〈Original Article〉

Protective effect of Brazilian propolis ethanol extract against stress-induced gastric mucosal lesions in rats. Its evaluation using oxidative stress markers

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Summary We evaluated the protective effect of Brazilian propolis ethanol extract (BPEE) against gastric mucosal lesions in rats with water-immersion restraint stress (WIRS) using oxidative stress markers. Exposure of Wistar rats to 6 h of WIRS caused lesion development, increased lipid peroxide (LPO) and NOx concentrations and xanthine oxidase and myeloperoxidase activities, and decreased non-protein SH, ascorbic acid, and vitamin E concentrations in the gastric mucosa. Pre-administration of BPEE (10, 50 or 100 mg/kg, p.o.) prevented the lesion development by the following order: 50 mg/kg > 100 mg/kg > 10 mg/kg. Each dose of BPEE attenuated the increased xanthine oxidase activity and the decreased non-protein SH concentration. BPEE (50 mg/kg) attenuated the increased LPO and NOx concentrations and the decreased ascorbic acid and vitamin E concentrations. BPEE (100 mg/kg) attenuated the increased LPO concentration. These results suggest that BPEE protects against WIRS-induced gastric mucosal lesions in rats through its antioxidant properties.

Key words: Brazilian propolis (ethanol extract), Water-immersion restraint stress (rat), Gastric mucosal lesion, Oxidative stress

1. Introduction

Propolis (bee glue) is a resinous hive product collected by honeybee from various plant sources. It has important pharmacological properties and it can be used for a wide range of purposes as anti-inflammatory, antioxidant, antibacterial, and immunomodulatory agents[1-3)]. Chemically, propolis obtained from different areas of the world is constituted by 50-60% of resin, 30-40% of wax, 5-10% of essential oils, 5% of pollen,

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besides microelements[4]. It contains various organic compounds such as phenols, tannins, polysaccharides, terpenes, aromatic acids, and aldehydes[1, 4-6].

Brazilian green propolis is known to exert anti-ulcer activity in experimental animal models. It has been reported that the hydroalcoholic extract of Brazilian green propolis protects against gastric mucosal lesions induced by ethanol, indomethacin or water-immersion restraint stress (WIRS) in rats[7]. It has also been reported that the main phenolic acids of Brazilian green propolis exert a protective effect against gastric mucosal lesions induced by ethanol, indomethacin or WIRS in rats[8]. In addition, it has been reported that *Baccharis dracunculifolia*, the main botanical source of Brazilian green propolis, protects against gastric mucosal lesions induced by ethanol, indomethacin or WIRS in rats[9]. These reports have suggested that the hydroalcoholic extract of Brazilian green propolis, the main phenolic acids of Brazilian green propolis, and *Baccharis dracunculifolia* could exert an anti-ulcer effect in rats with WIRS by reducing the volume and acidity of gastric juice and by increasing the pH of gastric juice[7-9]. However, it is still unclear how the ethanol extract of Brazilian propolis protects against experimentally induced gastric mucosal lesions in rats.

We have reported that oxidative stress associated with lipid peroxidation, inflammation associated with neutrophil infiltration, and an excessive nitric oxide ($NO^{\cdot}$) production contribute to the development of WIRS-induced gastric mucosal lesions in rats[10-16]. Other researchers have reported that oxidative stress associated with the enhanced production of reactive oxygen species (ROS) and lipid peroxide and inflammation play an important role in the pathogenesis of WIRS-induced gastric mucosal injury in rats[17-21].

Therefore, we evaluated the protective effect of the ethanol extract of Brazilian propolis against WIRS-induced gastric mucosal lesions in rats using oxidative stress markers.

2. Materials and Methods

1. Materials

3,3',5,5'-Tetramethylbenzidine (TMB) and xanthine were purchased from Sigma (St. Louis, MO, USA); artepillin C, *L*-ascorbic acid, corticosteone, dichloromethane, N,N-dimethylformamide, α, α'-dipyridyl, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteau reagent, gallic acid, quercetin, reduced glutathione (GSH), 2-thiobarbituric acid, tocopherol standards such as a-tocopherol (α-Toc) and δ-tocopherol, trichloroacetic acid (TCA), and other chemicals from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). All chemicals used were of reagent grade and were not further purified.

2. Preparation of the ethanol extract of Brazilian green propolis

Brazilian green propolis was collected in the area of Minas Gerais in Brazil by MN Propolis Ind., Comércio e Exportacëo, Ltda (Mogi das Cruzes, SP, Brazil). The collected propolis (Lot No. KA-02) was provided by Japan Beekeeping Co. Ltd. (Gifu, Japan). The quality of the provided propolis had been certificated as follows: artepillin C, 10.1%; flavonoids, 41.1 mg/g; and bee wax, 5.6%. Ethanol extraction of Brazilian green propolis was conducted as follows: approximately 35 g of crude propolis was added to 100 ml of 95% ethanol and the mixture was kept at room temperature for 7 days. The final concentration of ethanol in the Brazilian propolis ethanol extract (BPEE) prepared was 80%. When the prepared BPEE was completely dried at 40℃, the content of solid components was estimated to be13.2%. The contents of flavonoid, total polyphenol, and artepillin C, a main component of Brazilian green propolis, in the prepared BPEE were 6.7 mg/ml, 27.6 mg/ml, and 28.8 mg/ml, respectively. The content of flavonid in BPEE was determined according to the method of Dowd[22]. To 0.5 ml of BPEE solution was added 0.5 ml of 2% $AlCl_3$ ethanol solution. After 1 h at room temperature, the absorbance was measured at 420 nm. The content of flavonoid in BPEE is expressed as that of quercetin equivalents. The content of polyphenol in BPEE was determined by the Folin-Ciocalteau colormetric method as described in the report of Ahin *et al.*[23] BPEE solution (0.5 ml) was mixed with 0.5 ml of the Folin-Ciocalteau reagent

and 0.5 ml of 10% Na_2CO_3, and the absorbance was measured at 760 nm after incubation for 1 h at room temperature. The content of total polyphenol in BPEE is expressed as that of gallic acid equivalents. The separation and quantification of artepillin C in BPEE were performed based on the HPLC method of Hayashi et al.[24] with modifications as to the mobile phase and column oven temperature. The HPLC was performed on a reversed-phase Shim-Pack HRC-ODS (15 cm x 4.5 mm i.d., Shimadzu, Kyoto, Japan) column with water-acetonitrile-acetic acid (45 : 55 : 2, v/v) as a mobile phase at a flow rate of 1 ml/min at 40℃. The volume of the BPEE sample injected to the column was 5 ml. Artepllin C in BPEE was detected at 280 nm and the identification was performed by comparing the retention time with the authentic compound using ultraviolet-detection.

3. Experimental animals

Male Wistar rats aged six weeks were purchased from Nippon SLC Co. (Hamamatsu, Japan). The animals were housed in cages in a ventilated animal room with controlled temperature (23 ± 2℃) and relative humidity (55 ± 5%) with 12 h of light (7:00 to 19:00). The animals were maintained with free access to rat chow, Oriental MF (Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum for one week. All animals received humane care in compliance with the Guidelines of the Management of Laboratory Animals in Fujita Health University. This animal experiment protocol was approved by the Institutional Animal Care and Use Committee.

4. Induction and observation of gastric mucosal lesions

Seven-week-old rats were starved for 24 h prior to experiments, but were allowed free access to water. Rats were restrained in wire cages and immersed up to the depth of the xiphoid process in a 23℃ water bath to induce WIRS-induced gastric mucosal lesions, as described by Takagi and Okabe[25]. Rats were sacrificed under ether anesthesia at 6 h after the onset of WIRS. The observation of gastric lesions was performed as follows: 10 ml of 0.9% NaCl was infused into the stomachs of rats with and without WIRS through the duodenum after ligation of the esophagus at 5 mm proximal to the gastroesophageal junction and then the duodenum was ligated at 10 mm distal to the pylorus. The stomachs filled with 0.9% NaCl were removed, fixed with 10% formaldhyde for 10 min, and cut open along with the greater curvature. The gastric mucosa was carefully examined for lesions recognized as linear breaks (erosions) at the mucosal surface of the glandular part under a stereoscopic microscope (×10). The extent of the lesion (lesion index) is expressed as the sum of the length of these breaks per stomach.

5. Administration of BPEE

BPEE was diluted with distilled water in order to administer its dose corresponding to 10, 50 or 100 mg of solid components present in the extract per kg body weight. Rats with WIRS received a single oral administration of 1 ml of the diluted BPEE solution per 100 g body weight at 30 min before the onset of WIRS. Control rats without WIRS received a single oral administration of 1 ml of 6% ethanol as the vehicle per 100 g body weight at the same time point.

6. Assays of gastric mucosal components and enzymes and serum components

At 6 h after the onset of WIRS, all rats used for the assays of gastric mucosal components and enzymes and serum components were sacrificed under ether anesthesia at which time blood was collected from the inferior vena cava. Immediately after sacrifice, stomachs were isolated and cut open along the greater curvature. The gastric mucosa was removed using a pair of small scissors. Serum was separated from the collected blood by centrifugation. The collected gastric mucosa and serum were stored at -80℃ until use. Gastric mucosa was homogenized in 9 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA to prepare 10% homogenate using a Physcotron handy microhomogenizer (Microtec Co., Funabashi, Japan). The gastric mucosal homogenate was used for the assays of non-protein SH (NPSH), vitamin E, ascorbic acid, and lipid peroxide (LPO). NPSH in the gastric mucosal homogenate was assayed by the DTNB method of Sedlak and Lindsay[26] using DTNB and GSH as a standard. Vitamin E in the gastric mucosal homogenate was assayed by the

HPLC method with electrochemical detection using γ-tocopherol as an internal standard as described in our previous report[27]. The amount of gastric mucosal vitamin E is expressed as that of α-tocopherol (α-Toc). Ascorbic acid in gastric mucosal homogenate was assayed by the α,α'-dipyridyl method of Zannoni *et al.*[28] The concentration of ascorbic acid was assayed using the standard curve of authentic *L*-ascorbic acid. LPO in the gastric mucosal homogenate was assayed by the thiobarbituric acid method of Ohkawa *et al.*[29] using tetramethoxypropane as a standard except that 1 mM EDTA was added to the reaction mixture. The amount of gastric mucosal LPO is expressed as that of malondialdehyde (MDA) equivalents. NOx (nitrite/nitrate) in gastric mucosa was assayed by the Griess reaction-dependent method of Green *et al.*[30]. For this assay, gastric mucosal tissue was homogenized in 9 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.5) using a Physcotron handy microhomogenizer. The homogenate was sonicated two times on ice for 30 s using a Handy Sonic model UR-20P (Tomy Seiko Co., Tokyo, Japan). The sonicated homogenate was centrifuged at 10,000 × g for 20 min at 4℃ and the resultant supernatant was filtrated at 4℃ under centrifugation using a membrane filter Ultrafree-MC (Millipore Co., Bedford, MA, USA). NOx in the filtrate was determined using a nitric oxide assay kit (Roche-Diagnostics Co., Tokyo, Japan). Gastric mucosal xanthine oxidase (XO) was assayed by the ultraviolet method of Hashimoto[31]. For this enzyme assay, gastric mucosal tissues were homogenized in 9 volumes of ice-cold 0.25 M sucrose using a Physcotron handy microhomogenizer. The homogenate was sonicated as described above. The sonicated homogenate was centrifuged at 4℃ (10,000 × g, 20 min), and the resultant supernatant was dialyzed against 100 volumes of the same solution at 4℃ for 24 h. XO activity was assessed by measuring the increase in absorbance at 292 nm following the formation of uric acid at 30℃. One unit (U) of this enzyme is defined as the amount of enzyme forming 1 mmol uric acid per min. Gastric mucosal myeloperoxidase (MPO) was assayed by the method of Suzuki *et al.*[32]. For the assay of this enzyme, gastric mucosal tissues were homogenized in 9 volumes of ice-cold 0.05 M Tris-HCl buffer (pH 7.4) using a Physcotron handy microhomogenizer. After sonication on ice for 20 sec using a Handy Sonic model UR-20P, the homogenate was centrifuged at 4℃ (10,000 × g, 20 min), and the resultant supernatant was dialyzed against 100 volumes of the same buffer at 4℃ for 24 h. One unit (U) of this enzyme is defined as the amount of enzyme forming 1 mmol uric acid per min. MPO activity was assessed by measuring the hydrogen peroxide-dependent oxidation of TMB at 37℃. TMB was dissolved in *N*,*N*-dimethylformamide. One unit (U) of this enzyme is defined as the amount of enzyme causing a change in absorbance of 1.0 per min at 655 nm.

Serum adrenocorticotropic hormone (ACTH) was assayed using a commercial kit, ACTH EIA kit (Phonix Pharmaceutical Inc., Burlingame, CA, USA). Serum corticosterone was measured by the method of Guillemin *et al.*[33] using authentic corticosterone as a standard. Corticosterone in serum was extracted with dichloromethane and the resultant extract was mixed with a fluorescence reagent (ethanol and concentrated H_2SO_4 in the ratio of 3 : 7 v/v). The resulting fluorescence of the acid layer was fluorometorically measured. The excitation and emission wavelengths were 470 and 530 nm, respectively. Serum glucose was assayed using a commercial kit, Glucose C-Test Wako (Wako Pure Chemical Ind., Ltd., Osaka, Japan).

7. Statistical analysis

All results obtained are expressed as means ± standard deviation (S.D.) The statistical analyses of the results were performed using a computerized statistical package (StatView). Each mean value was compared by one-way analysis of variance (ANOVA) and Bonferroni/Dunn for multiple comparisons. The significance level was set at $P<0.05$.

3. Results

When BPEE (10, 50 or 100 mg/kg) was orally administered to rats with 6 h of WIRS at 30 min after the onset of stress, the lesion index of developed gastric mucosal lesions was significantly reduced by each dose of the extract (Fig. 1). The order of the

protective effect of BPEE against the development of WIRS-induced gastric mucosal lesions was as follows: 50 mg/kg > 100 mg/kg > 10 mg/kg (Fig. 1). Unstressed control rats orally given 6% ethanol as the vehicle showed no gastric mucosal lesion (data not shown). The gross features of typical gastric mucosal lesions in the stressed rats pre-administered with and without BPEE (50 mg/kg) are shown in comparison with that of the gastric mucosa of a control rat without WIRS in Fig. 2.

Rats with 6 h of WIRS had significantly higher gastric mucosal LPO and NOx concentrations than unstressed control rats (Fig. 3A and B). The increased gastric mucosal LPO concentration was significantly attenuated by BPEE pre-administered at a dose of 50 or 100 mg/kg but the attenuating effect of BPEE was significantly larger at its dose of 50 mg/kg than at its dose of 100 mg/kg (P<0.05) (Fig. 3A). The increased gastric mucosal NOx concentration was significantly attenuated by BPEE pre-administered at a dose of 50 mg/kg (Fig. 3B). Rats with 6 h of WIRS had significantly higher gastric mucosal MPO and XO activities than unstressed control rats (Fig. 3C and D). The

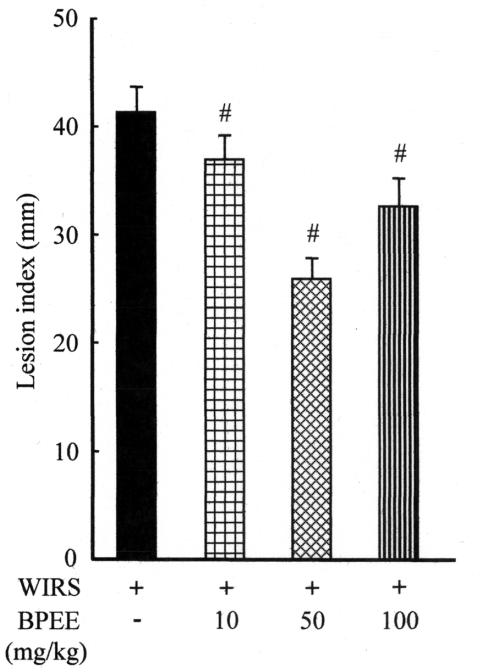

Fig. 1 Effect of BPEE on gastric mucosal lesion development in rats with WIRS. BPEE (10, 50 or 100 mg/kg) was orally administered to rats with 6 h of WIRS at 30 min before the onset of WIRS. Each value is a mean ± S.D. (n = 10 for each group). # Significantly different from rats with WIRS alone at P<0.05.

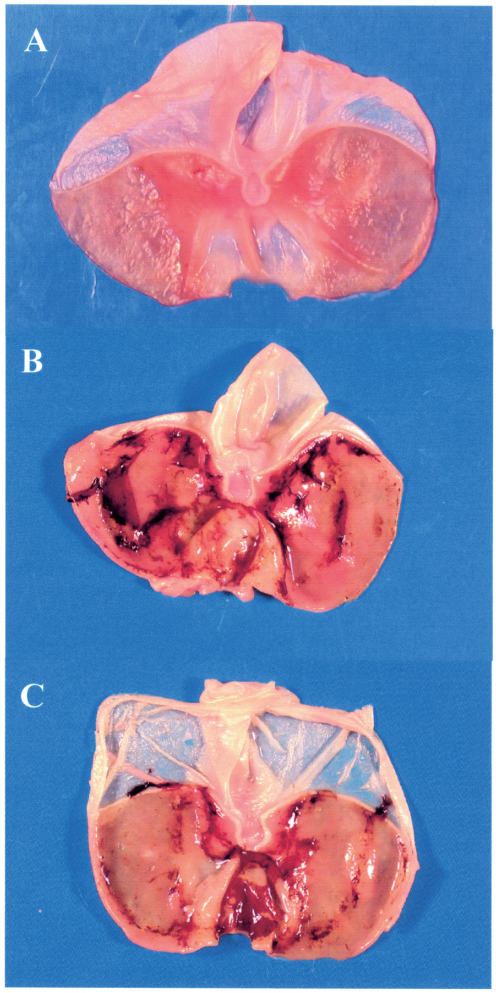

Fig. 2 Gross features of typical gastric mucosal lesions in WIRS-loaded rats with and without BPEE pre-administration. A, a control rat without WIRS; B, a WIRS-loaded rat without BPEE pre-administration; C, a WIRS-loaded rat pre-administered with BPEE (50 mg/kg).

increased gastric mucosal XO activity, but not gastric mucosal MPO activity, was significantly attenuated by BPEE pre-administration at a dose of 10, 50 or 100 mg/kg (Fig. 3C). The attenuating effect of BPEE was significantly higher at its dose of 50 or 100 mg/kg than at its dose of 10 mg/kg ($P < 0.05$), but there was no significant difference between its doses of 50 and 100 mg/kg (Fig. 3D).

Rats with 6 h of WIRS had significantly lower gastric mucosal NPSH, ascorbic acid, and vitamin E concentrations than unstressed control rats (Fig. 4). The decreased gastric mucosal NPSH concentration was significantly attenuated by BPEE pre-administered at a dose of 10, 50 or 100 mg/kg and the attenuating effect of BPEE occurred in a dose-dependent manner (Fig. 4A). In addition, there was no significant difference in gastric mucosal NPSH concentrations between the stressed rats pre-administered with BPEE at a dose of 10 or 50 mg/kg and unstressed control rats and the stressed rats pre-administered with BPEE at a dose of 100 mg/kg had significantly higher gastric mucosal NPSH concentration than unstressed control rats (Fig. 4A). The decreased gastric mucosal ascorbic acid and vitamin E concentrations were significantly attenuated by BPEE pre-administered at a dose of 50 mg/kg, but not 10 or 100 mg/kg (Fig. 4B and C).

Rats with 6 h of WIRS had significantly higher serum ACTH, corticosterone, and glucose concentrations than unstressed control rats (Fig. 5). The increased serum ACTH, corticosterone, and glucose concentrations were not affected by BPEE pre-administered at a dose of 10 or 50 mg/kg but their concentrations were further increased significantly by BPEE pre-administered at a dose of 100 mg/kg (Fig. 5).

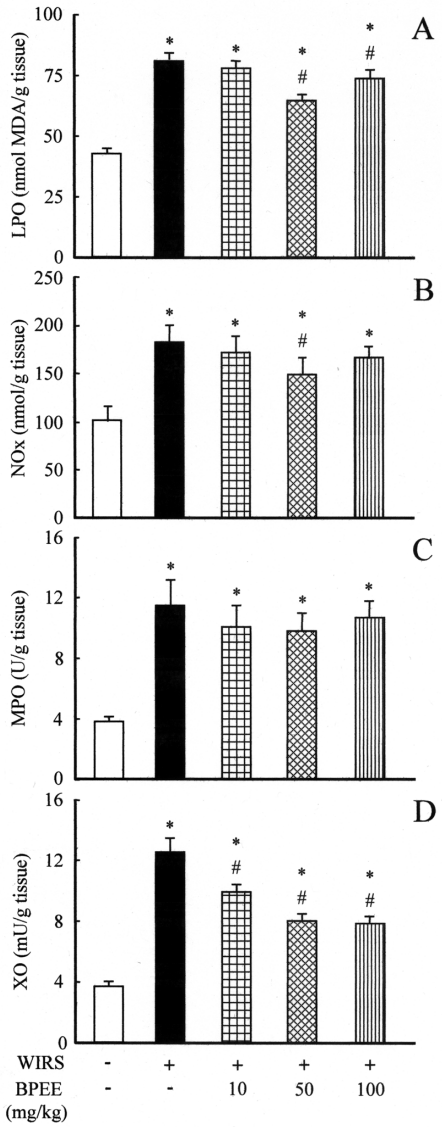

Fig. 3 Effect of BPEE on gastric mucosal LPO (A) and NOx (B) concentrations and MPO (C) and XO (D) activities in rats with 6 h of WIRS. BPEE (10, 50 or 100 mg/kg) was orally administered to rats with WIRS at 30 min before the onset of WIRS. Each value is a mean ± S.D. (n = 8 for each group). * Significantly different from control rats without WIRS at P<0.05; # Significantly different from rats with WIRS alone at P<0.05.

4. Discussion

In the present study, a single oral administration of BPEE, which was prepared by extraction of Brazilian green propolis with 95% ethanol, at a dose of 10, 50 or 100 mg/kg exerted a protective effect against gastric mucosal lesions induced by 6 h of WIRS at 23℃ in male Wistar rats weighing 200-230 g, although the protective effect of BPEE was the highest at a dose of 50 mg/kg and was reduced rather than enhanced at a

dose of 100 mg/kg. Thus, the protective effect of BPEE against WIRS-induced gastric mucosal lesions was found to be diminished at its high dose. de Barros et al.[7] have reported that a single oral administration of the 70% ethanol extract of Brazilian green propolis protects against gastric mucosal ulcer induced by 17 h of WIRS at 25℃ in male Wistar rats weighing 200-250 g at a dose of 250 or 500 mg/kg, but not 50

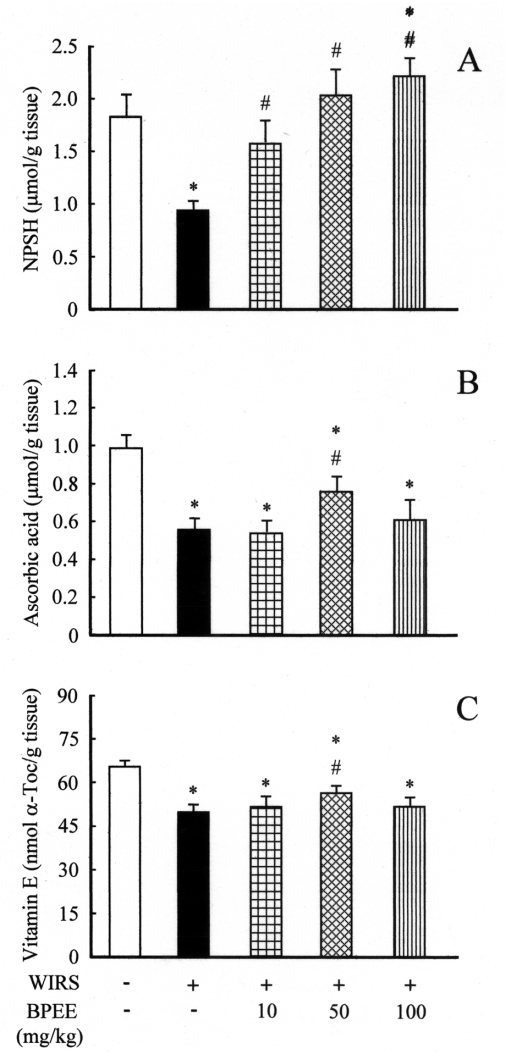

Fig. 4 Effect of BPEE on gastric mucosal NPSH (A), ascorbic acid (B), and vitamin E (C) contents in rats with WIRS. BPEE (10, 50 or 100 mg/kg) was orally administered to rats with 6 h of WIRS at 30 min before the onset of WIRS. Each value is a mean ± S.D. (n = 8 for each group). * Significantly different from control rats without WIRS at P<0.05; #,Significantly different from rats with WIRS alone at P<0.05.

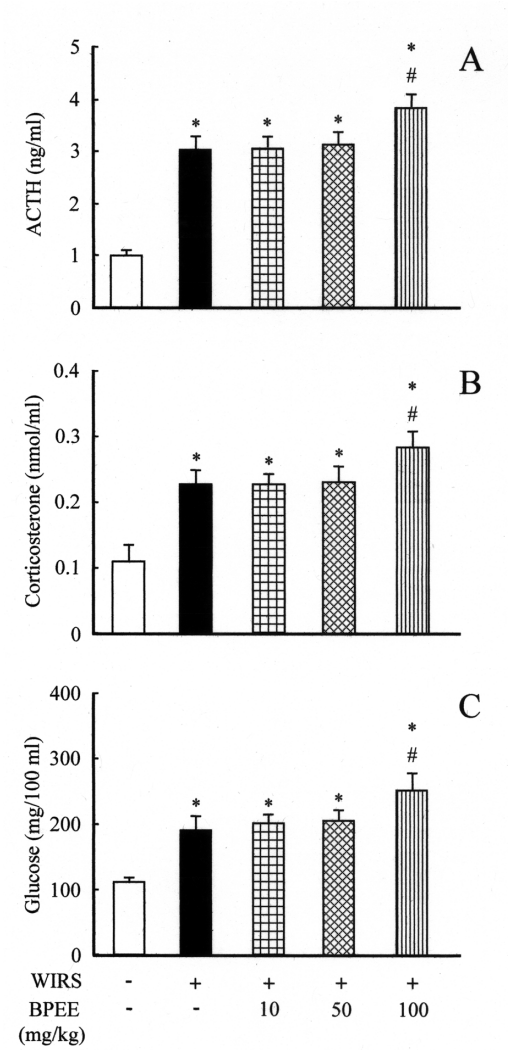

Fig. 5 Effect of BPEE on serum ACTH (A), corti costerone (B), and glucose (C) concentra tions in rats with WIRS. BPEE (10, 50 or 100 mg/kg) was orally administered to rats with 6 h of WIRS at 30 min before the onset of WIRS. Each value is a mean ± S.D. (n = 8 for each group). * Significantly different from control rats without WIRS at P<0.05; # Significantly different from rats with WIRS alone at P<0.05.

mg/kg, although there is little difference in the protective effect between doses of 250 and 500 mg/kg. Thus, the report of de Barros *et al.*[7] indicates that a high dose of the hydroalcoholic extract of Brazilian green propolis does not reduce its protective effect against stress-induced gastric mucosal lesions. Furthermore, de Barros *et al.*[8] have reported that caffeic acid, cinnamic acid, *p*-coumaric acid, and ferulic acid, the main phenolic acids found in Brazilian green propolis, protects against gastric ulcer induced by 17 h of WIRS in male Wistar rats weighing 200-250 g. However, the same authors have demonstrated that cinnamic acid exerts less of a protective effect against the stress-induced gastric ulcer at a dose of 250 mg/kg than at a dose of 50 mg/kg[7]. Accordingly, there seems to be a possibility that the above-described difference in a protective effect against WIRS-induced gastric mucosal lesions between BPEE used in the present study and the 70% ethanol extract of Brazilian green propolis used in the study of de Barros *et al.*[7] is due to the differences in the composition and/or the content of phenolic acids in the extract used. In addition, there are some differences in the stress-inducing conditions used, such as water temperature and the duration of WIRS, between the present study and the study of de Barros *et al.*[7].

It has been implicated that oxidative stress associated with enhanced lipid peroxidation and ROS production and impaired antioxidant defense systems associated with depletion of antioxidants such as NPSH, ascorbic acid, and vitamin E, an excessive $NO^{\cdot}$ production, and inflammation associated with neutrophil infiltration contribute to gastric mucosal lesion development in rats subjected to WIRS[10-21]. In the present study, rats with 6 h of WIRS showed increases in gastric mucosal LPO and NOx concentrations and MPO and XO activities and decreases in gastric mucosal NPSH, ascorbic acid, and vitamin E concentrations. A single oral pre-administration of BPEE at a dose of 10, 50 or 100 mg/kg attenuated the increased gastric mucosal XO activity significantly and the order of its attenuating effect was as follows: 100 mg/kg $\geqq$ 50 mg/kg > 10 mg/kg. The pre-administration of BPEE at a dose of 10, 50 or 100 mg/kg attenuated the decreased gastric mucosal NPSH concentration significantly in a dose-dependent manner. In addition, the gastric mucosal NPSH concentration in the stressed rats pre-administered with 100 mg/kg of BPEE was significantly higher than that in unstressed control rats. BPEE pre-administered at a dose of 50 or 100 mg/kg, but not 10 mg/kg, attenuated the increased gastric mucosal LPO concentration significantly but the attenuating effect was larger at a dose of 50 mg/kg than at a dose of 100 mg/kg. BPEE pre-administered at a dose of 50 mg/kg, but not 10 or 100 mg/kg, attenuated the increased gastric mucosal NOx concentration significantly. However, BPEE pre-administered at a dose of 10, 50 or 100 mg/kg did not attenuate the increased gastric mucosal MPO activity. Thus, any dose of BPEE used was found to be ineffective in preventing the infiltration of neutrophils into the gastric mucosa of rats with 6 h of WIRS. BPEE pre-administered at a dose of 50 mg/kg, but not 10 or 100 mg/kg, attenuated the decreased gastric mucosal ascorbic acid and vitamin E concentrations significantly. Accordingly, BPEE pre-administered at a dose of 50 mg/kg was found to be the most effective in suppressing oxidative stress and an excessive $NO^{\cdot}$ production in the gastric mucosa of rats with 6 h of WIRS which could contribute to the most effective protection against WIRS-induced gastric mucosal lesions.

Yoshizumi *et al.*[34] have reported that the 80% ethanol extract of Brazilian propolis inhibits XO activity in vitro and that artepillin C and *p*-coumaric acid are very weak in inhibiting XO activity in vitro in comparison with caffeic acid phenetyl ester (CAPE) and flavonids such as galangin, and chrysin. The same authors have shown that the contents of CAPE, galangin, and chrysin are in trace amount in comparison with those of artepillin C and *p*-coumaric acid in the ethanol extract of Brazilian propolis[34]. We have observed that 40 μg or more of BPEE used in the present study inhibits XO activity in vitro (unpublished data). Therefore, the effect of BPEE to attenuate the increased gastric mucosal XO activity in rats with WIRS seems to be due to its direct inhibitory effect on the enzyme activity which may be caused by unknown component(s) rather than CAPE, galangin, and chrysin present in the extract.

It has been reported that the ethanol extracts of Brazilian propolis and artepillin C, a major component present in the extract, exerts antioxidant activity by inhibiting lipid peroxidation and by scavenging reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical, and hydrogen peroxide and free radicals[35-39]. Therefore, it is suggested that pre-administered BPEE could attenuate the increased gastric mucosal LPO concentration in rats with 6 h of WIRS through its antioxidant activity, which is possibly mainly due to artepillin C present in the extract, although the attenuating effect of BPEE was less at a dose of 100 mg/kg than at a dose of 50 mg/kg.

As described above, BPEE pre-administered at a dose of 50 mg/kg attenuated the increased gastric mucosal NOx concentration in rats with 6 h of WIRS, although the same dose of the extract did not reduce the increase in gastric mucosal MPO activity. We have shown that an excessive $NO^{\cdot}$ production in the gastric mucosa of rats with WIRS is mediated by inducible nitric oxide synthase (iNOS) increasing in the gastric mucosal tissue and have suggested that this excessive $NO^{\cdot}$ generation mediated by iNOS could be associated with an increase in neutrophils infiltrating into the gastric mucosal tissue[12, 14]. Besides, we have reported that iNOS activity increasing in the gastric mucosa of rats with WIRS is closely related with increased LPO production and NPSH depletion in the tissue, which contributes to the development of WIRS-induced gastric mucosal lesions[13]. It has been shown in rats infused with $NO^{\cdot}$ donors that $NO^{\cdot}$ derived from $NO^{\cdot}$ donors increases lipid peroxidation in the gastric mucosal tissue possibly through the formation of peroxynitrite by the reaction between $NO^{\cdot}$ and $O_2^{\cdot-}$[40]. Paulino et al.[41] have reported that the 95% ethanol extract of Brazilian green propolis decreases $NO^{\cdot}$ production in lipopolysaccharide-stimulated RAW 264.7 cells possibly by inhibiting the gene expression of iNOS (Paulino and others 2006). Tan-no et al.[42] have reported that the ethanol extract of Brazilian propolis inhibits $NO^{\cdot}$ production in mice with carrageenan-induced paw edema. Furthermore, Paulino et al.[43] have demonstrated that artepillin C inhibits $NO^{\cdot}$ production in RAW 264.7 cells treated with lipopolysaccharide. Thus, the ethanol extract of Brazilian propolis and aetepillin C inhibit iNOS-mediated $NO^{\cdot}$ production under inflammatory conditions. In addition, it is known that the methanol extract of Brazilian propolis scavenges not only $O_2^{\cdot-}$ but also $NO^{\cdot}$ directly[44]. It is also known that the ethanol extract of Brazilian green propolis inhibits the production of ROS by rabbit neutrophils stimulated with opsonized zymozan[45]. We have observed that 10 μg or less of BPEE used in the present study inhibits the production of $O_2^{\cdot-}$ in human neutrophils stimulated with *N*-formyl-methionyl-leucyl-phenylalanine or phorbol myristate without scavenging the generated $O_2^{\cdot-}$ (unpublished data). Accordingly, it is suggested that BPEE administered at a dose of 50 mg/kg could protect against WIRS-induced gastric mucosal lesions in rats through its inhibitory effect on excessive $NO^{\cdot}$ and ROS production via infiltrated neutrophils and/or through its direct action to scavenge $O_2^{\cdot-}$ and $NO^{\cdot}$ generated via infiltrated neutrophils in the gastric mucosal tissue.

As described above, BPEE exerted a protective effect against WIRS-induced gastric mucosal lesions in rats less effectively at a dose of 100 mg/kg than at a dose of 50 mg/kg. This finding may be explained based on the phenomenon that the sensitivity to stress response was enhanced by BPEE pre-administered at a dose of 100 mg/kg, but not at a dose of 50 mg/kg, judging from the serum levels of stress markers such as ACTH, corticosterone, and glucose. It has been shown in pylorus-ligated rats with and without rotational stress that the sensitivity to stress response affects the formation of gastric ulcer, judging from the level of plasma corticosterone[46]. Carolina *et al.*[47] have shown that mice administered orally with the 70% ethanol extract of Brazilian propolis (200 mg/kg) just before the onset of restraint stress have higher serum corticosterone concentrations than mice without the extract administration after a short period of restraint stress.

It has been implicated that an increase in histamine-mediated gastric acid secretion contributes to the development of WIRS-induced gastric mucosal

lesions in rats[48, 49]. de Barros *et al.*[7] have shown that pre-administration of the 70% ethanol extract of Brazilian green propolis (250 or 500 mg/kg, per os) to pylorus-ligated rats reduces the total volume, total acidity, and pH of the gastric juice and have suggested that the ethanol extract (250 or 500 mg/kg, per os) could exert a protective effect against gastric ulcer in rats with 17 h of WIRS through its anti-histamine activity. In the present study, however, BPEE administered orally at a dose of 50 mg/kg exerted the highest protective effect against gastric mucosal lesions in rats with 6 h of WIRS. Therefore, there seems to be little possibility that BPEE exerts a protective effect against WIRS-induced gastric mucosal lesions in rats by inhibiting acid secretion.

In conclusion, the results obtained from the present study suggest that a single oral administration of BPEE protects against gastric mucosal lesions in rats with 6 h of WIRS through its antioxidant properties in the gastric mucosa, although a high dose of BPEE reduces the protective effect possibly by enhancing the sensitivity to stress response.

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