

# Benexate hydrochloride betadex modulates nitric oxide synthesis and cytokine expression in gastric ulcers

JAE MIN LEE<sup>1\*</sup>, JI-YOUN LIM<sup>2\*</sup>, YOONJIN KIM<sup>2</sup>, YE JI KIM<sup>1</sup>, HYUK SOON CHOI<sup>1</sup>, EUN SUN KIM<sup>1</sup>, BORA KEUM<sup>1</sup>, YEON SEOK SEO<sup>1</sup>, YOON TAE JEEN<sup>1</sup>, HONG SIK LEE<sup>1</sup>, SOON HO UM<sup>1</sup>, CHANG DUCK KIM<sup>1</sup>, HO SANG RYU<sup>1</sup>, DONGGEUN SUL<sup>2</sup>, JUNGHWA HONG<sup>3</sup> and HOON JAI CHUN<sup>1</sup>

<sup>1</sup>Division of Gastroenterology and Hepatology, Department of Internal Medicine, College of Medicine;

<sup>2</sup>Graduate School of Medicine, Korea University, Seoul 02841; <sup>3</sup>Department of Control and Instrumentation Engineering, Korea University, Sejong 30019, Republic of Korea

Received March 19, 2015; Accepted February 11, 2016

DOI: 10.3892/etm.2016.3384

**Abstract.** The present study investigated benexate hydrochloride betadex (BHB)-mediated ulcer healing, and changes to microcirculation modulated through nitric oxide synthase (NOS) and anti-inflammatory activity. A rat model of gastric mucosal injury was established through injection of a 60% acetic acid solution into the stomach. Following ulcer induction, the rats were administered BHB orally for 5 days at doses of 0, 100, 300 or 1,000 mg/kg. The highest dose of BHB was also administered with or without L-NG-nitroarginine methyl ester (L-NAME). The area of gastric ulcers was determined by planimetry, and expression of cyclooxygenases (COX), cytokines and NOS in stomach tissues were measured using western blotting. Compared with the control group, gastric ulcer size was significantly decreased in the 1,000 mg/kg BHB-treated group ( $P<0.05$ ). Administration of BHB led to a significant increase in endothelial (e)NOS expression ( $P<0.05$ ). Although acetic acid co-treatment with L-NAME induced more severe mucosal damage, BHB decreased COX expression and tumor necrosis factor- $\alpha$  levels when administered with the nitric oxide inhibitor, L-NAME ( $P<0.05$ ). BHB exhibited protective effects in a rat model of gastric ulcers, which were associated

with a decrease in pro-inflammatory cytokine levels and the activation of eNOS.

## Introduction

Benexate hydrochloride betadex (BHB) is used clinically as an antiulcer agent. In the gastric mucosa, BHB promotes prostaglandin synthesis, inhibits acid secretion and increases mucosal blood flow (1). The effects of BHB are proposed to be mediated by nitric oxide (NO), through inhibition of cyclooxygenases (COXs) and inflammatory cytokines. However, the mechanism by which BHB promotes mucosal angiogenesis and ulcer healing remains unclear.

NO is involved in numerous physiological functions, including healing processes (2). NO acts as a local vasodilator in the gastric mucosal microvasculature, and stimulates mucus and bicarbonate secretion in the stomach. Previous studies have suggested that the inhibition of NO synthase (NOS) significantly delays ulcer healing by reducing gastric blood flow around the ulcer (3). NO inhibitors, including L-NG-nitroarginine methyl ester (L-NAME), have an inhibitory effect on gastric ulcer healing.

COX may also be involved in gastrointestinal mucosal integrity. COX is expressed in inflammatory cells and fibroblasts of the gastric mucosa; in a previous study, COX mRNA expression increased rapidly in injured mucosa, and the level of expression was correlated with the severity of the gastric mucosal injuries (4). mRNA expression levels of certain inflammatory cytokines, including interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), are also significantly increased in gastric ulcers (5).

Despite numerous studies investigating anti-gastric ulcer agents, the molecular mechanism of BHB is not fully understood. In the present study, the anti-gastric ulcer effects of BHB with respect to the modulation of NOS, COX and inflammatory cytokine expression have been investigated.

## Materials and methods

**Materials.** BHB was purchased from Ildong Pharmaceutical Co., Ltd. (Seoul, Korea). Protein expression was detected

*Correspondence to:* Dr Hoon Jai Chun, Division of Gastroenterology and Hepatology, Department of Internal Medicine, College of Medicine, Korea University, 73 Inchon-ro, Seoul 02841, Republic of Korea  
E-mail: drchunhj@chol.com

Dr Junghwa Hong, Department of Control and Instrumentation Engineering, Korea University, 2511 Sejong-ro, Sejong 30019, Republic of Korea  
E-mail: hongjh32@korea.ac.kr

\*Contributed equally

**Key words:** benexate hydrochloride betadex, stomach, ulcer, nitric oxide, cytokine

using the following primary antibodies from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA): Rabbit polyclonal IL-1 $\beta$  (dilution, 1:500; cat. no. sc-7884); goat polyclonal IL-6 immunoglobulin G (IgG) (dilution, 1:500; cat. no. sc-1265); goat polyclonal TNF- $\alpha$  IgG (dilution, 1:500; cat. no. sc-1351); rabbit polyclonal cyclooxygenase-1 (COX-1; dilution, 1:1,000; cat. no. sc-7950); and goat polyclonal cyclooxygenase-2 IgG (COX-2; dilution, 1:1,000; cat. no. sc-1745) (all purchased from Santa Cruz Biotechnology, Inc.). The following primary antibodies were also used: Rabbit polyclonal inducible NOS (iNOS; dilution, 1:1,000; cat. no. ab15323); rabbit monoclonal neuronal NOS (nNOS; dilution, 1:1,000; cat. no. ab76067) (both purchased from Abcam, Cambridge, UK); and rabbit monoclonal endothelial NOS (eNOS; dilution, 1:1,000; cat. no. 32027; Cell Signaling Technology, Inc., Beverly, MA, USA). Horseradish peroxidase (HRP-conjugated goat anti-mouse IgG (dilution, 1:2,000; cat. no. sc-2005), HRP-conjugated goat anti-rat IgG (dilution, 1:2,000; cat. no. sc-2006) and HRP-conjugated donkey anti-goat IgG (dilution, 1:2,000; cat. no. sc-2020) were used (all purchased from Santa Cruz Biotechnology, Inc.).

**Animals.** A total of 30 male Sprague-Dawley rats, weighing between 240–250 g, were purchased from Samtako Laboratory Animal Company (Osan, Korea) and housed for 1 week in the animal facility for acclimation. Constant environmental conditions were maintained with a temperature of 23 $\pm$ 1 $^{\circ}$ C, humidity of 55% and a 12-h light/dark cycle. Following acclimation, the rats underwent a fast for the 24 h prior to the experiments. The present study was approved by the Institutional Animal Care & Use Committee of Korea University (approval number, KUIACUC-2013-181).

**Study methods.** Gastric ulcers were induced in all rats by direct injection of acetic acid. The rats were anesthetized using tiletamine/zolazepam (10 mg/kg; intramuscular injection; Zoetis, Inc., Florham Park, NJ, USA) and xylazine (5 mg/kg; intraperitoneal injection; Bayer AG, Leverkusen, Germany). A longitudinal incision of 2 cm was made in the upper abdomen. The stomach was then exposed and directly injected with 2 cm<sup>3</sup> of 60% acetic acid solution, as described by Okabe *et al* (6). After 45 sec, the gastric contents were aspirated by syringe. The abdomen was sutured and oral intake of food and water was permitted following closure. Following gastric ulcer induction, the rats were randomly divided into 6 groups, with 5 rats per group. The groups were organized as follows: Control, no BHB treatment; BHB 100; 300; or 1,000 mg/kg treatment; L-NAME (Sigma-Aldrich, St. Louis, MO, USA) 70 mg/kg treatment; and L-NAME 70 mg/kg treatment with BHB at 1,000 mg/kg. The drugs were dissolved in 2 ml 5% dextrose water (DW; JW Pharmaceutical Corporation, Seoul, Korea) and administered orally once per day for 5 days. The rats in the control group were administered 2 ml 5% DW without BHB.

**Assessment of the gastric lesions.** A total of 5 days after the induction of gastric ulcers, the rats were sacrificed using CO<sub>2</sub>. The stomachs were dissected, gently incised along the longer curvature, opened and rinsed with phosphate-buffered saline (PBS) to remove the gastric contents. The gastric mucosa lesions were macroscopically examined with a magnifier using

a metric measurement scale. The areas of the ulcerous lesions were measured in mm<sup>2</sup> using the lesion index (7).

**Western blot analysis.** The expression levels of COXs (COX-1 and COX-2), cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) and NOS (nNOS, eNOS and iNOS) were measured using western blot analysis. The gastric tissues were frozen using liquid nitrogen and stored at -80 $^{\circ}$ C. Samples were pulverized by a mortar and pestle, then mixed with radioimmunoprecipitation assay buffer (a lysis buffer) and centrifuged at 14,200  $\times$  g for 15 min. The supernatants were collected and the protein content was determined using a Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A similar mass of total protein was loaded from each sample onto a 5–12% sodium dodecyl sulfate gel and transferred to polyvinylidene fluoride membranes using electrophoresis. The membranes were blocked with a blocking buffer (5% skimmed milk in PBS) for 1 h at room temperature, then incubated with the primary antibody. Following several washes with PBS-Tween 20 over 30 min, the membranes were incubated with the secondary antibody specific to the primary antibody for 1 h at room temperature. Following several additional washes with PBS-Tween 20 over 30 min, detection was performed using an enhanced chemiluminescence kit (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The intensity of each band was compared with that of the internal control,  $\beta$ -actin.

**Statistical analysis.** Data were processed and analyzed using SPSS, version 20.0 (IBM SPSS, Armonk, NY, USA). Statistical comparisons were performed using a Student's t-test.  $P \leq 0.05$  was considered to indicate a statistically significant difference.

## Results

**Gastric ulcers.** The control group developed ulcerous lesions (Fig 1). Rats that received BHB at doses of 100, 300 and 1,000 mg/kg demonstrated reductions in mucosal injury of 7.8, 10.7 and 19.3%, respectively, compared with the control (Fig. 2); the area of ulcerous lesions significantly decreased in the group treated with 1,000 mg/kg BHB (Fig. 2). L-NAME aggravated the acetic acid-induced ulcerous lesions, observed macroscopically (Fig. 1E), but the effect of L-NAME was somewhat reversed when it was administered with 1,000 mg/kg BHB (Fig. 1F). The L-NAME + BHB group exhibited significantly reduced lesion area compared with the L-NAME group ( $P < 0.05$ ; Fig. 2).

**COX and cytokine levels of gastric ulcers.** The expression levels of COX-1 and -2 are reported in Fig. 3. COX-2 expression was decreased in the BHB groups compared with the control group; however, a significant difference was only observed at 1,000 mg/kg ( $P < 0.05$ ; Fig. 3C). No significant difference in COX-1 expression was observed between the BHB treatment and control groups.

The expression levels of pro-inflammatory cytokines are reported in Fig. 4; and the expression levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were not observed to be significantly different following BHB treatment.

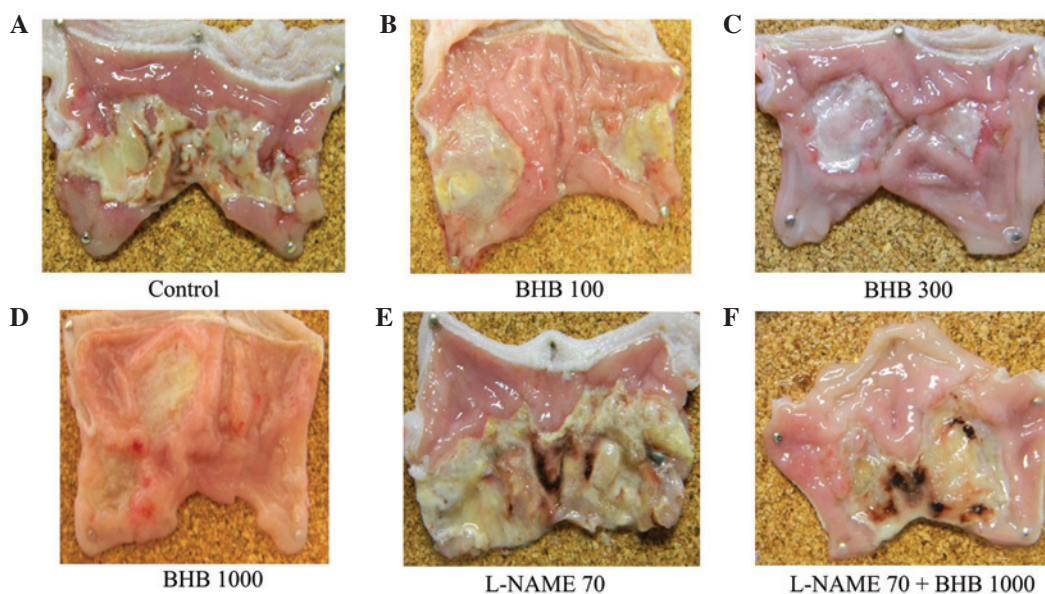


Figure 1. Macroscopic images of gastric lesions in a rat model, demonstrating acetic acid-induced mucosal damage. Treatment with (A) control, (B) 100, (C) 300 and (D) 1,000 mg/kg BHB, (E) 70 mg/kg L-NAME and (F) 70 mg/kg L-NAME with 1,000 mg/kg BHB. BHB, benexate hydrochloride betadex; L-NAME, L-NG-nitroarginine methyl ester.

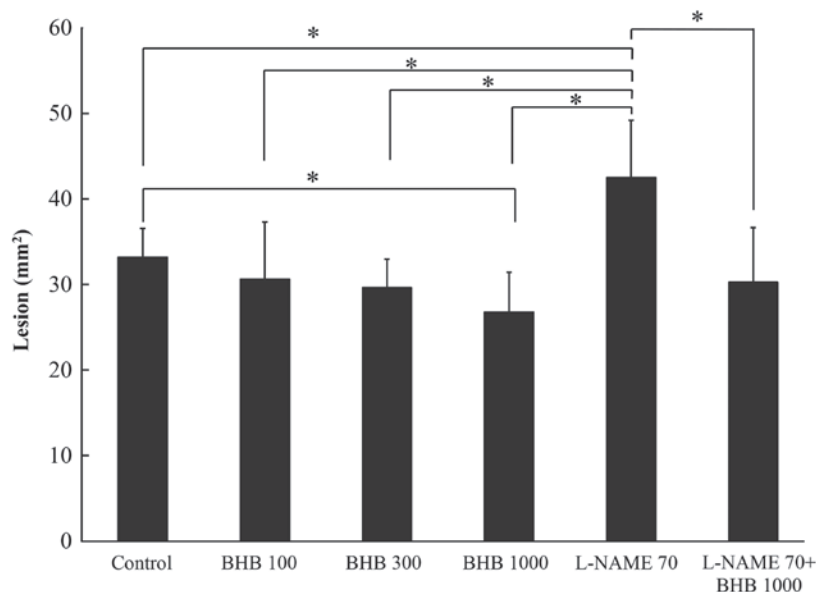


Figure 2. Pathological ulcerous lesion size in the each of the groups, following treatment with BHB and/or L-NAME, with doses provided in mg/kg. \*P<0.05, comparisons shown by brackets. BHB, benexate hydrochloride betadex; L-NAME, L-NG-nitroarginine methyl ester.

**NOS levels in the gastric ulcers.** The effect of BHB on eNOS, iNOS and nNOS protein expression was also assessed by western blot (Fig. 5A). All BHB treatments significantly increased eNOS expression compared with the control group (P<0.05; Fig. 5B), but no significant differences were identified in the expression levels of nNOS or iNOS between the groups (Fig. 5C and D).

**COX and inflammatory cytokine levels of gastric ulcers in the presence of an NO inhibitor.** As presented in Fig. 6, COX-1 protein expression levels increased in rats administered L-NAME compared with the control group (P<0.05), and the co-treatment with BHB significantly decreased the expression

of COX-1 compared with the L-NAME group (P<0.05). COX-2 protein expression levels increased in rats in the control group, and the administration of BHB significantly decreased COX-2 protein expression in both groups, with or without L-NAME (P<0.05). As presented in Fig. 7, L-NAME administration significantly increased the expression of the inflammatory cytokine TNF- $\alpha$  (P<0.05 vs. control group), whereas combined treatment suppressed its expression (P<0.05 vs. L-NAME group). No significant difference was observed in the expression of IL-1 $\beta$  and IL-6 with either drug.

**NOS levels of gastric ulcers in the presence of an NO inhibitor.** In the presence of L-NAME, eNOS levels did not differ

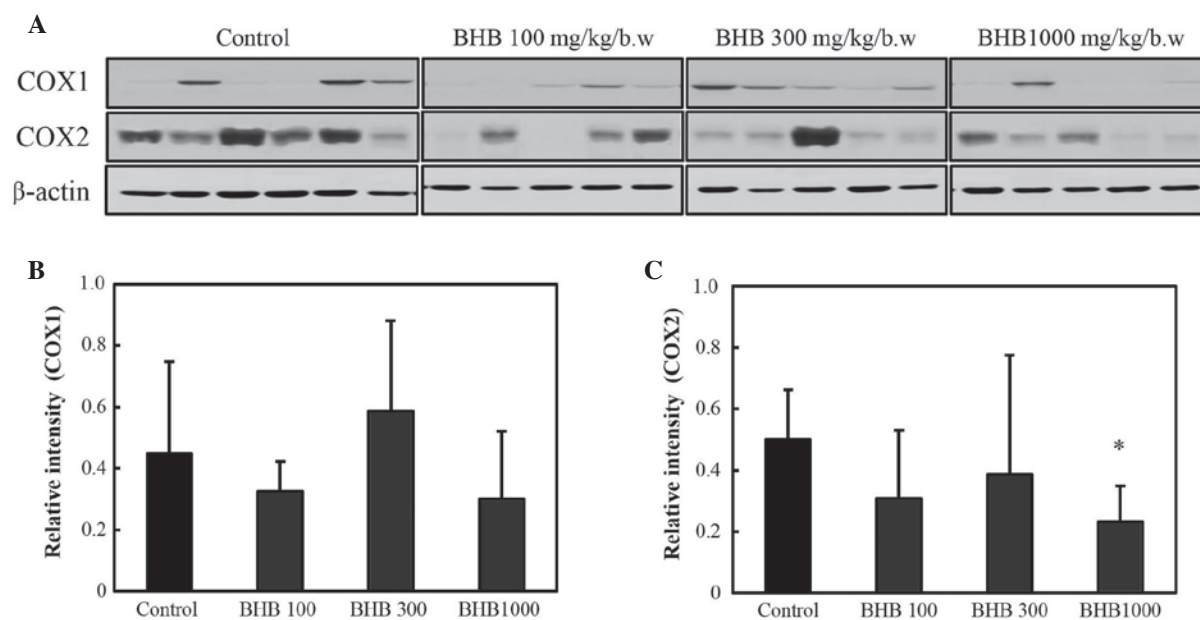


Figure 3. COX expression following treatment with BHB, with doses provided in mg/kg. (A) Western blot analysis of COX expression; (B) COX-1 and (C) COX-2 expression. \* $P < 0.05$  vs. the control group. BHB, benexate hydrochloride betadex; COX, cyclooxygenase.

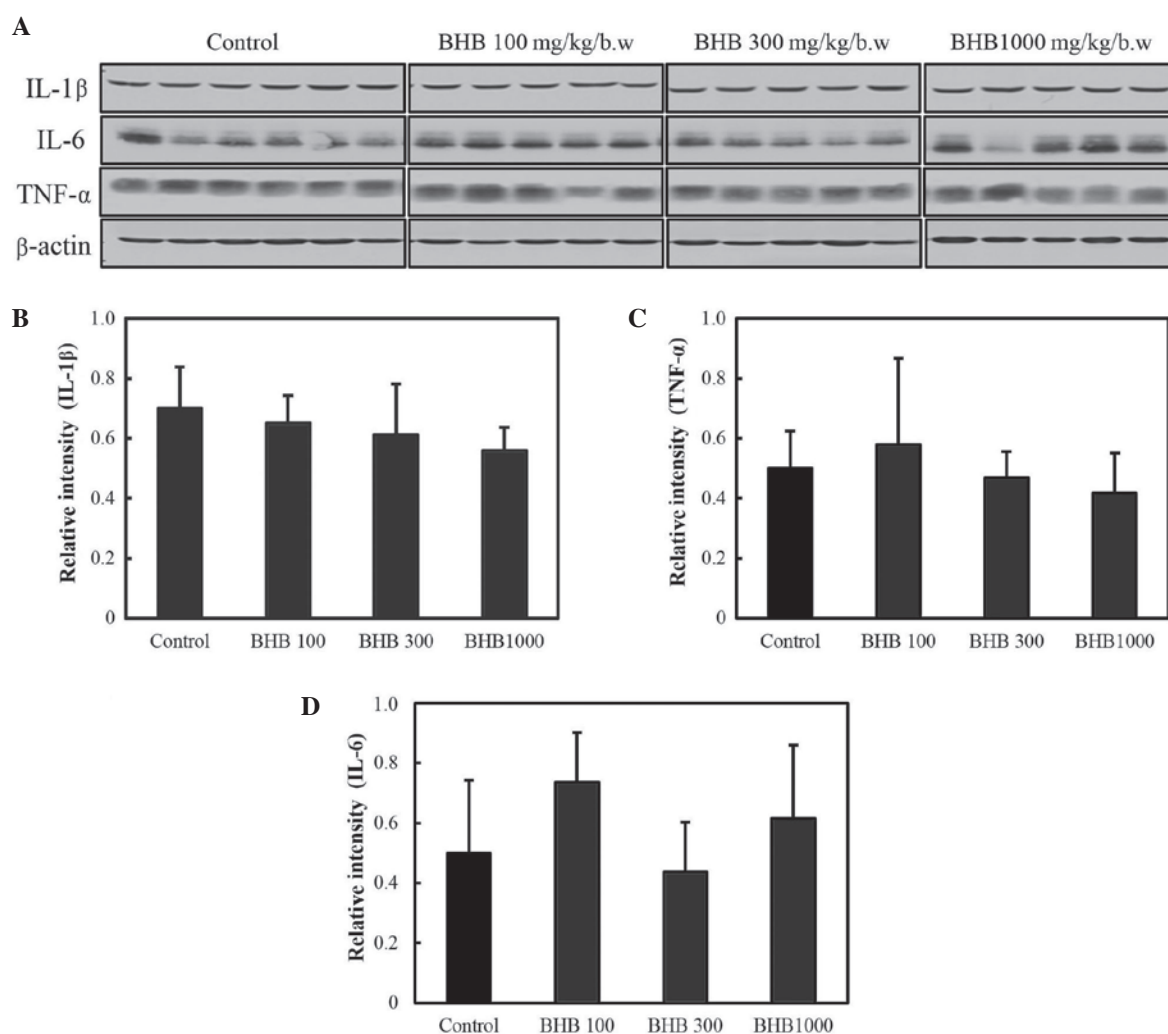


Figure 4. Expression levels of pro-inflammatory cytokines following treatment with BHB, with doses provided in mg/kg of body weight. (A) Western blot analysis of pro-inflammatory cytokines; (B) IL-1β, (C) TNF-α and (D) IL-6 expression. BHB, benexate hydrochloride betadex; IL, interleukin; TNF-α, tumor necrosis factor-α.



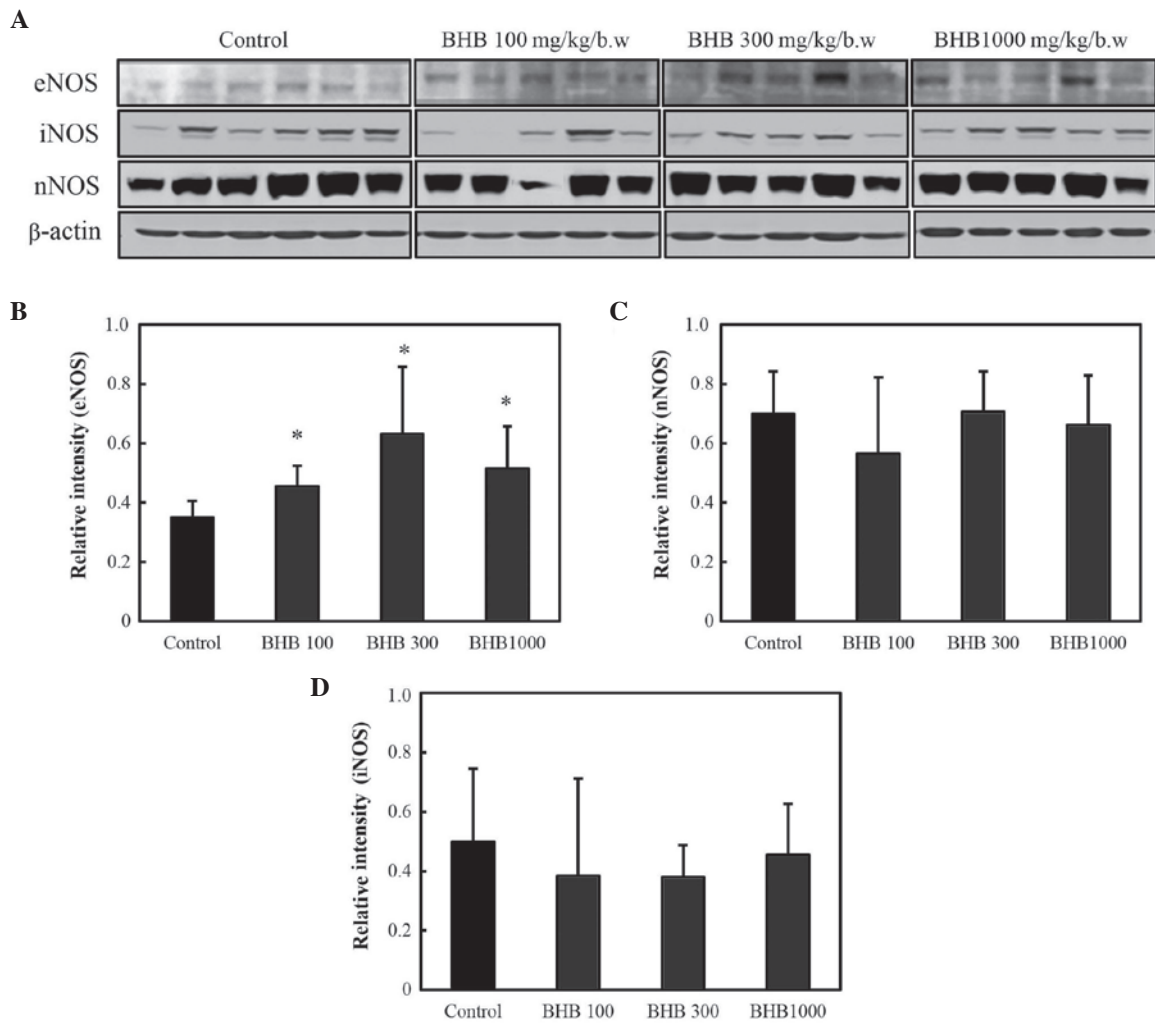


Figure 5. NOS expression following treatment with BHB, with doses provided in mg/kg. (A) Western blot analysis of NOS expression; (B) eNOS, (C) nNOS and (D) iNOS expression. \* $P < 0.05$  vs. the control group. NOS, nitric oxide synthase; BHB, benexate hydrochloride betadex; eNOS, endothelial NOS; nNOS, neuronal NOS; iNOS, inducible NOS.

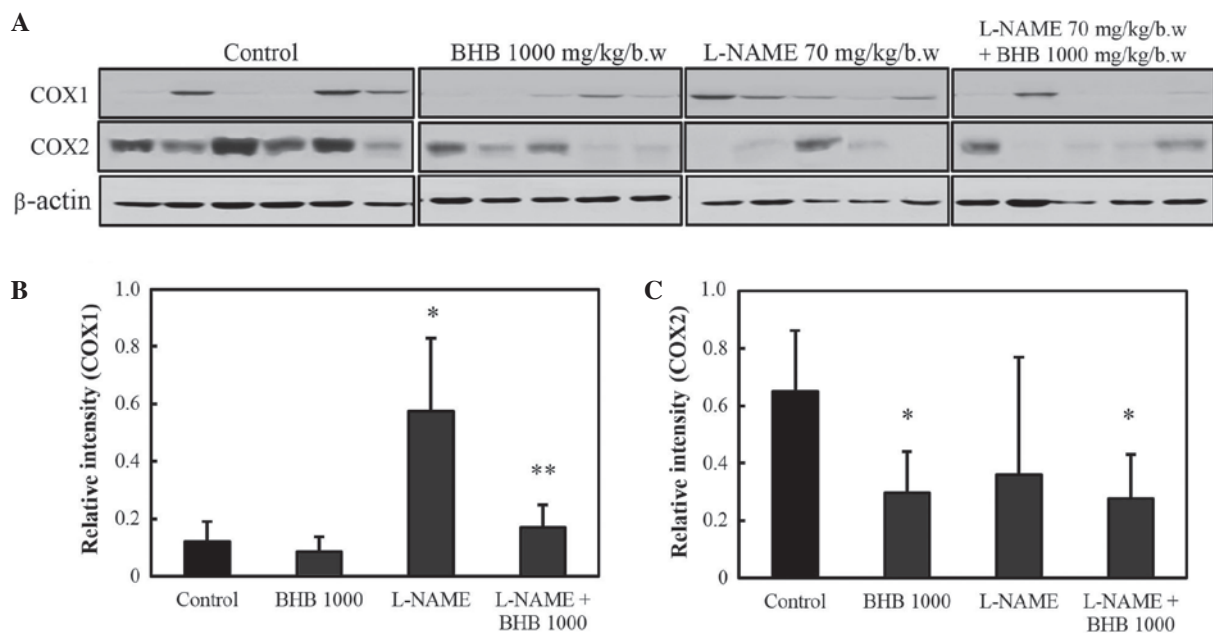


Figure 6. COX expression in rats administered L-NAME with or without BHB. (A) Western blot analysis of COX expression; (B) COX-1 and (C) COX-2 expression. \* $P < 0.05$  compared with control; and \*\* $P < 0.05$  compared with L-NAME treatment only. BHB, benexate hydrochloride betadex; L-NAME, L-NG-nitroarginine methyl ester; COX, cyclooxygenase.

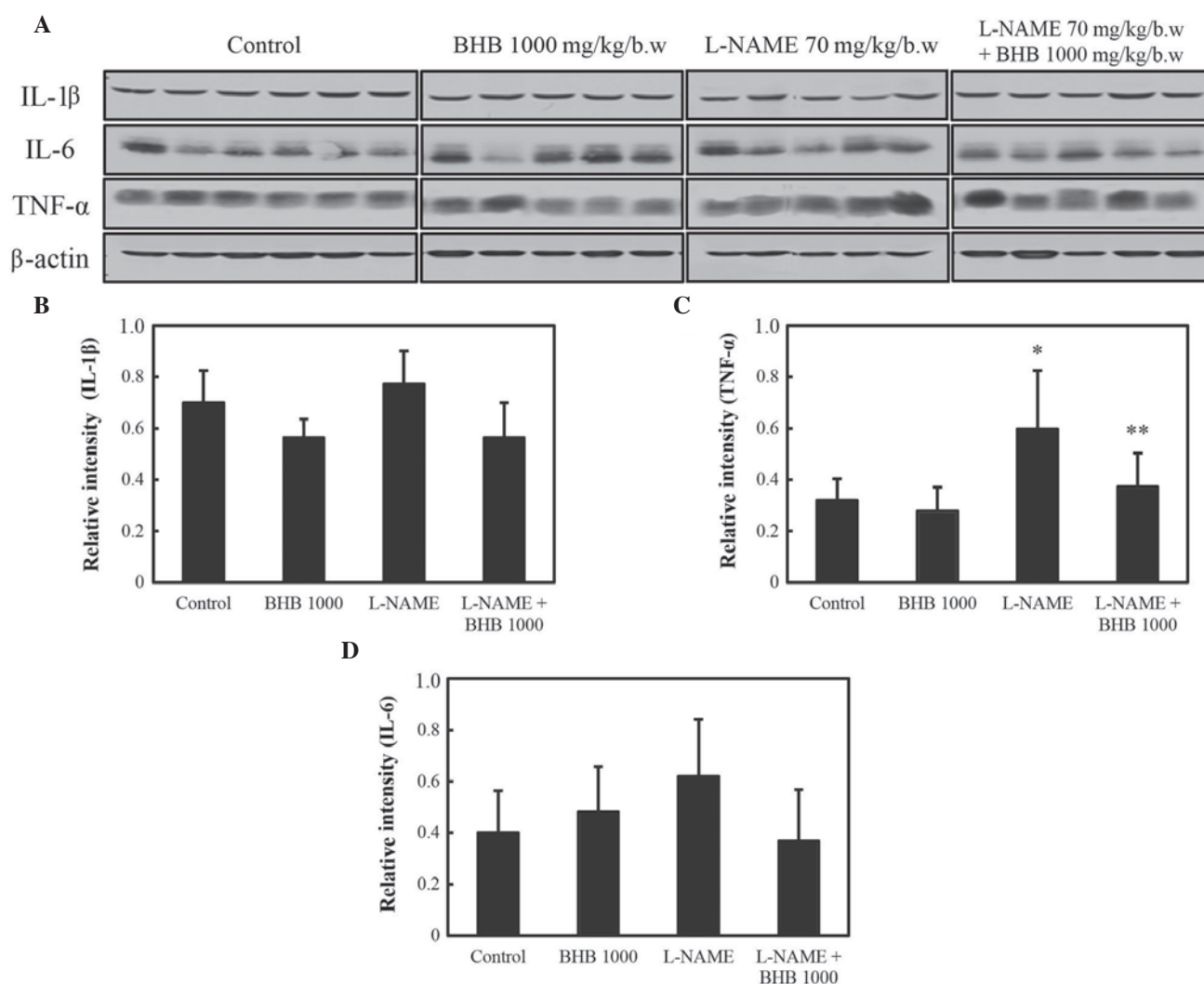


Figure 7. Expression of pro-inflammatory cytokines in rats administered L-NAME with or without BHB. (A) Western blot analysis of pro-inflammatory cytokine expression. Expression of (B) IL-1 $\beta$ , (C) IL-6 and (D) TNF- $\alpha$ . \*P<0.05 vs. the control; and \*\*P<0.05 vs. L-NAME treatment only. BHB, benexate hydrochloride betadex; L-NAME, L-NG-nitroarginine methyl ester; IL, interleukin; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

between those groups that were or were not administered BHB (P<0.05; Fig. 8A and B). The expression levels of nNOS were decreased in the L-NAME group compared with levels in the control group; and compared with the L-NAME group, these levels were significantly increased in the L-NAME + BHB group (P<0.05; Fig. 8A and C). By contrast, the expression levels of iNOS were suppressed by BHB + L-NAME co-treatment compared with L-NAME treatment alone (P<0.05; Fig. 8A and D).

## Discussion

The gastric microcirculation has an essential role in healing gastric ulcers. Local vasodilators, including NO, are important in the maintenance of mucosal integrity and in mucosal defense mechanisms (2). Previous studies have reported that increased levels of NO significantly enhance ulcer healing by the maintenance of gastric blood flow; NO also promotes angiogenesis and endothelial cell proliferation and migration (8-10). It has previously been proposed that the anti-ulcer effects of NO result from gastric mucosal NOS activation (3). The calcium-dependent isoforms of NOS, eNOS and nNOS

are constitutively expressed in the endothelium of blood vessels and in the brain, respectively. However, expression of iNOS, the calcium-independent isoform, is induced by pro-inflammatory agents (11,12). eNOS is known to promote ulcer healing through angiogenesis, enhancing gastric blood flow and stimulating mucus and bicarbonate secretion (13-15). iNOS is associated with acute and chronic inflammation, and it has previously been reported that changes in iNOS expression and activity levels are correlated with the severity of tissue inflammation (16).

Iwasaki and Matsunaga (17) reported that the vasorelaxant effects of BHB, which were blocked by an NO inhibitor in defective endothelium, were associated with NOS activation. By contrast, Arimoto *et al* (18) demonstrated that the *in vitro* activity of NOS was suppressed by BHB. The current study, therefore, hypothesized that a specific NOS may be activated through BHB administration, leading to gastric mucosal healing. In the present study, BHB significantly increased eNOS expression in the rat gastric ulcer model, suggesting that the protective effect of BHB against gastric ulcers may involve an increase in eNOS. iNOS and nNOS were not significantly increased following BHB administration. These

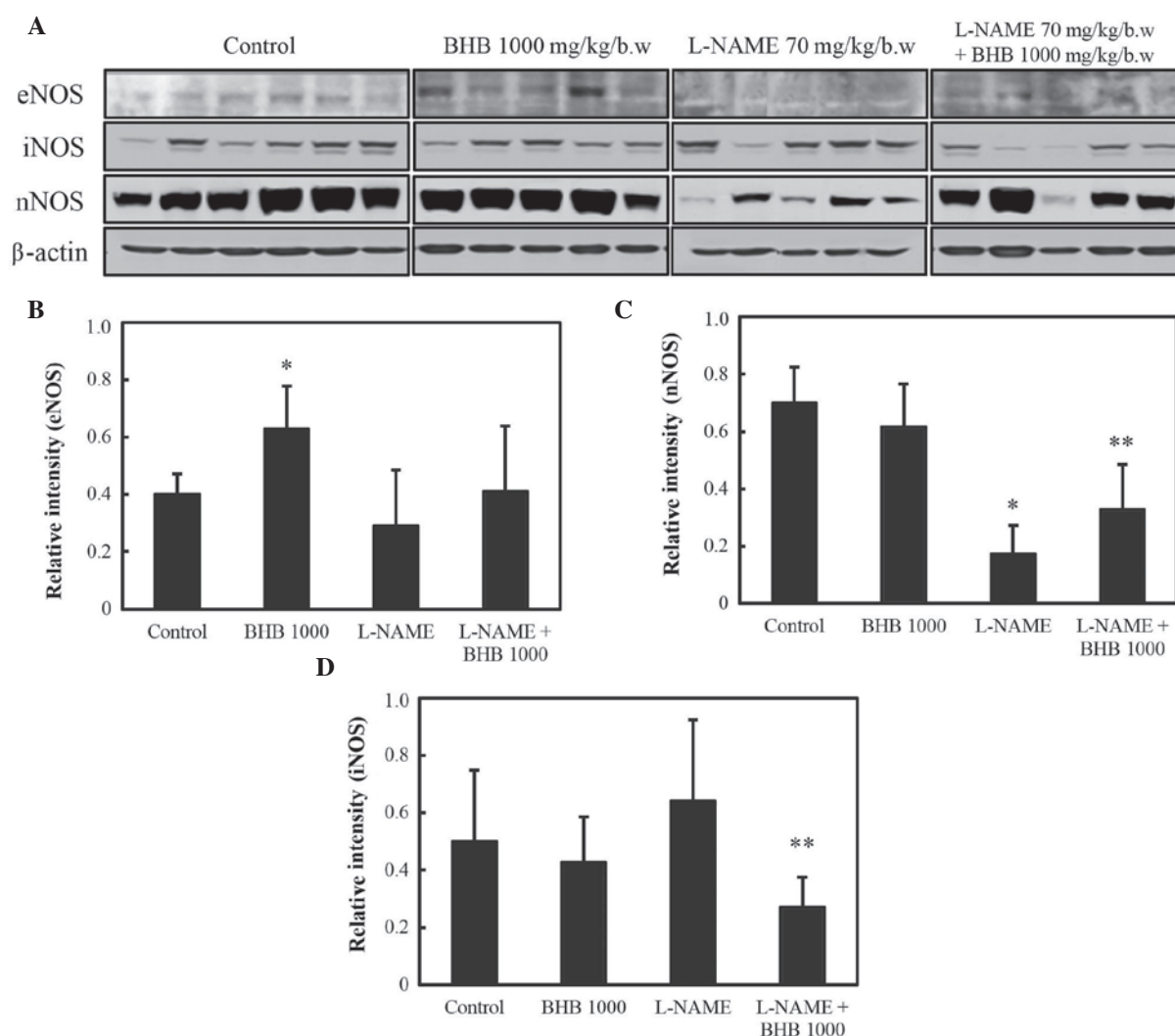


Figure 8. NOS expression in rats administered L-NAME with or without BHB. (A) Western blot analysis of pro-inflammatory cytokine expression. (B) eNOS, (C) nNOS and (D) iNOS expression. \* $P < 0.05$  vs. the control; and \*\* $P < 0.05$  vs. L-NAME treatment only. NOS, nitric oxide synthase; L-NAME, L-*NG*-nitroarginine methyl ester; BHB, benxate hydrochloride betadex; eNOS, endothelial NOS; nNOS, neuronal NOS; iNOS, inducible NOS.

results are partially in agreement with the hypothesis that the anti-ulcer effects of BHB are associated with increased blood flow through the activation of the specific NOS isoform, eNOS. However, BHB did not increase eNOS in the presence of L-NAME, which aggravated the ulcerous lesion. Additional study is therefore required in order to establish the mechanisms of the BHB-induced increased blood flow in gastric ulcers.

An increase in COX-2 expression has previously been demonstrated to be a major contributor to inflammation (10). Previous studies have reported that COX-1 and COX-2 levels are positively correlated with the severity of gastric mucosal damage (19-21). COX-2 mRNA expression is induced during the acute stage of gastric ulceration (22); the early phase of inflammation is primarily mediated by constitutive COX-1 expression, whilst the late and acute phases are mediated by COX-1 and COX-2. COX-2 expression is typically observed 1-2 h after stimulation (4,23). In the present study, BHB administration at a dose of 1,000 mg/kg significantly decreased COX-2 levels, which may indicate that the protective effect of BHB is associated with modulation of COX-2 expression.

Inflammatory cytokines mediate the upregulation of COX-2 expression and inhibit growth factors that are responsible for

recovery from mucosal damage (24,25). The current results suggest that BHB enhances ulcer healing through a reduction of gastric inflammation and of the inflammatory cytokine TNF- $\alpha$ ; however, no association was observed between BHB and interleukin levels.

There are a number of differences between the mechanism of action of BHB and of other mucoprotective agents, including rebamipide. Rebamipide reportedly suppresses COX-2 expression and NF- $\kappa$ B activation (22,26). In the present study, however, BHB only decreased COX expression whilst upregulating eNOS. Together, the current results suggest that the anti-ulcer effects of BHB are, in part, associated with decreased COX-2 and pro-inflammatory cytokine levels and with eNOS activation. In conclusion, BHB administration ameliorates acetic acid-induced mucosal injury in a rat model of gastric ulcers by BHB-induced decreases to TNF- $\alpha$  and increases to eNOS expression levels.

#### Acknowledgements

This study was supported by the Industrial Core Technology Development Program (grant no. 10060251; Development

of the diagnostic device for functional dyspepsia based on Korean-Western medicine fusion abdominal diagnosis) and the Basic Science Research Program (grant no. NRF2012R1A2A2A01016829) funded By the Ministry of Trade, Industry and Energy (MI, Korea) and the Ministry of Science, ICT and Future Planning.

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