In this study, we first established that oral intake of BS decreased gastric mucosal inflammation *in vivo* in a well-established *H. pylori*-infected animal model. Oral dosing of animals in this model (approximately 3 μmol/mouse/day) was consistent with the SFN dosages in a variety of other mouse experiments investigating carcinogenesis [21,32-35].

Second, the findings strongly suggest that mitigation of gastritis by SFN was at least partially due to the induction of antioxidant enzymes via the Nrf2 signaling pathway. SFN is a well-known activator of antioxidant enzymes and we have shown that these enzymes are upregulated in BS-treated animals.

Third, we determined that *H. pylori* colonization decreased in SFN-treated wild-type mice but not in Nrf2-/- mice. This *in vivo* finding suggests that SFN may have a direct antibiotic effect on the level of *H. pylori* colonization. The primary effect may occur via the upregulation of the host's systemic protection against oxidative stress and inflammation, which results in reduced *H. pylori* colonization. The mechanisms for the detoxification effects of SFN have been studied extensively [1, 17, 36-38]. SFN induces cytoprotective, antioxidant, and anti-inflammatory enzymes via the transcription factor Nrf2, which activates the genes that control these endogenous protective responses [20]. *H. pylori* infection generates a variety of reactive oxygen species within the mucosa that enhance gastric mucosal injury and inflammation. Our data show that *H. pylori*-induced gastritis in human subjects is mitigated by BS treatment. The data suggest that this effect is induced either by the inhibition of *H. pylori* colonization (*in vitro*, SFN is a potent antibiotic against *H. pylori*), the upregulation of Nrf2-dependent antioxidant enzyme activity (a number of *in vitro*, animal, and clinical studies have reported this), or by a combination of these two factors. Nonetheless, the SFN-induced enhancement of Nrf2-dependent antioxidant enzyme activity in gastric mucosal cells reduced reactive oxygen species from gastric mucosa, which resulted in the mitigation of *H. pylori* infection-induced gastritis.

Fourth, daily intake of BS (70 g) for 2 months decreased serum levels of PGI and PGII and increased the PGI/PGII ratio during the 2-month intervention period, which was consistent with clinical observations correlating to increased PGI/PGII ratio with reduced inflammation of gastric mucosa [30, 31]. We also measured reductions in HpSA (an indicator of recent infection) after the intake of BS. All biomarkers returned to baseline levels after the intervention was discontinued.

In summary, our data on *H. pylori*-infected mice and humans clearly suggested that SFN may have a direct antibacterial effect on *H. pylori*, leading to reduced gastritis, and a systemic effect by increasing the antioxidant response. It is not possible to determine the relative contributions of these two mechanisms from this study; however, in light of other evidence which suggests a strong anti-*H. pylori* effect of SFN *in vitro* [2], the findings in this study suggest that SFN has promise both as an antibacterial agent directed against *H. pylori* and a dietary preventive agent against the development of human gastric cancer.

Roles of SFN in Protection of the Small Intestine against Aspirin/NSAID-induced Mucosal Damage

The current prevalence of the prescription of aspirin and/or other NSAIDs to prevent cardiovascular events and relieve pain from osteoarthritis [39-41] is rising. It is well-established that aspirin and/or NSAIDs frequently cause peptic ulcer disease and bleeding from the upper GI tract. In order to prevent aspirin and/or NSAID-induced GI mucosal injuries, potent acid inhibitors that significantly ameliorate these injuries, such as proton pump inhibitors (PPI), have been prescribed [42]. However, recent advances in capsule video endoscopy have revealed that the ulcers caused by aspirin and/or NSAIDs were not only localized to the upper GI tract, but also the small intestine [43-45]. Studies in human volunteers using capsule endoscopy have shown PPIs do not offer effective protection of the small intestinal mucosa against injury [46]. A recent study in rat has shown that PPI exacerbates NSAID-induced ulcers in the small intestine [47]. Although misoprostol has some beneficial effects on NSAID-induced gastrointestinal ulcers [48,49], it causes several adverse reactions such as abdominal pain, diarrhea, and abortion [50]. A recent study in rats has shown that geranylgeranylacetone (GGA), a mucosal protective agent known to induce HSP70 in gastrointestinal mucosa, prevents small intestinal mucosa from injury induced by loxoprofen [51]. A few clinical trials using a small number of human volunteers have shown that aspirin/NSAID-induced small intestinal injuries detected by capsule video endoscopy are ameliorated by GGA [52] or rebamipide [53,54], both of which have been used as gastric mucosal protective agents. Currently, however, there is no direct evidence that such agents are clinically effective for protection against NSAID-induced small intestinal ulcers. In the present study, we investigated the phytochemical sulforaphane (SFN) as a possible candidate drug for prevention of NSAID-induced small intestinal injuries. Since activation of antioxidant enzymes by SFN persists for 48-72 h, the protective effects of SFN against oxidative stress may be more potent than those of other antioxidant substances are. As NSAIDs induce oxidative stress in GI mucosa, it is reasonable to assume that SFN may be useful for protection of the small intestinal mucosa against oxidative stress induced by NSAIDs. Recent studies have shown that NSAID exaggerates small intestinal injuries, at least in part by causing overgrowth of anaerobic bacteria in the intestinal lumen [55,56]. Therefore, it seems reasonable to assume that SFN may inhibit colonization of anaerobic bacteria in the intestinal lumen, thereby preventing invasion of the bacteria into the mucosa and consequently mitigating small intestinal injuries. Therefore, we decided to determine if SFN could protect small intestinal mucosa from NSAID-induced small intestinal injuries.

1. Role of SFN in Protection of IEC6 cells against Aspirin-induced Injury *in vitro*

IEC6 cells, derived from rat small intestinal mucosa, were incubated with or without various doses of SFN. After incubation for 12 h, the cells were treated with various dosages of aspirin. In some experiments, the effect of zinc protoporphyrin-IX (ZnPP), an inhibitor of HO-1, was also examined. The viability of the IEC6 cells was assessed by the measurement of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) incorporation into the cells. Expression of HO-1 protein by IEC6 cells were evaluated by western blot analysis, using a polyclonal antibody to HO-1 (Hsp 32) as the primary antibody.

1) Effects of Aspirin on Viability of IEC-6 Cells

Aspirin, at doses between 10 to 40 mM, dose-dependently decreased viability of IEC-6 cells after incubation for 12 h (data not shown). Therefore, in the next series of experiments, we decided to use 20 mM aspirin, which is a submaximal dose to cause injury in IEC-6 cells.

2) Effect of Pretreatment with SFN on Aspirin-Induced Injury in IEC6 Cells

We confirmed that incubation of the cells with 5 µM SFN for 6 h did not cause injury to IEC-6 cells, but did induce HO-1 expression, as discussed later. Therefore, we decided to use 5 µM SFN in the following experiments. In this series of experiments, the cells were initially exposed to 5 µM SFN for 6 h, followed by incubation without SFN for a further 12 h. Then, the cells were exposed to various doses of aspirin. Pretreatment of the cells with SFN attenuated the 20 mM-aspirin-induced death of IEC6 cells (Fig 9).

3) Effect of Pretreatment with SFN on HO-1 Expression in IEC-6 Cells

The cells were exposed to 5 µM SFN for 6 h, followed by incubation without SFN for a further 12 h. Western blot analysis showed that HO-1 expression in IEC-6 cells increased significantly after incubation with SFN for 6 h, and the effects persisted at 12 h after removal of SFN from the medium (Fig 10). These results indicated that pretreatment with SFN upregulated HO-1 expression, and that the effect persisted during the subsequent exposure to aspirin.

4) Effects of HO-1 Inhibitor on the Protective Effects of SFN Against Aspirin-Induced Cell Injury

The cells were exposed for 6 h to 5 µM SFN in combination with 0.1 µM ZnPP, an HO-1 inhibitor, followed by incubation without SFN and ZnPP for a further 12 h. After this, the cells were exposed to 20 mM aspirin. Co-administration of ZnPP with SFN tended to attenuate the protective effects of SFN on the aspirin-induced IEC6 cell injury (Fig 11), which indicated that HO-1 may contribute to the protective effect of SFN against aspirin-induced cell injury. Further experiments are required to confirm this assumption.

2. Role of SFN in Protection of the Small Intestinal Mucosa against Indomethacin (IND)-Induced Injury *in vivo*

Male ddY mice aged 7 weeks were used in this study. Small intestinal injuries were induced by two subcutaneous injections of IND (20 mg/kg), as described elsewhere [57]. The time interval between the first and second IND injections was 24 h. The mice were sacrificed 6 h after the second IND injection. The small intestinal mucosa was carefully examined for lesions under a dissecting microscope.

MPO activity was measured by a previously described method [58]. Vascular permeability was assessed by measuring the amount of leakage of Evans blue into the small intestinal tissue, after intravenous injection of the dye at 1 h before sacrifice [59]. Enterobacteria within the small intestinal mucosa were quantified by a previously described method [60]. In brief, the homogenates of the small intestinal mucosa were placed on Gifu anaerobic medium agar and incubated for 24 h under anaerobic conditions. The number of enterobacteria was expressed as log CFU/g of tissue. SGS was orally administered at 0.5 h before and 6 h after the administration of IND.

1) Effects of IND on Small Intestinal Mucosae

Treatment of the mice with IND induced small bowel shortening, increases in the lesion score (Fig 12), vascular permeability (Fig 13), and MPO activity (Fig 14); this suggested that treatment with IND caused small intestinal mucosal injury in mice.

2) Effect of SGS on the IND-induced Injuries in Small Intestinal Mucosa

Oral administration of SGS did not affect the lesion score, vascular permeability, or mucosal MPO activity in the IND-untreated negative control mice. In contrast, pretreatment of the mice with orally administrated SGS inhibited the increases in lesion score, vascular permeability, and MPO activity in the IND-treated mice (Fig. 12, 13, 14), suggesting that SGS attenuates IND-induced small intestinal injuries in mice.

3) Effect of SGS on Anaerobic Enterobacterial Count in Small Intestinal Mucosa of IND-Treated Mice

Oral administration of SGS significantly inhibited the overgrowth of anaerobic enterobacteria in IND-untreated mice. Furthermore, pretreatment of the mice with SGS completely abolished the increase in the overgrowth of anaerobic enterobacteria the IND-treated mice (Fig. 15).

3. Discussion

The data from *in vitro* experiments show adaptive cytoprotection by SFN against aspirin in IEC6 cells. We have confirmed that SFN enhanced HO-1 expression in small intestinal mucosa in this experimental system. Furthermore, the present finding that the protective effects of SFN are mitigated by an HO-1 inhibitor, ZnPP, strongly support our hypothesis that SFN affords adaptive cytoprotection against aspirin via Nrf2-dependent pathways. It has been reported that aspirin causes oxidative stress in cells by damaging mitochondrial respiration [61]. Thus, we assume that pretreatment with SFN enhances the antioxidant system in the cells, thereby eliminating oxidative stress induced by aspirin. Our data from *in vivo* studies also showed that pretreatment with orally administrated SGS protects small intestinal mucosa against IND-induced injury, suggesting that SFN also provides adaptive cytoprotection to the small intestinal mucosa against IND-induced injury *in vivo*. The present study showed that SFN enhanced HO-1 expression *in vitro*. We also confirmed that SGS treatment enhanced HO-1 expression in small intestinal mucosa in mice *in vivo* (unpublished observations). In addition, the present study also indicated that SGS inhibited invasion of anaerobic enterobacteria into the mucosa *in vivo*. Thus, it seems reasonable to assume that SFN and/or SGS have twofold beneficial effects on small intestinal mucosa against IND-induced small intestinal injury.

It has been reported that mild irritants protect gastric mucosa from the ensuing severe stress by induction of endogenous prostaglandins, a phenomenon named “adaptive cytoprotection” by Andre Robert [62]. However, a number of other studies have shown that “adaptive cytoprotection” cannot be explained by endogenous prostaglandins. More recent studies have suggested possible candidates for adaptive cytoprotection. For example, it has been shown that mild stress induces heat shock proteins in gastric mucosa, thereby protecting the mucosa from severe stress, which also accounts for “adaptive cytoprotection” [63]. Alternatively, Yamamoto *et al.* have recently demonstrated that mild oxidative stress induces antioxidant enzymes via the Nrf2-Keap1 system, which strengthen antioxidant activity of the cells against more severe oxidative stress [64]. Since NSAIDs cause oxidative stress in cells [62,65], it is reasonable to assume that SFN protects GI mucosa from NSAID-induced oxidative stress by strengthening Nrf2-Keap1 mediated antioxidant systems. Our findings strongly support this hypothesis [5]. In contrast, recent studies have suggested a role of anaerobic enterobacteria in the pathogenesis of NSAID-induced intestinal mucosa [47,55,56], which indicated that anaerobic bacteria generally exacerbate NSAID-induced injury in the small intestine, although the exact mechanisms remain to be elucidated. As SFN shows antibacterial activity against *H. pylori* [1,4], it may be possible that SFN also demonstrates antibacterial activity against anaerobic enterobacteria in the small intestine, thereby ameliorating NSAID-induced small intestinal injury. In conclusion, we propose that SFN is a strong candidate for the prevention or treatment of aspirin/NSAID-induced small intestinal ulcers in clinical practice. Clinical trials of the effects of SFN on NSAID-induced injury in the small intestine of humans should be conducted imminently.

In summary, our data suggest that the activation of Nrf2-Keap1 dependent antioxidant enzyme activity by SFN contributed to protection of small intestinal mucosa against NSAID-induced oxidative stress. Our data also indicated that SFN ameliorates NSAID-induced small intestinal injuries by suppression of the invasion of anaerobic bacteria into the mucosa.

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Figure Legends

Figure 1. Broccoli Sprouts Contain High Concentration of Sulforaphane (SFN)

SFN, a member of the isothiocyanate (ITC) family, is abundant in cruciferous vegetables, especially broccoli sprouts (BS). (A) Fifty grams of raw BS contain 128 mg of sulforaphane glucosinolate, a precursor of SFN. (B) SFN possesses -N=C=S, which is a common chemical structure found in the ITC family. This molecular structure accounts for the pungency of the cruciferous vegetables.

Figure 2. Sulforaphane (SFN) Enhances Antioxidant Activity via Nrf2-Keap1 System.

SFN potently induces various antioxidant (or phase 2) enzymes, such as glutathione S-transferase (GST), heme oxygenase-1 (HO-1), and NAD(P)H: quinone oxidoreductase 1 (NQO1), via Nf-E2 related factor 2 (Nrf2) - Kelch-like ECH-associated protein 1 (Keap1) dependent pathways. These are responsible for the enhanced antioxidant activity of the cells.

Figure 3. Sulforaphane (SFN) Markedly Inhibits Urease Activity and Viability of *H. pylori*

The *H. pylori* strain Sydney Strain-1 (SS-1) was used in this study. The urease activity of *H. pylori* was assessed by measurement of the concentration of ammonia released into the medium during a 1-h incubation period with 5 mM urea. The viability of *H. pylori* was determined by the number of colony forming units (CFU) after incubation of the *H. pylori* in the absence or presence of various concentrations of SFN for 3 h. The effects of SFN on *H. pylori* viability and urease activity were examined at ambient pH 7.4 *in vitro*. At doses of 1-100 µg/mL, SFN dose-dependently decreased the viability of *H. pylori*. The representative trace in left panel shows that SFN dose-dependently inhibits *H. pylori* urease activity. The right panel shows that SFN dose-dependently decreased *H. pylori* viability. n: number of experiments. *$P < 0.05$; significant difference from the corresponding values in the absence of SFN.

Figure 4. Broccoli Sprouts Markedly Attenuate Corpus Gastritis in *H. pylori*-infected Mice Fed High Salt Diet

Gastric mucosal infections with *H. pylori* Sydney strain-1 were established in 6-week-old female C57BL/6 mice of both the wild-type (Nrf2+/+) and knockout (Nrf-/-) mice by inoculation of 5 × 10^7 CFU of *H. pylori* [24]. The *H. pylori*-infected mice were fed for 2 months with a high-salt diet (7.5% NaCl) in order to exacerbate inflammation in gastric corpus mucosa. The mice were fed with the homogenized broccoli sprouts (+BS), or without BS (−BS). Approximately 3 µmol/mouse/day of SGS were administered into the +BS group. All the mice were sacrificed at 8 weeks later. Gastric mucosal preparations, fixed with formalin and stained with hematoxylin and eosin, were examined by light microscopy.

A representative histology of gastric mucosa of the *H. pylori*-infected mice without BS treatment shows massive infiltration of inflammatory cells (upper panel), while the histology of the mice fed with BS shows less inflammation (lower panel).

Scale bar: 200 µm.

Figure 5. Broccoli Sprouts Attenuate Corpus Gastritis and Inhibits *H. pylori* Colonization in Nrf2+/+, but not in Nrf2-/- mice

The wild-type (Nrf2+/+) and the Nrf2 knockout (Nrf2-/-) mice infected with *H. pylori* were fed with broccoli sprouts (BS) (+BS; ■), or without BS (-BS; □) (as described in Fig 4 legend). The degree of inflammation in gastric corpus mucosa was expressed as the inflammation score as defined in the updated Sydney system [25]. *H. pylori* colonization was expressed as the number of *H. pylori* colony forming units (CFU) after incubation of the mucosal homogenates in the specific medium for *H. pylori* culture, described in our previous report [4]. The left panel shows that feeding with BS significantly mitigated both the inflammation score (left panel) and the colonization (right panel) in Nrf2+/+, but not in Nrf2-/- mice (*$P < 0.05$; significant difference from the corresponding values in the absence of BS; n=number of animals).

Figure 6. Protocol for the Broccoli Sprouts Clinical Trial on *H. pylori* Infection

Fifty *H. pylori*–positive volunteers were randomized to either the broccoli sprouts (BS) group (n=25), or the alfalfa sprouts (AS) group (n=25). In this study, AS was used as the placebo, since AS do not contain any sulforaphane glucosinolates (SGS), the precursor of SFN. Subjects were instructed to consume 70 g/day of either the SGS-rich 3-day-old BS that contains SGS of approximately 6 μmol/g dose [27, 28], or an equivalent amount of AS for 8 weeks. All participants were instructed to attend the hospital for collection of blood and stool samples at 0, 4, 8, and 16 weeks. Levels of serum pepsinogens I and II (PGI and PGII) were measured by a commercially available ELISA kit, and PGI/PGII ratios were calculated [30, 31]. Stool samples were analyzed for *H. pylori* stool antigen (HpSA) using a HpSA-ELISA kit, as previously described (29) All tests results were compared using a Student's *t* test for paired comparison. Error bars on all figures represent ±1 SD from the mean.

Figure 7. Effect of Broccoli Sprouts (BS) and/or Alfalfa Sprouts (AS) on Serum Pepsinogen I/II Ratio

There were significant reductions in both PGI and PGII compared with baseline levels during the 8-week intervention period in the BS group, and there was a return to baseline values at eight weeks after the intervention. Since the magnitude of the reduction in PGII was greater than PGI, the ratio of PGI to PGII (PGI/PGII) rose significantly during the intervention in the BS group, suggesting that BS treatment mitigates gastric inflammation. In contrast, there were no changes in PGI/PGII throughout the entire study period in AS group. Rectangular overlays represent means for each time period. n=number of participants.

$P < 0.05$; Significant difference from the corresponding values at week 0.

$P < 0.05$; Significant difference from the corresponding values at week 8.

Figure 8. Effects of Broccoli Sprouts (BS) and/or Alfalfa Sprouts (AS) on *H. pylori*-Specific Stool Antigen (HPSA)

There was a significant reduction in of *H. pylori*-Specific Stool Antigen (HpSA) compared with baseline levels during the 8-week intervention period in the BS group, and there was a return to baseline values at eight weeks after the intervention, suggesting that BS treatment reduces *H. pylori* colonization, but does not eradicate *H. pylori* completely. In contrast, there were no changes in the HpSA levels in the AS group, throughout the whole study period. Rectangular overlays represent means for each time period. n=number of participants.

$P < 0.05$; Significant difference from the corresponding values at week 0.

$P < 0.05$; Significant difference from the corresponding values at week 8.

Figure 9. Sulforaphane (SFN) attenuates Aspirin-Induced Injury in IEC6 Cells

The cells were initially exposed to 5 μM SFN for 6 h, followed by incubation without SFN for a further 12 h. The cells were subsequently exposed to various doses of aspirin. Pretreatment with 5 μM SFN attenuated aspirin (20 mM)-induced decrease in the viability of IEC6 cells. The data are expressed as Mean ± SEM. n= number of experiments; * $P < 0.05$; statistically significant difference from the corresponding value in the absence of SFN.

Figure 10. Sulforaphane (SFN) Up-Regulates Heme Oxygenase (HO)-1 Expression in IEC6 Cells

The cells were exposed to 5 μM SFN for 6 h, followed by incubation without SFN for a further 12 h. Western blot analysis shows that HO-1 expression increased significantly at 6 h after incubation with 5 μM SFN, and that this effect still persists at 12 h after removal of SFN from the medium. The data are expressed as Mean ± SEM. n=number of experiments; * $P < 0.05$; significant difference from the corresponding value in the absence of SFN.

Figure 11. ZnPP, an Heme Oxygenase (HO)-1 inhibitor, Tends to Attenuate Protective Effects of Sulforaphane (SFN) against Aspirin-Induced Injury of IEC6 Cells

The cells were initially exposed for 6 h to 5 μM SFN in combination with 0.1 mM ZnPP, an HO-1 inhibitor, followed by incubation without either agent for a further 12 h. The cells were then exposed to 20 mM aspirin. Co-administration of ZnPP with SFN attenuated the protective effects of SFN on aspirin-induced IEC6 cell injury. The data are expressed as Mean ± SEM. n=number of experiments; a, $P < 0.05$; significant difference from the corresponding value in the absence of SFN; b, $P = 0.09$; difference from the corresponding value in the absence of ZnPP.

Figure 12. Oral Intake of Sulforaphane Glucosinolates (SGS) Ameliorates Indomethacin (IND)-Induced Injury of Small Intestine

Oral administration of 17 mg/kg SGS did not affect the lesion score in the IND-untreated mice. In contrast, pretreatment with SGS inhibited the increase in the lesion score in the IND-treated mice. The data are expressed as mean ± SEM. n=number of experiments; a, $P<0.05$; significant difference from the corresponding value without IND; b, $P<0.05$; significant difference from the corresponding value without SGS.

Figure 13. Oral Intake of Sulforaphane Glucosinolates (SGS) Ameliorates Indomethacin (IND)-Induced Increase in Mucosal Permeability of Small Intestine in Mice

Oral administration of 17 mg/kg SDS did not influence the mucosal content of Evans blue in the IND-untreated negative control mice. In contrast, pretreatment of with SGS inhibited the increase in the mucosal content of Evans blue in the IND-treated mice. The data are expressed as mean ± SEM. n=number of experiments; a, $P < 0.05$; significant difference from the corresponding value without IND; b, $P < 0.05$; significant difference from the corresponding value without SGS.

Figure 14. Oral Intake of Sulforaphane Glucosinolates (SGS) Blocks Indomethacin (IND)-Induced Increase in Myeloperoxidase (MPO) Activity of Small Intestinal Mucosa in Mice

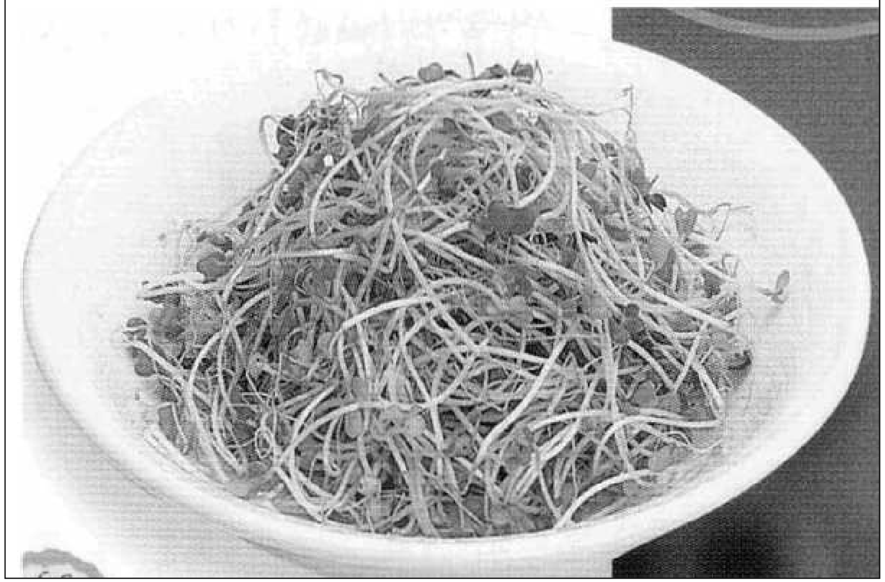
Oral administration of 17 mg/kg SDS did not affect the mucosal MPO activity in the IND-untreated mice. In contrast, pretreatment with SGS inhibited the increase in the mucosal MPO activity in the IND-treated mice. The data are expressed as Mean ± SEM. n=number of experiments; a, $P < 0.05$; significant difference from the corresponding value without IND; b, $P < 0.05$; significant difference from the corresponding value without SGS.

Figure 15. Oral Intake of Sulforaphane Glucosinolates (SGS) Inhibits Indomethacin (IND)-Induced Increase in Anaerobic Bacteria of Small Intestinal Mucosa in Mice

Oral administration of 17 mg/kg SDS significantly inhibited amount of mucosal anaerobic enterobacteria in the IND-untreated mice. Pretreatment of the mice with SGS totally abolished the increase in the mucosal amount of anaerobic enterobacteria the IND-treated mice. The data are expressed as Mean ± SEM. n=number of experiments; a, $P < 0.05$; significant difference from the corresponding value without IND; b, $P < 0.05$; significant difference from the corresponding value without SGS.

Fig 1.
Broccoli Sprouts Contain a High Concentration of SFN

Broccoli Sprouts



Sulforaphane

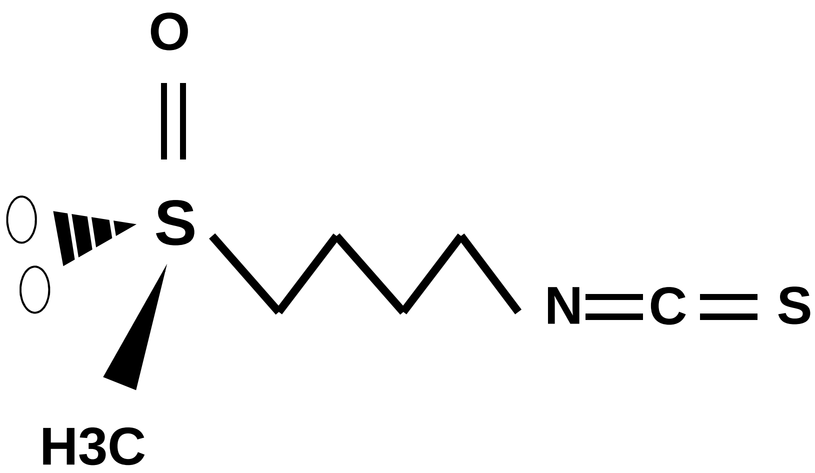


Fig 2.

SFN Enhances Antioxidant Activity via the Nrf2-Keap 1 Pathway

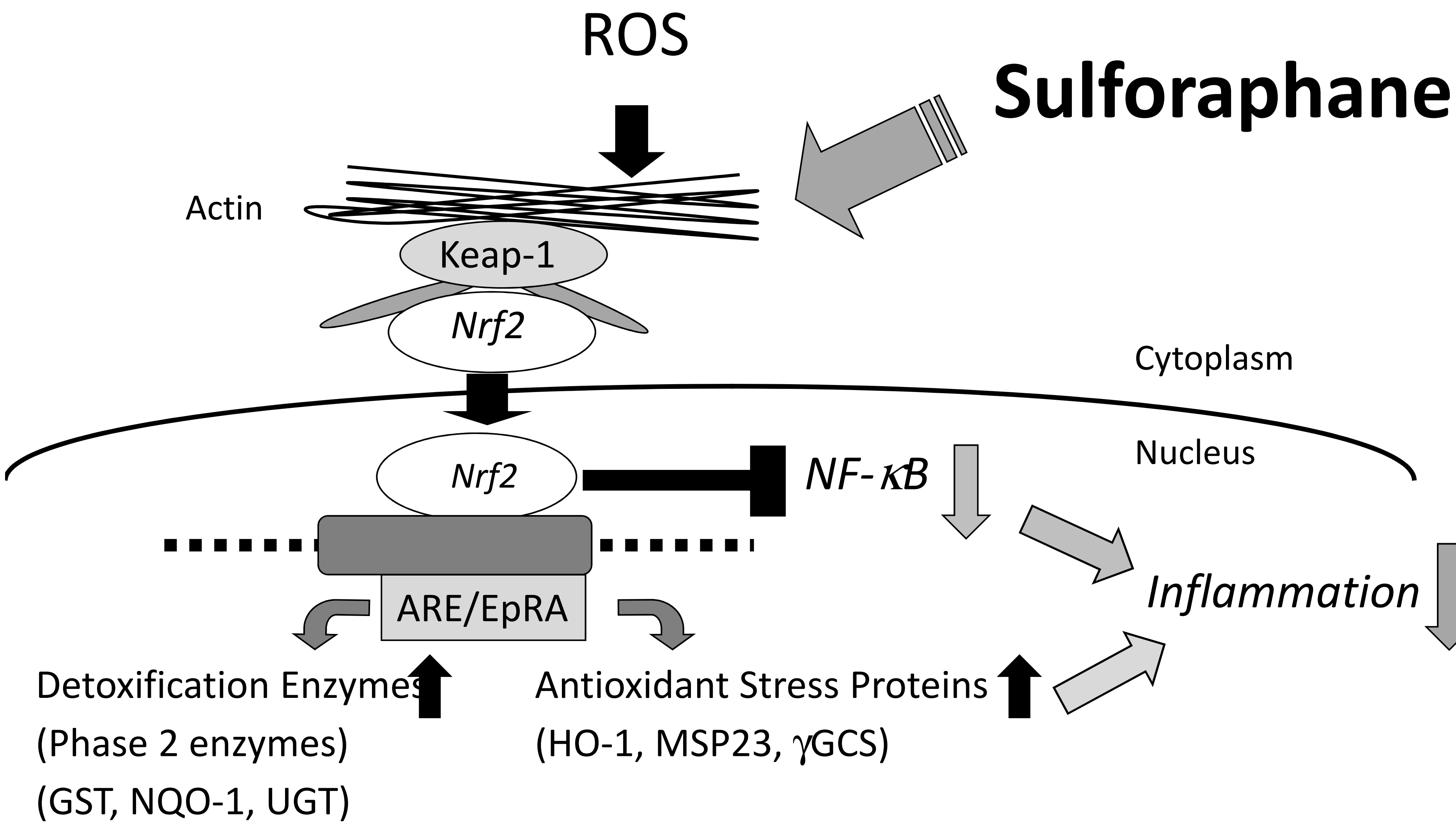
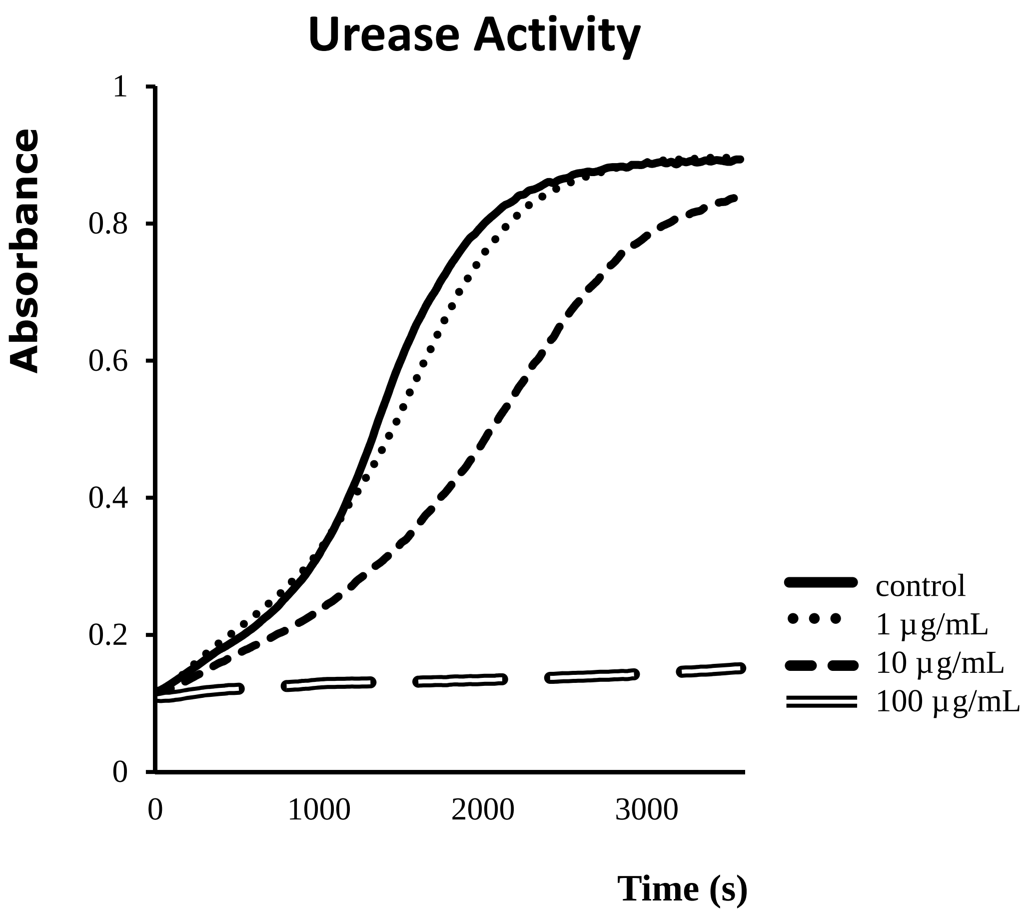


Fig 3.
SFN Markedly Inhibits Urease Activity and Viability of *H. pylori*



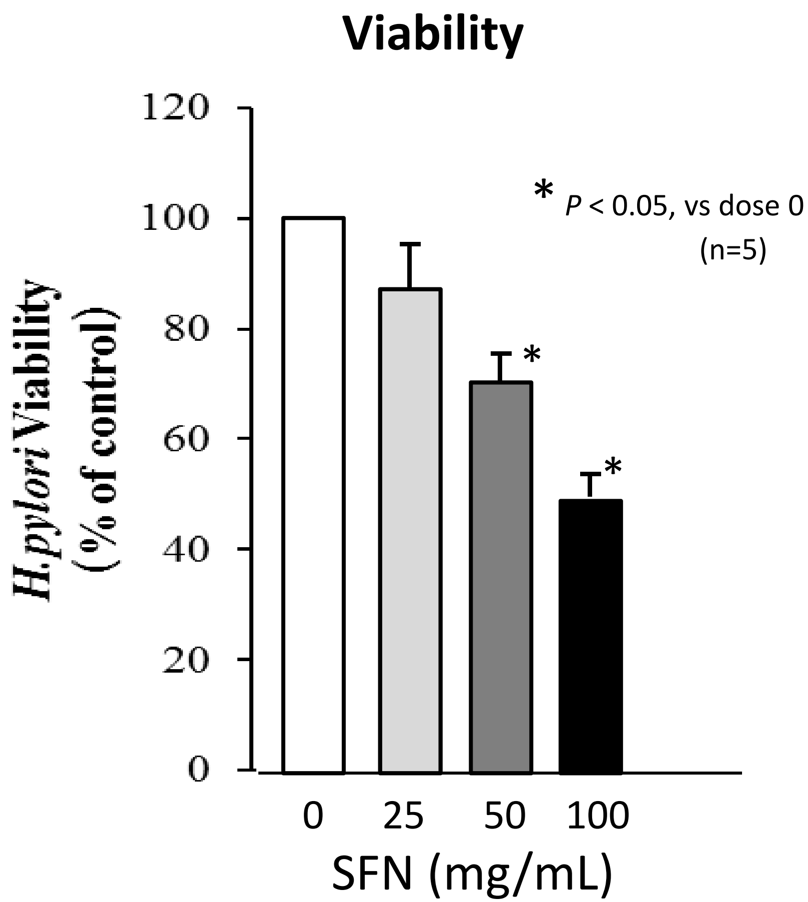


Fig 4. Broccoli Sprouts Markedly Attenuate Corpus Gastritis in *H. pylori*-infected Mice Fed a High Salt Diet

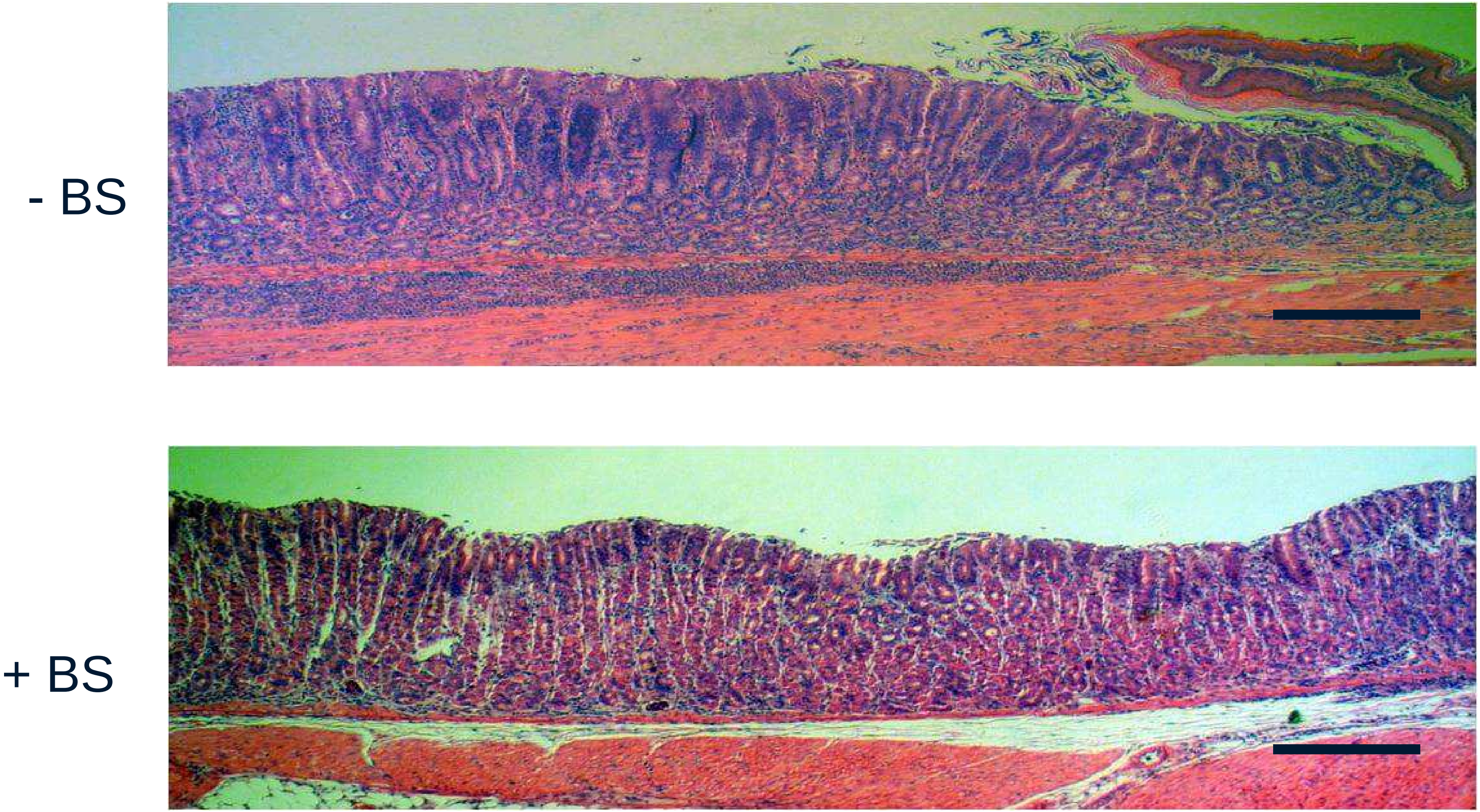


Fig 5.

Broccoli Sprouts Attenuate Corpus Gastritis and Inhibit *H. pylori* Colonization in Nrf2+/+, but not in Nrf2-/- mice

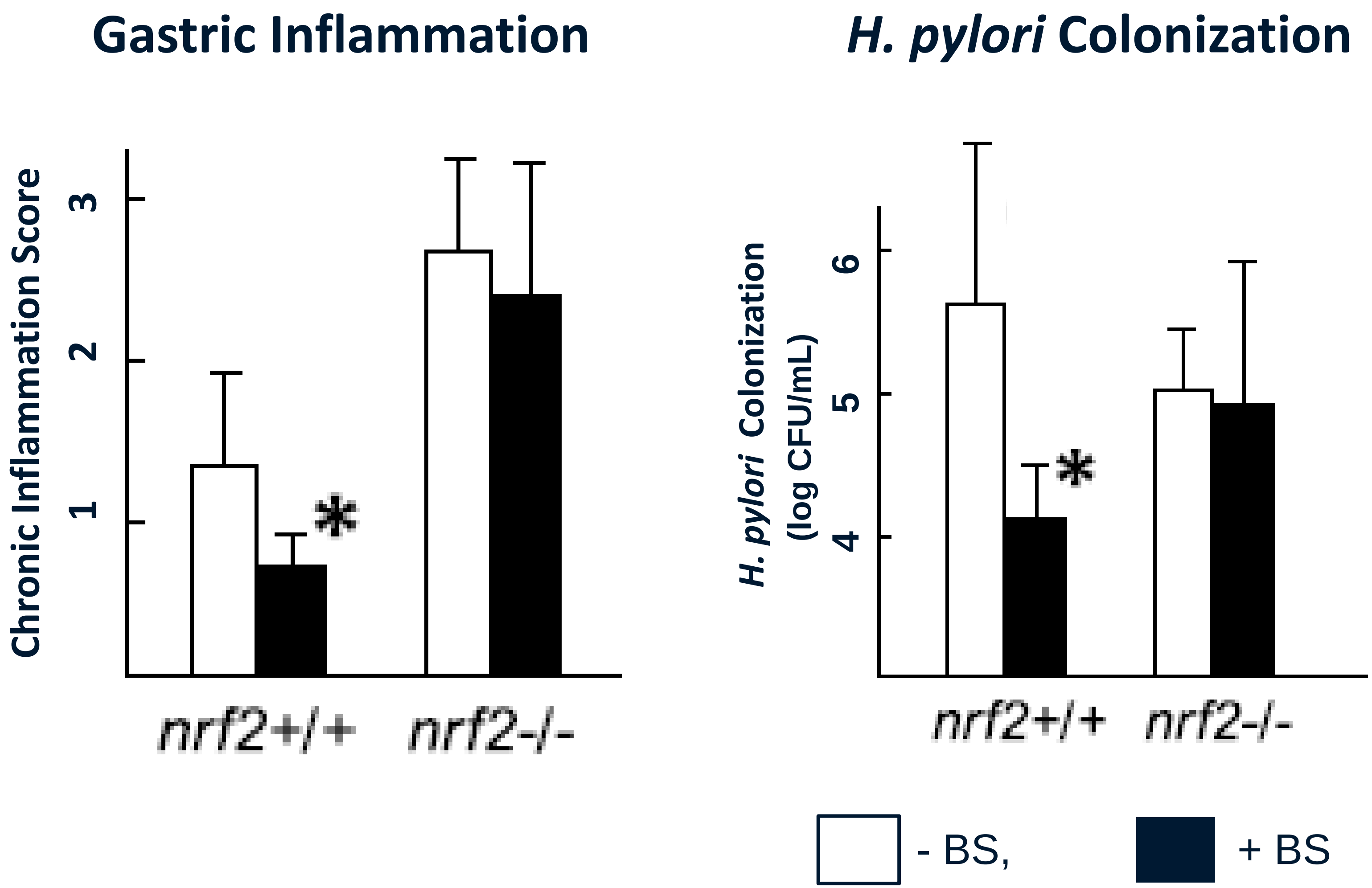
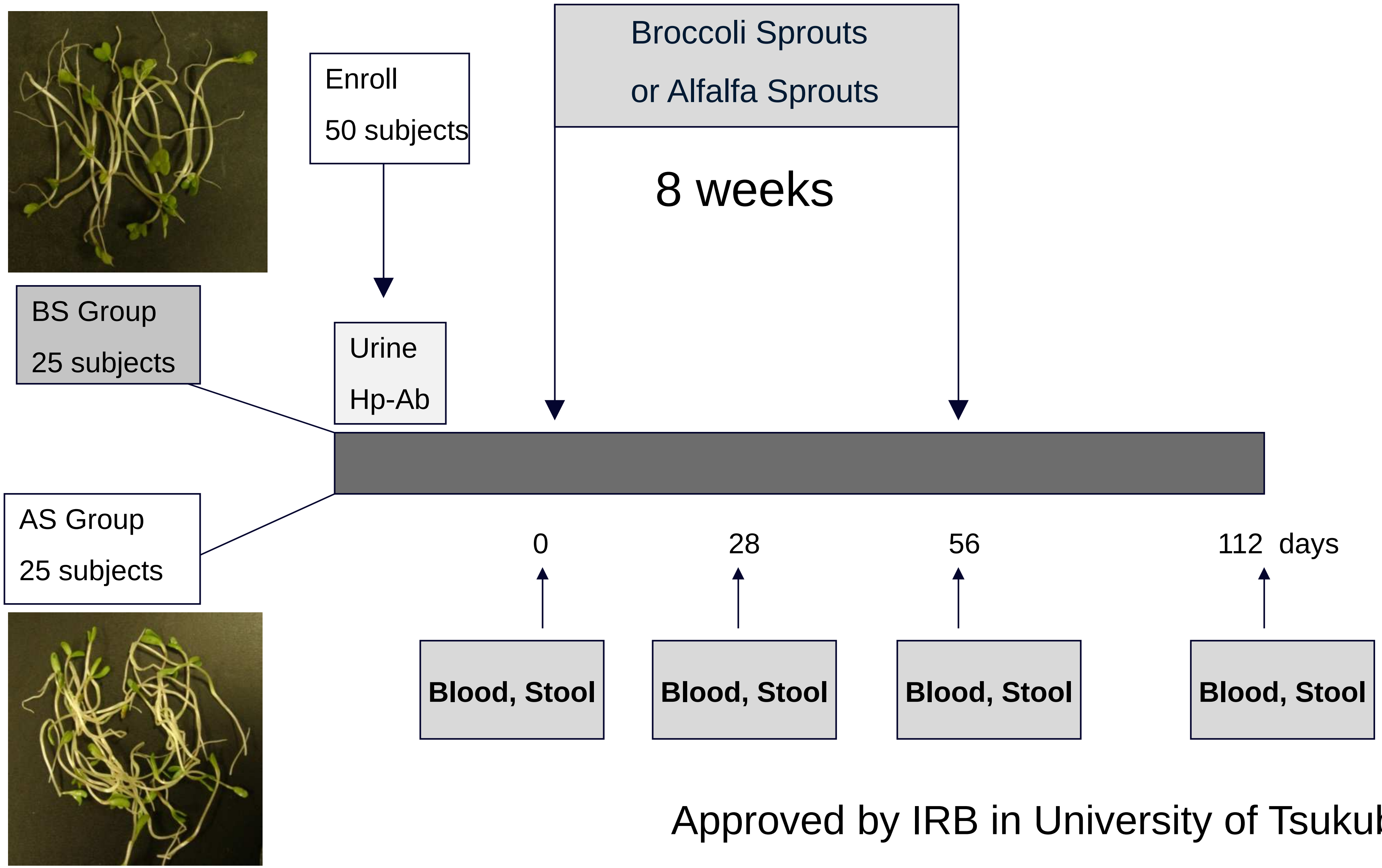


Fig 6.

Protocol for the Broccoli Sprouts Clinical Trial on *H. pylori* Infection



Approved by IRB in University of Tsukuba

Fig 7.
Effects of Broccoli/Alfalfa Sprouts on Serum Pepsinogen I/II Ratio

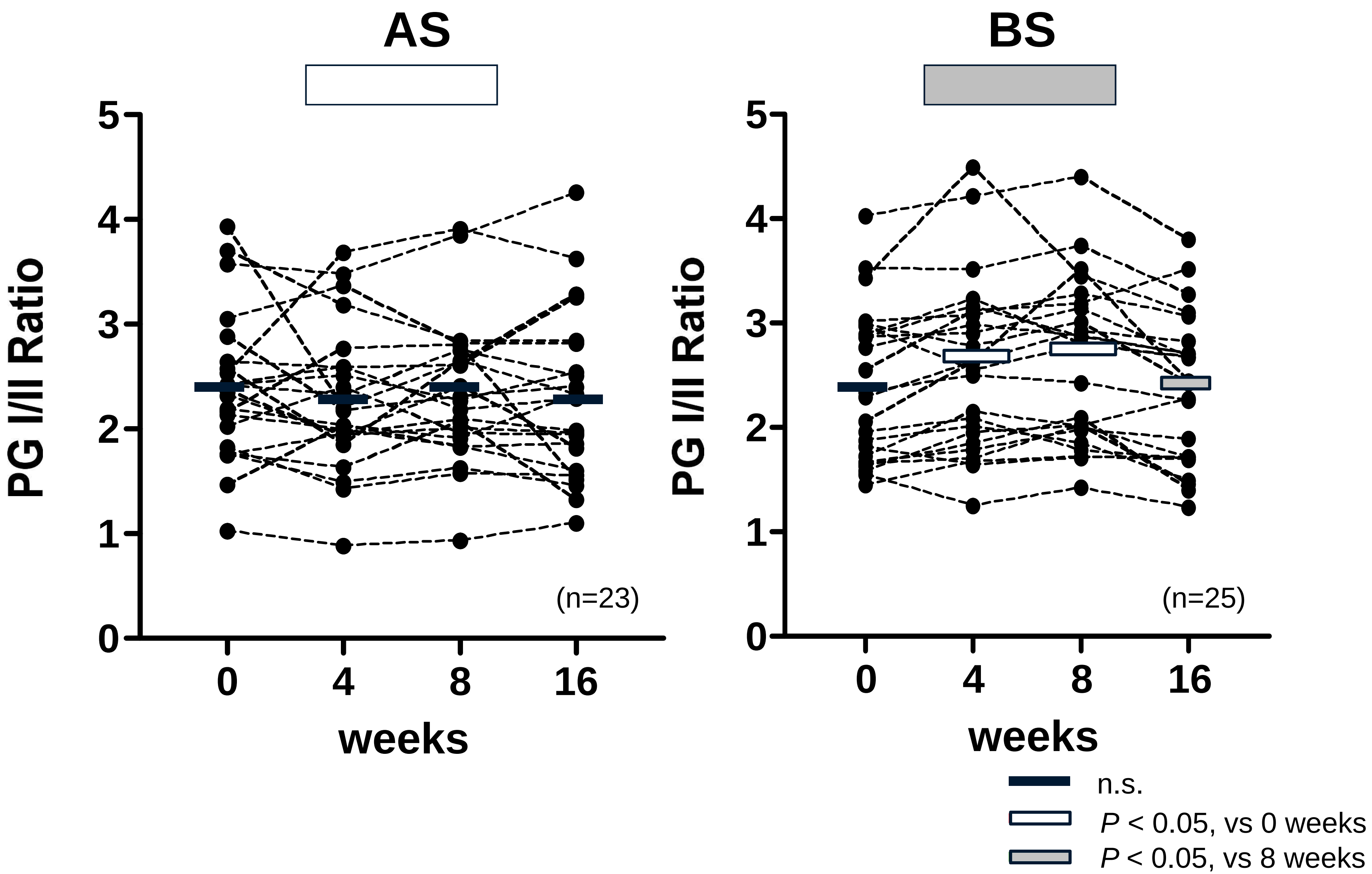


Fig 8.
Effects of Broccoli/Alfalfa Sprouts on *H. pylori*-Specific Stool Antigen

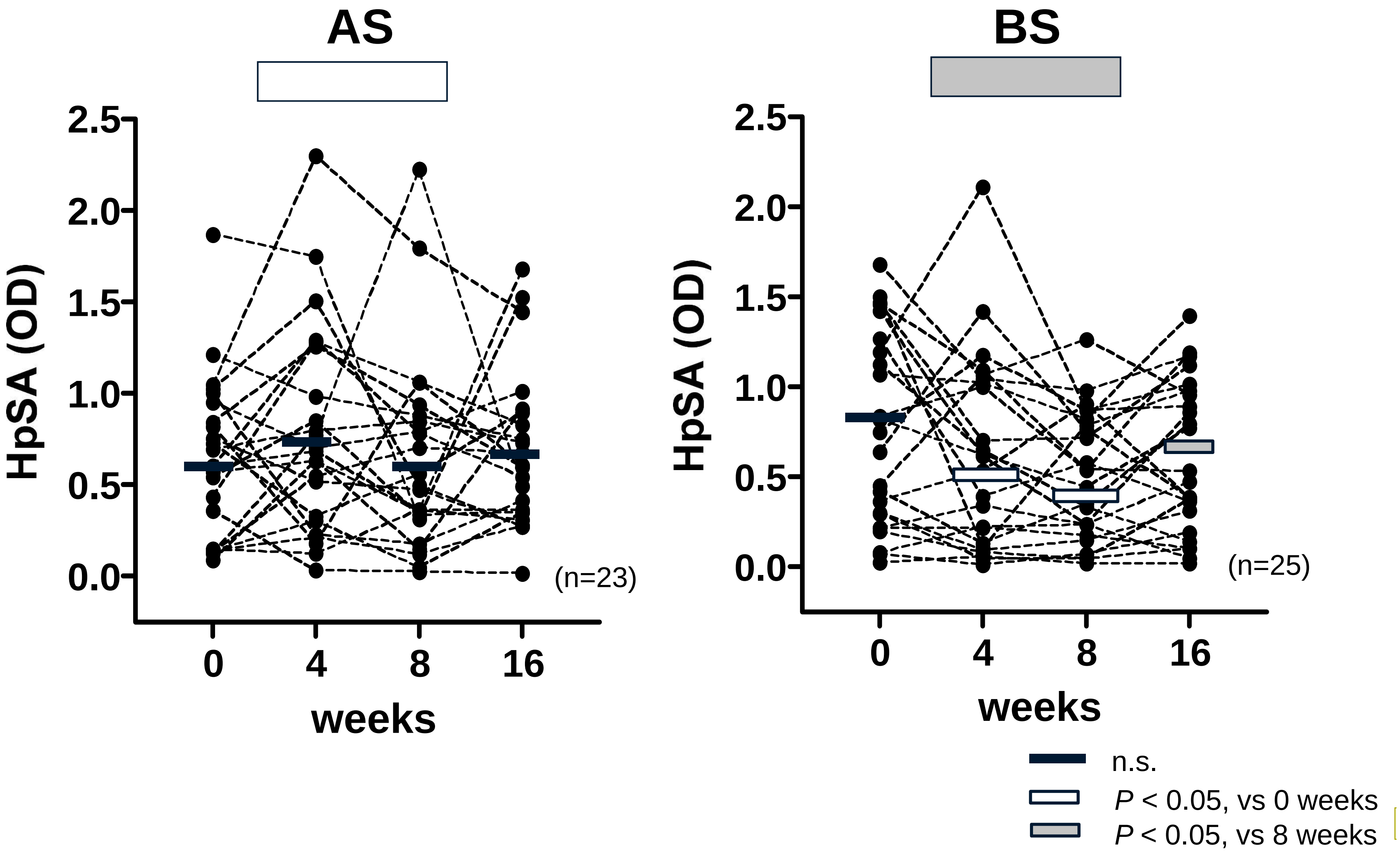


Fig 9.

SFN attenuates Aspirin-Induced Injury in IEC6 Cells

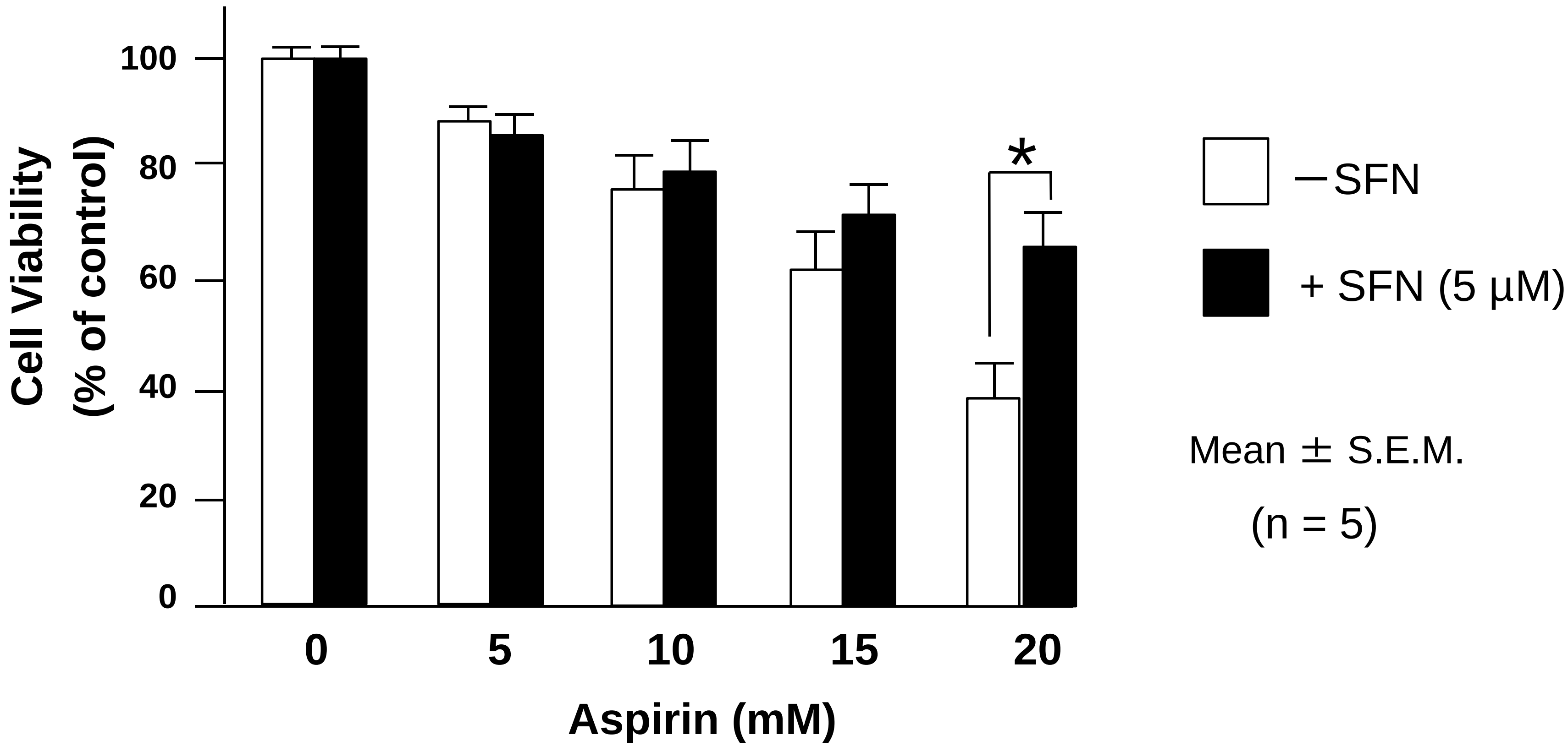


Fig 10.

SFN Upregulates HO-1 Expression in IEC6 Cells

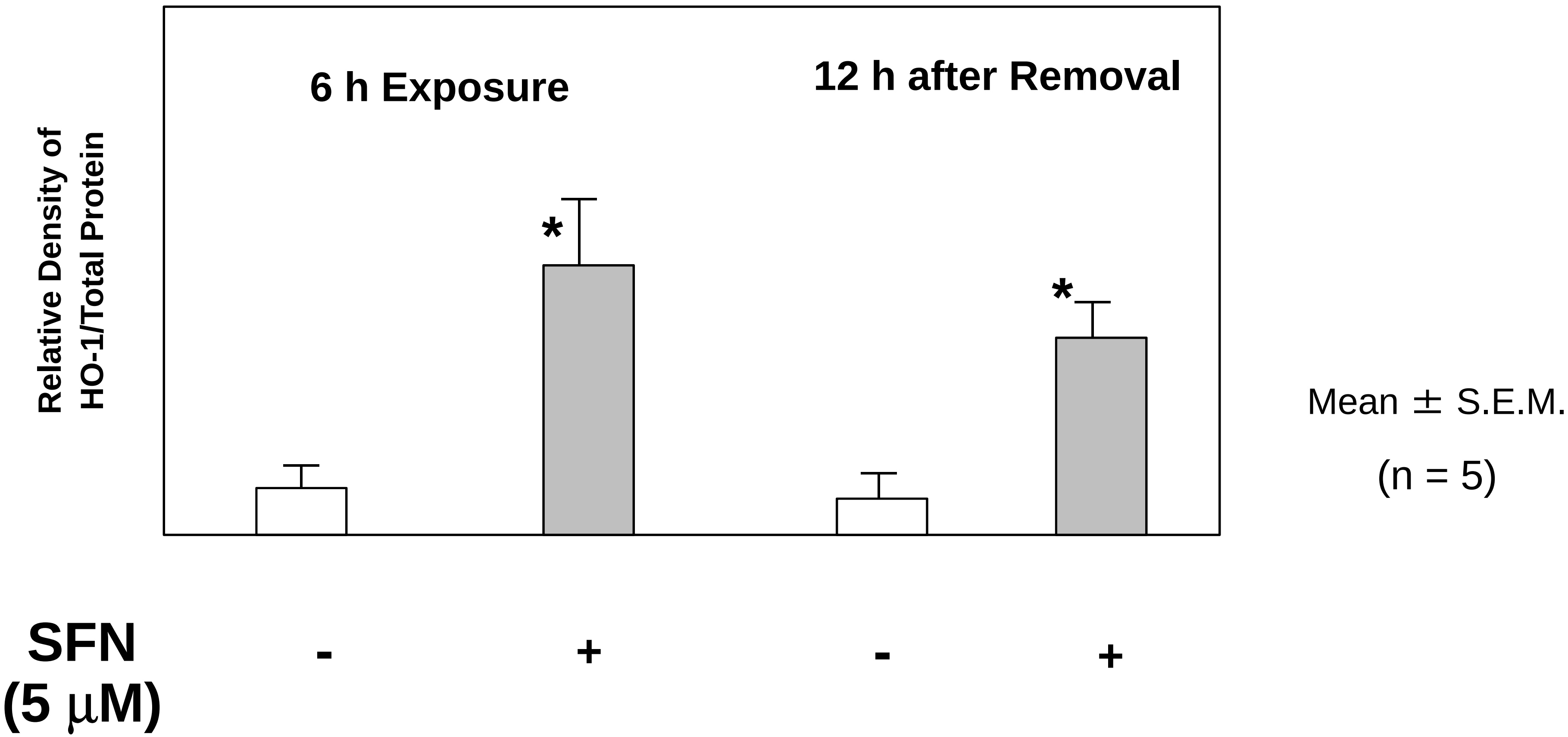


Fig 11.

ZnPP, an HO-1 inhibitor, tends to Attenuate Protective Effect of SFN against Aspirin-Induced Injury of IEC6 Cells

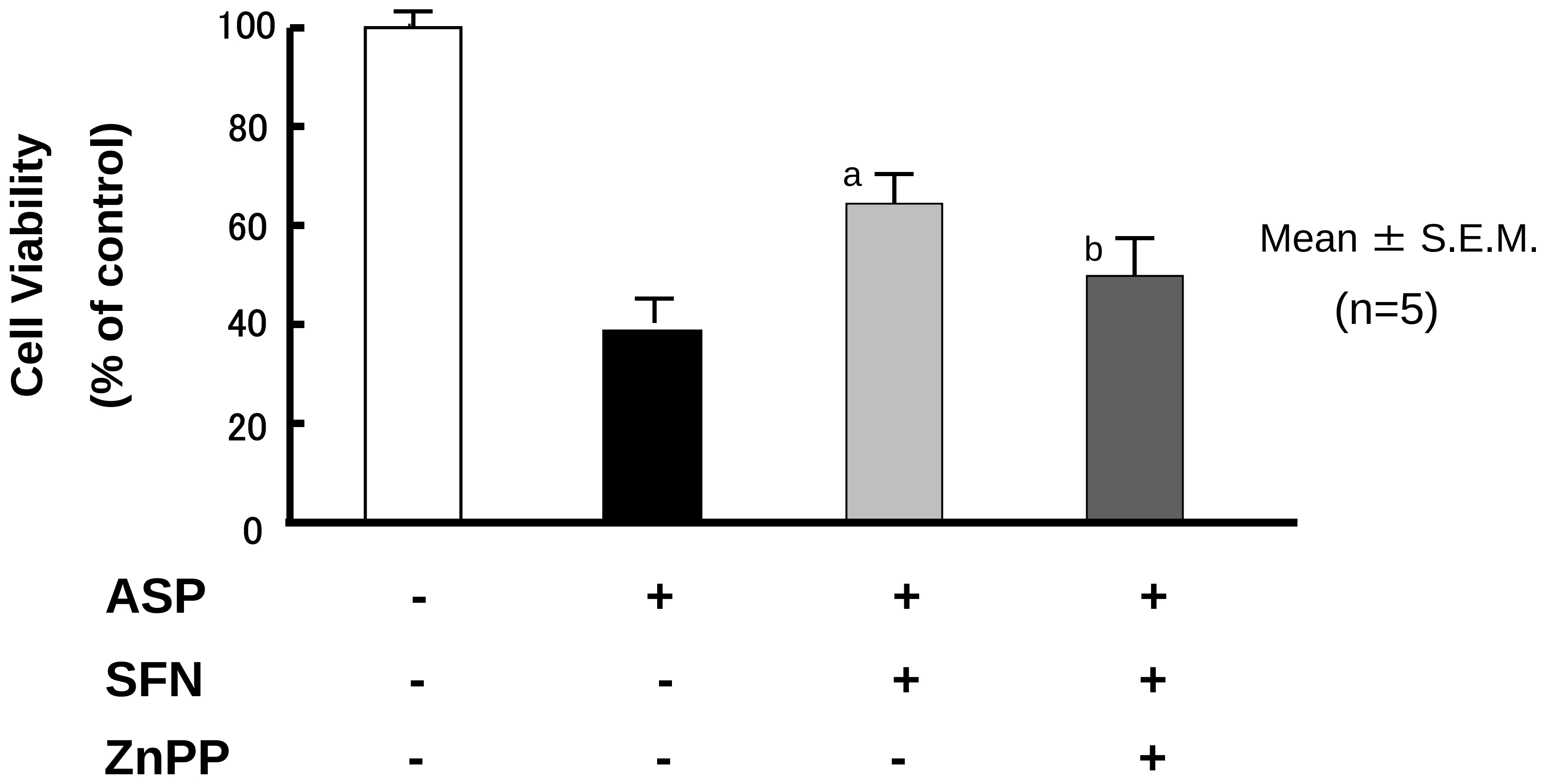


Fig 12.

Oral Intake of SGS Ameliorates IND-Induced Injury of the Small Intestine

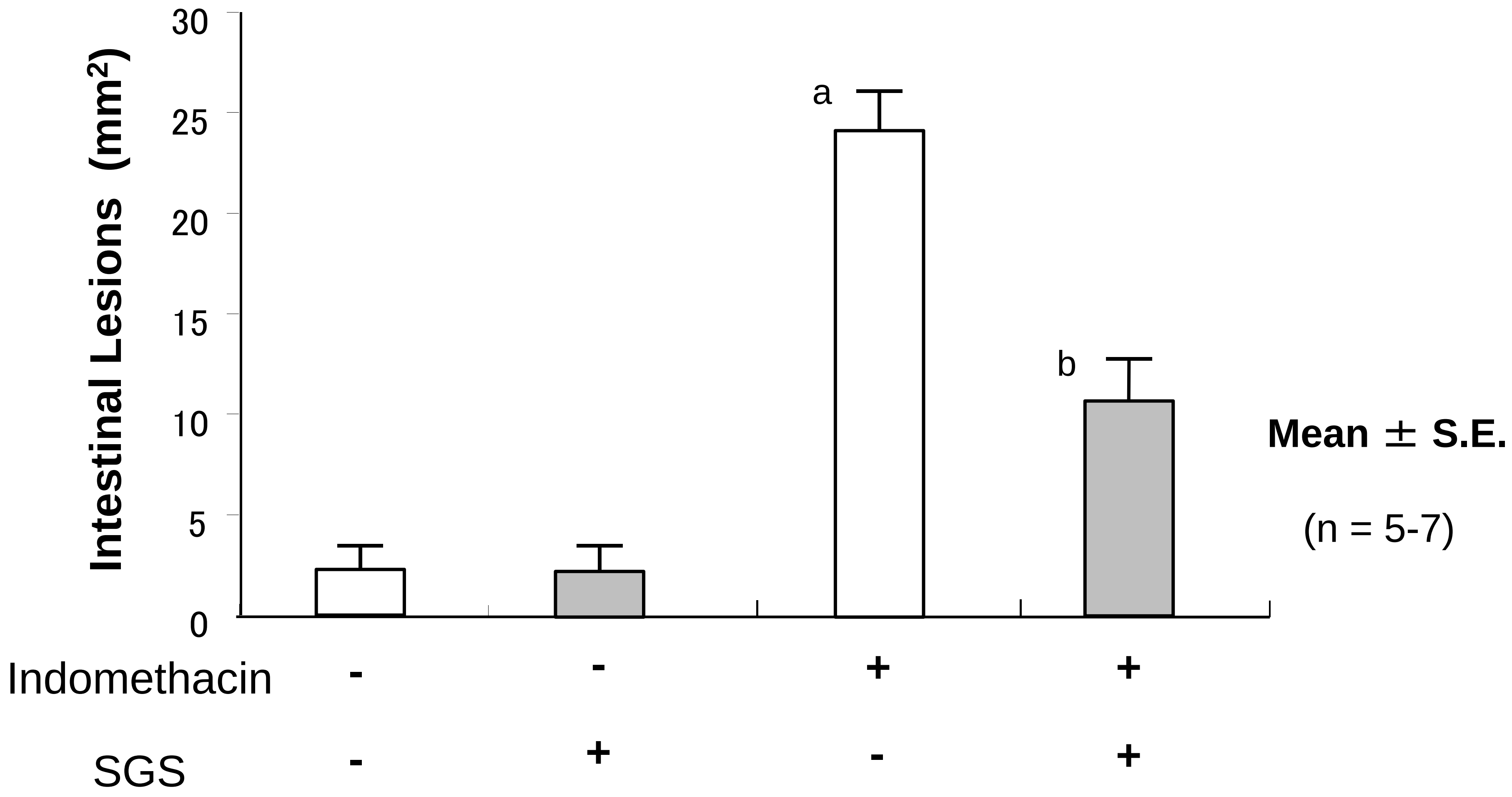


Fig 13. Oral Intake of SGS Ameliorates IND-Induced Increase in Mucosal Permeability of Small Intestine in Mice

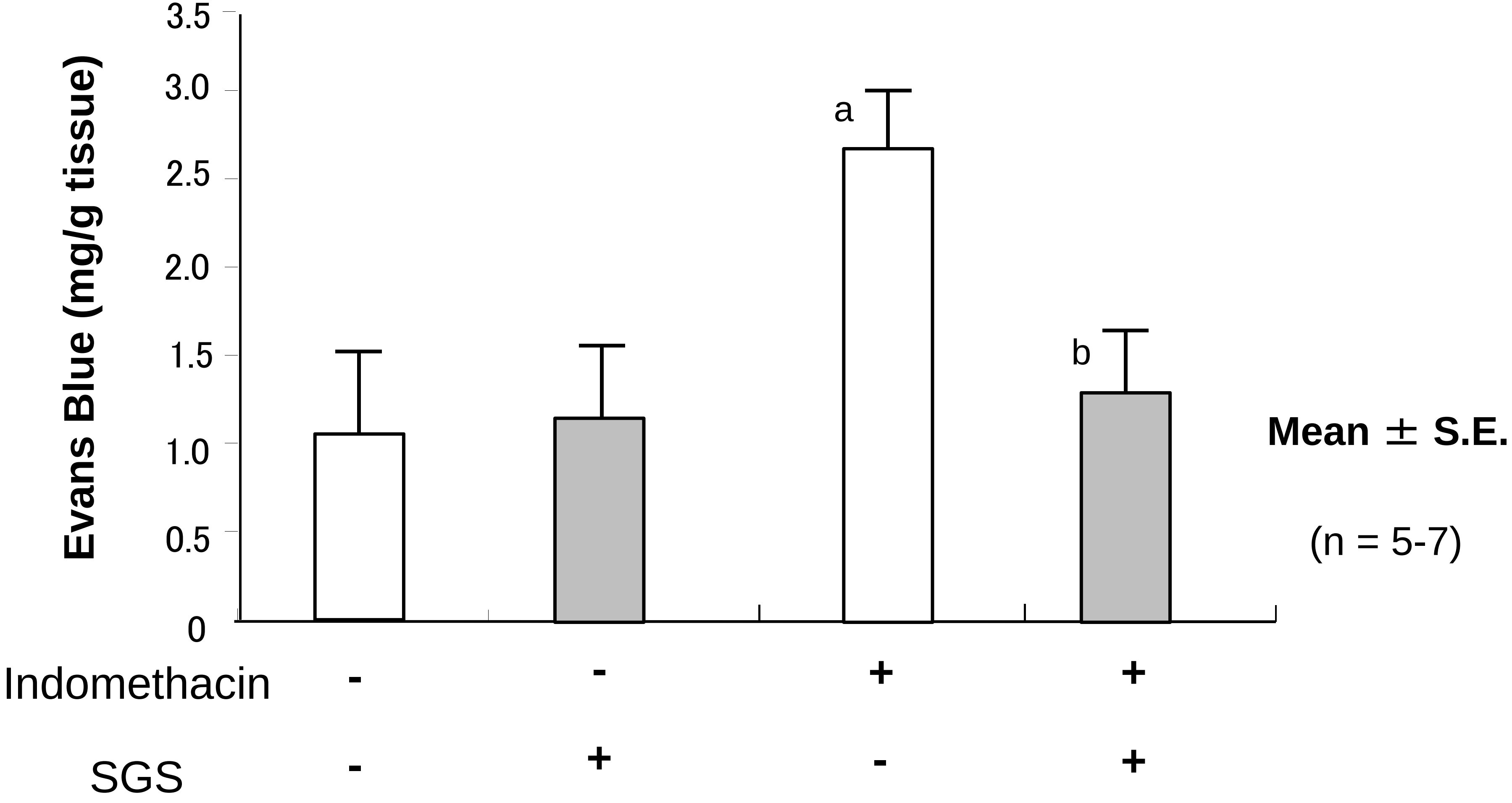


Fig 14. Oral Intake of SGS Blocks IND-Induced Increase in MPO Activity of Small Intestinal Mucosa in Mice

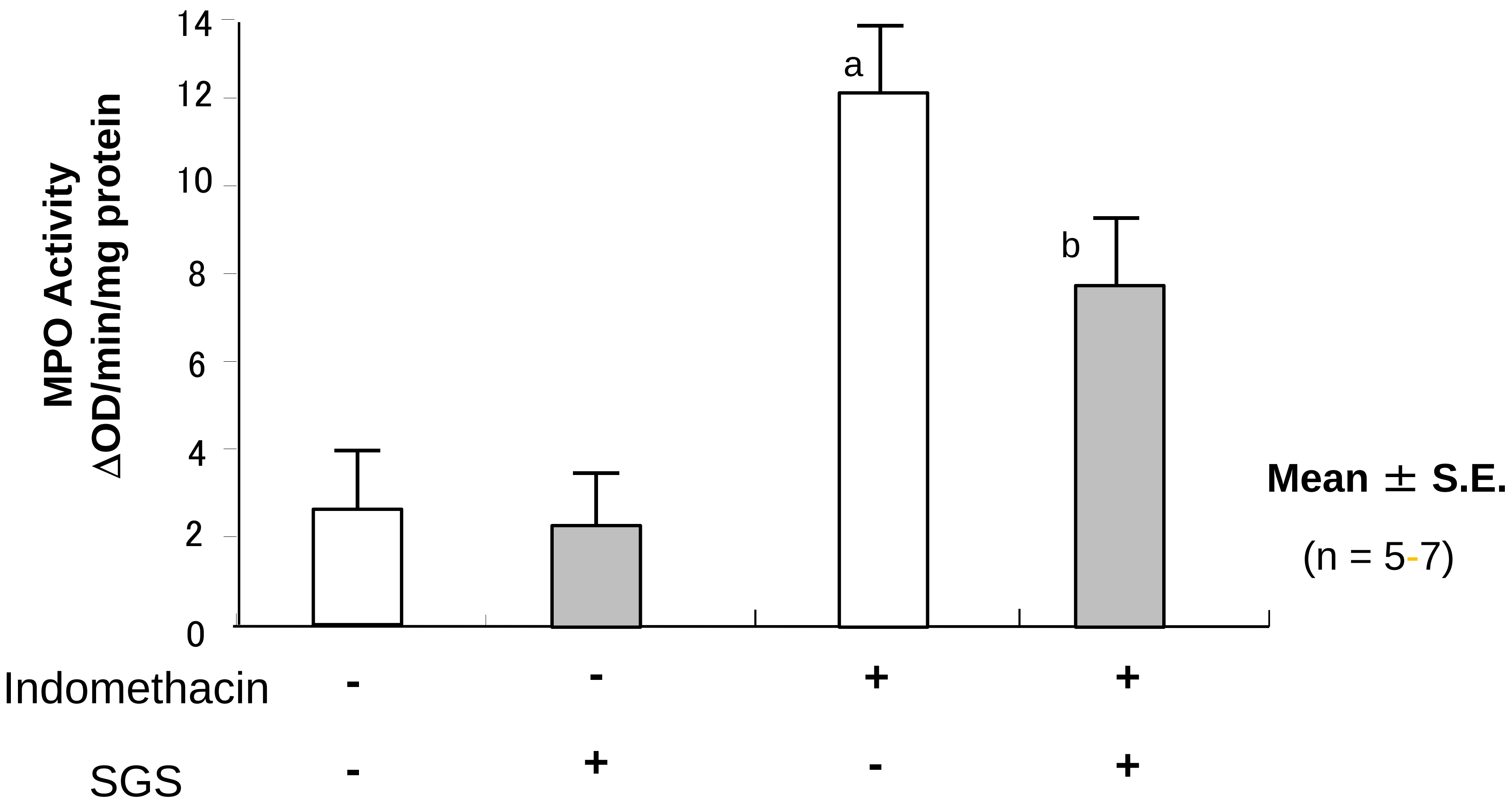


Fig 15. Oral Intake of SGS Inhibits IND-Induced Increase in Anaerobic Bacteria of Small Intestinal Mucosa in Mice

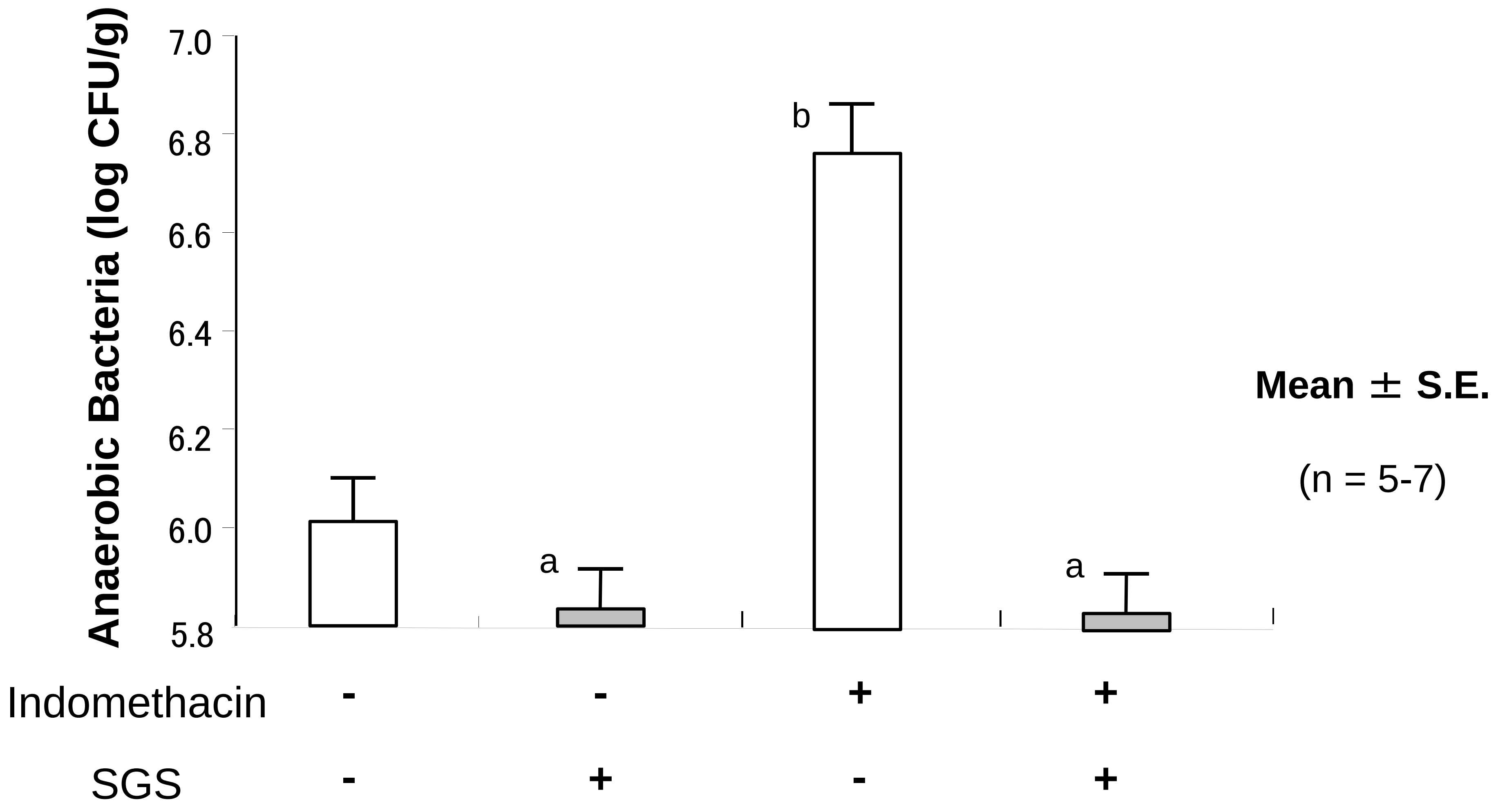


Table 1

Table 1. Baseline data for *H. pylori*–infected subjects (n = 50) after randomization to broccoli sprout and alfalfa sprout supplemented diets

	Broccoli sprout (n = 25)		Alfalfa sprout (n = 23)	
	Mean ± SD	**Range**	**Mean ± SD**	**Range**
Age (y)	53.4 ± 11.6	25-70	55.7 ± 12.9	23-73
UBT (‰)	32.7 ± 14.4	10.0-56.8	35.7 ± 25.3	6.7-106.1
PGI (ng/mL)	70.3 ± 21.4	33.3-118	69.4 ± 26.7	31.5-135
PGII (ng/mL)	30.5 ± 14.8	14.7-43.7	29.4 ± 8.63	18.3-52.5
PGI/PGII (ratio)	2.41 ± 1.00	0.68-4.10	2.38 ± 0.70	1.03-3.93

NOTE: Note that there were two dropouts, both in the alfalfa group, one due to proton pump inhibitor use and one due to antibiotic use after enrollment, and these subjects are not included in the table or the data presented in Figs. 5 to 7.