

Gastrodia elata Blume protects against stress-induced gastric mucosal lesions in mice

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Abstract. *Gastrodia elata* Blume (GEB) is a traditional herbal plant that has been used in Asian countries for centuries as an anticonvulsant, analgesic, and also as a sedative for treating general paralysis, epilepsy, vertigo, and tetanus. Although numerous reports have addressed the effects of GEB against degenerative diseases, no previous study has examined the possible gastroprotective effects of GEB. Here, we examined the effects of pretreatment with GEB (0.02 ml/g, p.o.) in a mouse water immersion restraint (WIR) stress-induced gastric lesion model. Our results revealed that mice pretreated with GEB had significantly fewer gastric lesions than their respective controls. Moreover, GEB-treated mice showed significant decreases in serum and gastric nitric oxide (NO) levels to 50 and 28%, respectively. To examine one possible mechanism underlying this effect, we used reverse transcription-polymerase chain reaction (RT-PCR) to examine NOS mRNA expression in gastric lesion tissues. Our results revealed that the mRNA expression of inducible nitric oxide synthase (iNOS) was reduced by ~50% in GEB-pretreated mice versus the controls, whereas the mRNA expression levels of endothelial nitric oxide synthase (eNOS) and

neuronal nitric oxide synthase (nNOS) remained unchanged. These findings collectively suggest that GEB significantly protects the gastric mucosa against WIR-induced gastric damage, at least in part by decreasing NO levels via suppression of iNOS mRNA expression.

Introduction

Acute gastric mucosal lesions represent a serious clinical problem worldwide. In an effort to facilitate the study of acute gastric damage, researchers have developed a number of gastric lesion animal models, including those induced by water immersion restraint (WIR) stress in rodents (1). This model has previously been used to examine various factors affecting the formation and healing of gastric mucosal lesions, including sulphhydryls, endogenous prostaglandins, growth factors and polyamines (2). WIR stress-induced gastric lesions have also been used to examine the roles of apoptosis and gastric acid secretion in ulcerogenesis (3). In general, gastric lesions are disturbances in the gastric barrier function that normally protects the mucosa against deep damage by hydrogen ions and other noxious substances produced in the gastric lumen (4). One major component of gastric barrier function is gastric microcirculation; disturbance of gastric mucosal perfusion results in the formation of erosions and ulcers, such as those seen in experimental models of ischemic gastric lesions (5). Gastric blood flow is typically regulated by signaling molecules such as prostaglandins and nitric oxide (NO), the latter of which plays numerous roles in maintaining mucosal integrity (6). NO is primarily produced by nitric oxide synthase, which is generally divided into two classes: constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS). The cNOS isoforms include endothelial nitric oxide synthase (eNOS), which regulates vascular homeostasis in endothelial cells, platelets and mesangial renal cells (7), and neuronal nitric oxide synthase (nNOS), which produces NO to act as a neurotransmitter in the central and peripheral nervous system (8). The third major isoform, iNOS, is a calcium-independent enzyme located in macrophages, hepatocytes, smooth vascular muscles, neutrophils and endothelial cells (9). These NOS isoforms have been characterized in the gastrointestinal tract, and NO generated

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Abbreviations: GEB, *Gastrodia elata* Blume; WIR, water immersion restraint; RT-PCR, reverse transcription-polymerase chain reaction; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; cNOS, constitutive nitric oxide synthase; i.p., intraperitoneal; p.o., peroral

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from cNOS and iNOS has been shown to play an important role in the formation of gastric lesions (10).

Numerous studies have investigated methods for treating and/or preventing gastric lesions with synthetic drugs (11). More recently, researchers have begun examining the possible usefulness of traditional Asian herbal remedies (12). One such traditional herbal agent, *Gastrodia elata* Blume (GEB), has been used in Asian countries for centuries as an anti-convulsant, analgesic and sedative against vertigo, general paralysis, and tetanus. Previous *in vitro* and *in vivo* studies have confirmed that constituents from GEB have anticonvulsive activity (13-15), but no prior study has investigated whether GEB has gastroprotective effects.

Here, we examined whether GEB has gastroprotective effects against WIR stress-induced gastric lesions in mice, and we further tested whether the identified gastroprotection acted via changes in NO content and NOS mRNA expression.

Materials and methods

Chemicals. Trizol[®] reagent was obtained from Invitrogen (Carlsbad, CA). L-arginine and diethyl ether were purchased from Junsei Chemicals (Tokyo, Japan). Sodium nitrate was obtained from Janssen Pharmaceutica (Mississauga, Ontario, Canada). Sodium chloride, phosphoric acid, formaldehyde, ethidium bromide (EtBr), sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride (NED) were purchased from Sigma (St. Louis, MO). QA-Agarose was purchased from Qbiogene, Inc. (Irvine, CA). The Maxime RT-PCR PreMix kit was purchased from iNtRON Biotechnology (Sunngnam, Korea). All other chemicals were of analytical grade.

Preparation of the GEB extract. Three-year-old rhizomes of GEB were obtained from Gastrodia Farm in YoungYang, KyoungBuk Province, Korea. The washed and chopped fresh GEB was deep-frozen at -70°C until freeze-dried to get power. GEB was extracted with methanol and the methanol extract was re-suspended in 30% methanol. After centrifugation, the supernatants were dried in a rotary evaporator. The color of the fraction was dark brown.

Animals and care. Eight-week-old male BALB/c SPF mice, weighing between 22-25 g, were obtained from Samtaco (Suwon, Korea), and were fed a commercial diet (Samtaco, Korea) and water *ad libitum*. The animals were housed under a 12-h light/dark cycle at a temperature of 22±1°C and a humidity of 50±5%. All animal care was carried out according to the in-house guidelines of the university, and animals were allowed to acclimate to the laboratory environment for at least 1 week prior to the experiments.

Induction of gastric mucosal lesions and experimental treatments. The WIR stress-induced gastric lesion mouse model was generated as described previously (1). The mice were randomly divided into control (no treatment), L-arginine (300 mg/kg) and GEB (20 ml/kg) groups (n=6 mice per group, as described below). L-arginine was used as a positive control and comparative agent, since previous reports indicated that exogenously-administered L-arginine had

Table I. Effect of pretreatment with L-arginine and *Gastrodia elata* Blume on WIR stress-induced development of gastric mucosal lesions in mice.

Pretreatment	Ulcer index (points)
Control (i.p.)	23.0±4.1
Control (p.o.)	23.3±7.8
L-arginine (300 mg/kg, i.p.)	8.0±5.5 ^{ab}
GEB (0.02 ml/g, p.o.)	2.5±3.3 ^{ab}

Data are the mean ± SD (n=6 animals per group). ^aP<0.05 compared with the i.p.-injected control group; ^bP<0.05 compared with the p.o.-injected control group. The results were analyzed by one-way ANOVA followed by Dunnett's test.

protective effects against acute gastric mucosal lesions (16). Prior to injections, food was withheld for 24 h, but the mice were allowed free access to water. The mice in the L-arginine treatment group (n=6) each received one intraperitoneal (i.p.) injection of L-arginine (300 mg/kg) dissolved in isotonic saline; those in the GEB treatment group (n=6) each received one peroral (p.o.) injection of GEB (0.02 ml/g); and those in the two respective control groups (n=6) each received a single volume-matched i.p. or p.o. injection of isotonic saline. After the injections, the animals were returned to their cages for 1 h to allow drug absorption, and then placed in individual restraint cages and vertically immersed in a water bath (temperature held at 20-23°C) to the level of the xyphoid process. The mice were not allowed access to food or drink during immersion. After a 6-h exposure to immersion stress, the mice were euthanized with diethyl ether and their stomachs were removed. Each stomach was cut open along the greater curvature, and the gastric mucosa was removed using a pair of small scissors. The gastric mucosa was carefully examined for lesions and/or linear breaks (erosions) at the glandular part of the mucosal surface. Blood was collected from the inferior vena cava of each mouse shortly after removal of the stomach, and serum samples were obtained by centrifugation.

Determination of ulcer index. Mucosal lesions were scored as previously reported (17). Briefly, 1 point was given for small round hemorrhagic erosions, 2 points when the length of the hemorrhagic erosion was <1 mm, 3 points when the length was 1-2 mm, 4 points when the length was 2-3 mm, and 5 points when the length was >4 mm. This score was then doubled when the width of the erosion was >1 mm.

Measurement of NO production. The NO contents in the serum were measured via a slight modification of Griess assay (18). Briefly, the serum was added to a 96-well plate with activators (LPS 10 ng/well), and GEB extract (final concentration 30 µg/ml) was added. After 24 h, the supernatant was collected (100 µl) and an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride and 5% phosphoric acid) was added and incubated at room temperature for 10 min. The optical density of each sample was read at 550 nm by a microplate reader Victor3

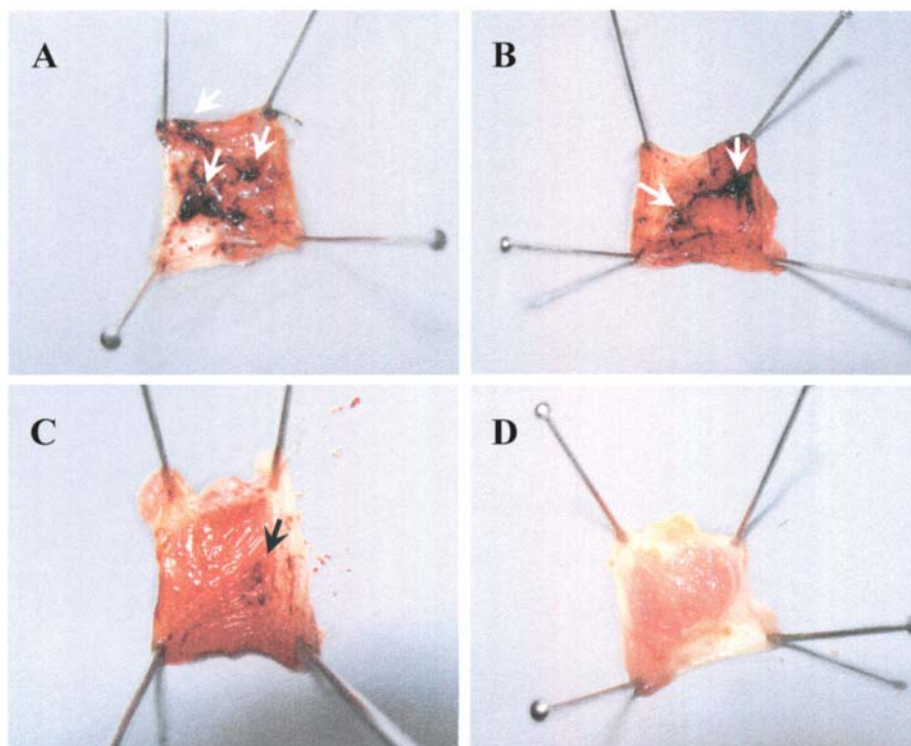


Figure 1. WIR stress-induced gastric mucosal lesions in mice. One hour prior to initiation of WIR stress, negative control mice received a single saline injection either i.p. (A) or p.o. (B); positive control mice received a single i.p. injection of L-arginine (300 mg/kg) (C); and experimental mice received a single p.o. injection of GEB (0.02 ml/g) (D). After a 6-h exposure to WIR stress, mice were euthanized and their stomachs were examined for gastric lesions, which were visualized as either punctuate or linear forms (black and white arrows).

(Wallac, Turku, Finland). The calibration curve was prepared using sodium nitrite as a standard.

Reverse-transcription polymerase chain reaction analysis of iNOS mRNA expression. Frozen gastric tissue (~100 mg) was homogenized in 1 ml Trizol reagent, and total RNA was prepared as described elsewhere (19). The total RNA was quantified by spectrophotometry at 260 nm (Victor3 spectrophotometer; Perkin-Elmer, Wellesley, MA) and the RNA quality was examined by 1% formaldehyde-agarose gel electrophoresis and EtBr staining. The RNA samples were then stored at -80°C until use. Single-step RT-PCR was performed using the Maxime RT-PCR PreMix, along with specific primer sets. These primers were designed using the Primer3 software (Whitehead Institute, MIT Center for Genome Research, Cambridge, MA) to analyze sequences deposited in the NCBI GenBank database. The primers, which were synthesized by Bioneer Co. (Daejeon, Korea), are shown in Table II. Briefly, first-strand cDNA was reverse-transcribed from 500 ng of total RNA at 45°C for 30 min, and the samples were denatured for 5 min at 95°C . PCR amplification was performed with 32 cycles (eNOS, nNOS and iNOS) or 27 cycles (GAPDH) of 45 sec at 95°C , 45 sec at 54°C for eNOS, 55°C for nNOS, 53°C for iNOS or 54°C for GAPDH, and 60 sec at 72°C . Following amplification, 20 μl of each RT-PCR product was resolved by 1.5% agarose gel electrophoresis and visualized by EtBr staining. The location of the predicted product was confirmed by comparison to a 1-kb DNA ladder (Solgent, Daejeon, Korea). For semi-quantitative analysis, the relative amounts of cDNA

in each matched set were normalized with regard to GAPDH expression using the Molecular Imager Gel Doc XR system and the Quantity One 1-D analysis software (Bio-Rad, Philadelphia, PA).

Statistical analysis. All data were presented as means \pm standard deviation (SD). Statistical analyses were performed with one-way analysis of variance (ANOVA), followed by Dunnett's test. P-values <0.05 were considered statistically significant.

Results

The effect of GEB on WIR stress-induced gastric mucosal lesions. One hour prior to initiation of WIR-induced stress, negative control mice received a single saline injection either i.p. or p.o., whereas positive control mice received a single i.p. injection of L-arginine at a concentration of 300 mg/kg. Experimental mice received a single p.o. injection of GEB (0.02 ml/g). After a 6-h exposure to WIR-induced stress, all mice were euthanized and their stomachs were examined for gastric lesions, which were visualized as either punctuate or linear forms. In samples from both control groups (i.p.- and p.o.-injected), we observed numerous lesions (Fig. 1A and B), most often 1-2 mm in size. In contrast, samples from the L-arginine-treated mice showed very few lesions as shown in Fig. 1. While control groups showed large-size blood-clotting clumps (Fig. 1A and B), in the positive control only medium-size clumps with minor numerous lesions were detected, which was not significant compared to the negative controls.

Table II. Oligonucleotide primers used for PCR reactions.

Name	Sequence of forward (F) or reverse (R) primer	Length (bp)
eNOS	5'-TACGCACCCAGAGCTTTTCT-3'	307
	5'-GCAGGATGCCCTAACTACCA-3'	
nNOS	5'-CCATCAGCTCCTCTCCAGAC-3'	511
	5'-ATATGGCCTCCTTGCTCCTT-3'	
iNOS	5'-AGACCTCAACAGAGCCCTCA-3'	305
	5'-GCAGCCTCTTGTCTTTGACC-3'	
GAPDH	5'-ATGTTCCAGTATGACTCCAC-3'	371
	5'-GCCAAAGTTGTCATCTGATGA-3'	

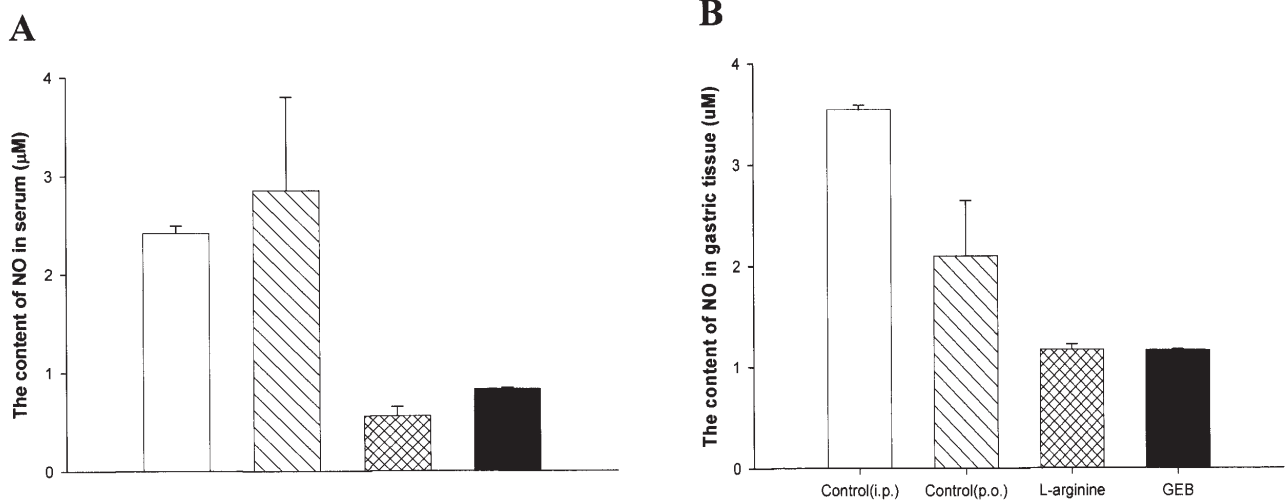


Figure 2. NO contents in serum and gastric tissue samples from mice exposed to WIR stress. One hour prior to initiation of WIR stress, negative control mice received a single saline injection either i.p. (white bar) or p.o. (bar with one set of diagonal lines); positive control mice received a single i.p. injection of L-arginine (300 mg/kg) (bar with a double set of diagonal lines); and experimental mice received a single p.o. injection of GEB (0.02 ml/g) (black bar). The presented data are the mean \pm SD of triplicate experiments. The results were analyzed by one-way ANOVA, followed by Dunnett's test. * $P < 0.05$ compared with the i.p. controls; † $P < 0.05$ compared with the p.o. controls.

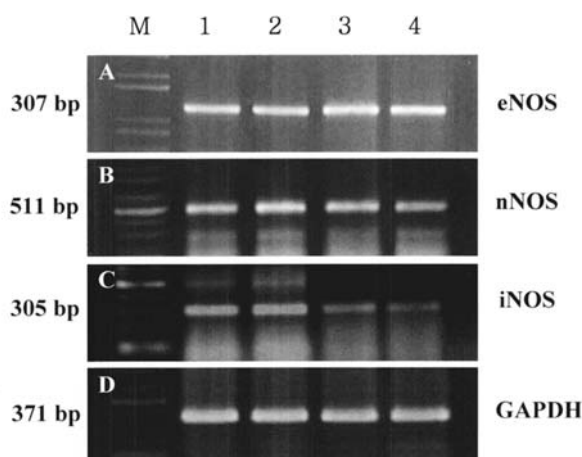


Figure 3. The mRNA expression levels of nitric oxide synthases in gastric tissue samples of mice exposed to WIR stress. mRNA expression levels of eNOS (A), nNOS (B), iNOS (C) and GAPDH (D) are shown. Lane M, PCR size marker; lane 1, i.p.-injected control group; lane 2, p.o.-injected control group; lane 3, L-arginine (300 mg/kg, i.p.)-treated positive control group; lane 4, GEB (0.02 ml/g, p.o.)-treated experimental group.

In Fig. 1D, GEB-treated mice showed no small-size clumps as well as large-size lesions. By scoring mucosal lesions with a slight modification of Nie *et al.* (17), we compared the ulcer index as shown in Table I. The ulcer indices of the negative controls were 23.0 ± 4.1 and 23.3 ± 7.8 , respectively, whereas that of the positive control was 8.0 ± 5.5 . Collectively, these findings indicate that GEB protects against WIR stress-induced gastric lesions in mice.

Changes in NO content. To begin addressing the mechanisms underlying the gastroprotective effects of GEB, we examined the NO content in serum and gastric tissue samples from the various groups of mice. As shown in Fig. 2, both L-arginine- and GEB-pretreated mice showed significant decreases in serum and gastric tissue NO levels with respect to their controls (51 and 50%, respectively, and 50 and 28%, respectively; all $P < 0.05$). These findings suggest that GEB treatment decreases NO levels in serum and gastric tissues, and further indicates that a similar NO level decrease is seen in mice treated with the known gastroprotective agent, L-arginine.

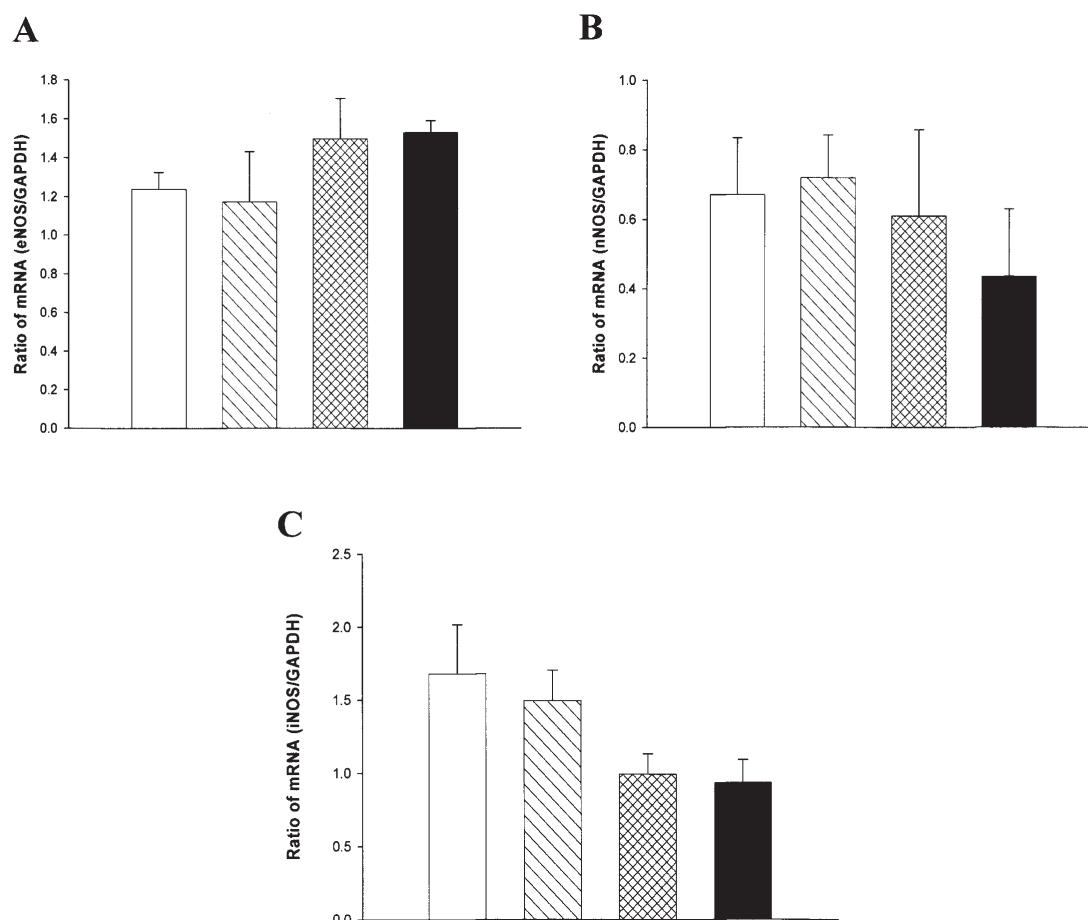


Figure 4. Comparison of nitric oxide synthase mRNA expression levels. The mRNA expression levels of eNOS (A), nNOS (B) and iNOS (C) mRNA were normalized versus that of GAPDH, using Quantity One version 4.6.1 (Bio-Rad, Philadelphia, PA). One hour prior to initiation of WIR stress, negative control mice received a single saline injection either i.p. (white bar) or p.o. (bar with one set of diagonal lines); positive control mice received a single i.p. injection of L-arginine (300 mg/kg) (bar with a double set of diagonal lines); and experimental mice received a single p.o. injection of GEB (0.02 ml/g) (black bar). The presented data are the mean \pm SD from three mice. The results were analyzed by one-way ANOVA, followed by Dunnett's test. * $P < 0.05$ compared with the i.p. controls; $^{\dagger}P < 0.05$ compared with the p.o. controls.

Expression profiles of eNOS, nNOS and iNOS mRNA. To test whether the observed changes in serum and gastric tissue NO levels were due to changes in the expression levels of eNOS, nNOS and iNOS, we used RT-PCR to examine the mRNA transcript levels of the genes encoding these enzymes. As shown in Figs. 3 and 4, the 305-bp product corresponding to the iNOS cDNA was markedly decreased in gastric tissues from WIR stress-induced lesions, whereas the levels of the 307-bp eNOS and 511-bp nNOS fragments were similar in all tested samples, regardless of treatment.

Discussion

We herein report for the first time that pretreatment with the traditional herbal plant, *Gastrodia elata* Blume (GEB), protects against WIR stress-induced gastric mucosal lesions in mice (Fig. 1 and Table I). This finding notably suggests that GEB can be applied for ulcer remedies.

We further show for the first time that the gastroprotective effects of GEB appear to involve suppression of nitric oxide levels via decreased mRNA expression of iNOS. Previous studies have shown that WIR-induced gastric mucosal lesions are associated with increased gastric acid production,

accelerated gastric peristalsis, microcirculation disturbances in the gastric mucosa, decreased prostaglandin levels, low turnover of gastric mucous cells, and NO-mediated changes in the homeostatic functions of the gastric mucosa (20-22). Of these mechanisms, NO-mediated regulation is particularly important, as it is involved in host defense and inflammatory responses (23), and also plays a critical role in gastric mucosal protection against injury induced by pressure, ethanol, stress and/or endotoxins (24). NOS activity is particularly high in gastric tissue (25), where NO helps maintain the integrity of the gastric epithelium (26), regulates gastric mucosal blood flow (27), and stimulates gastric mucus secretion and synthesis (25). The NO donor, glyceryl trinitrate, and the NOS substrate, L-arginine, have been shown to accelerate gastric ulcer healing in rats by increasing angiogenesis in the stomach (16). Consistent with this, inhibition of NOS activity was shown to attenuate the stimulatory effects of NO on mucus synthesis and secretion, inhibit angiogenesis, and impair ulcer healing (10,28).

In the present study, we showed that GEB-pretreated mice exposed to WIR stress had lower serum and gastric tissue NO contents than control stressed mice (Fig. 2). Similar results were obtained from L-arginine-pretreated mice, which

were used as a positive control based on a previous report that L-arginine pretreatment prevented the development of stress-induced gastric lesions (16). To test one possible mechanism underlying the observed decrease in NO content, we examined the expression levels of various NOS mRNAs. Our results revealed that the gastric mucosal expression of iNOS mRNA was significantly lower in GEB- and L-arginine-pretreated mice versus controls, whereas no such differences were observed in eNOS and nNOS mRNA expression levels (Figs. 3 and 4). These findings collectively suggest that the protective effects of NO against stress-induced gastric lesions in mice may be due, at least in part, to suppression of NO signaling via decreased iNOS mRNA expression by GEB.

Notably, previous studies have indicated the importance of cNOS in maintaining healthy gastric mucosa. Decreased cNOS activity has been associated with gastric erosion, while increased cNOS mRNA expression, activity and immunoreactivity has been seen in healing gastric mucosa, particularly in newly-formed vessels and neurons (29). Consistent with the notion that cNOS is a critical factor in gastric tissue repair, cigarette smoking-induced reductions in mucosal NOS activity have been associated with decreased gastric blood flow and suppression of angiogenesis at ulcer margins (10). However, we did not observe altered cNOS (eNOS and nNOS) mRNA expression in GEB- or L-arginine-pretreated mice exposed to WIR-induced stress. Instead, we observed changes in the mRNA expression levels of iNOS, suggesting that this isoform may be involved in the active mechanism of GEB-induced gastroprotection.

Large amounts of iNOS-generated NO have been implicated in gastric tissue injury during inflammatory reactions (30), and high iNOS expression and activity have been associated with severe inflammation in ulcerative mucosal tissues (31). Studies have shown that iNOS is usually induced under oxidative conditions, wherein high levels of NO can react with the superoxide anion ($O_2^{\cdot-}$), thus leading to peroxynitrite ($ONOO^-$) formation, protein tyrosine nitration, hydroxyl radical production, cell toxicity and tissue damage (31,32). In the present study, we observed high-levels of iNOS mRNA expression in the gastric mucosal lesions of control mice, but significantly lower levels in GEB-pretreated mice, suggesting that GEB might decrease NO production by inhibiting iNOS expression in WIR-induced gastric mucosal lesions. This may then prevent the abundant release of damaging NO, leading to gastroprotection.

In summary, we herein show for the first time that exogenously administered GEB protects against the development of WIR stress-induced gastric mucosal lesions in mice, and that this effect may be due, at least in part, to decreased iNOS mRNA expression and subsequent decreases in damaging NO levels. Although future studies will be needed to clarify the detailed mechanisms of this protective effect, these novel findings represent a new mode of action for GEB and may suggest that this traditional herbal plant or its extract could be developed for future use in ulcer prevention.

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