2004. Vol. 25. No. 4. 61-78
Korean Journal of Oriental Medicine

Original Articles

Experimental Studies on the Effect of *Gamibaegi-eum*

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Objective : This study was undertaken to determine whether Gamibaegi-eum (BGU) in vitro and in vivo exerts a beneficial effect against cell injury induced by reactive oxygen species (ROS) in the human intestine.

Methods : Effects of BGU in vitro on cell injury were examined using Caco-2 cells, cultured human intestinal cell line. Exposure of cells to H_2O_2 induced increases in the loss of cell viability in a time and dose-dependent fashion.

Results : BGU prevented H_2O_2-induced cell death and its effect was dose-dependent over a concentration range of 0.05-1%. H_2O_2-induced cell death was prevented by catalase, the hydrogen peroxide scavenger enzyme, and deferoxamine, the iron chelator. However, the potent antioxidant DPPD did not affect H_2O_2-induced cell death. H_2O_2 increased lipid peroxidation, which was inhibited by BGU and DPPD. H_2O_2 caused DNA damage in a dose-dependent manner, which was prevented by BGU, catalase, and deferoxamine, but not DPPD. BGU restored ATP depletion induced by H_2O_2. BGU inhibited generation of superoxide and H_2O_2 and scavenged directly H_2O_2. Oral administration of mepirizole in vivo at a dose of 200mg/kg resulted in ulcer lesions in the stomach and the proximal duodenum. Pretreatment of BGU(0.1%/kg, orally) and catalase (800Units/kg, i.v.) significantly decreased the size of ulcers. Mepirizole increased lipid peroxidation in the mucosa of the duodenum, suggesting an involvement of ROS. Pretreatment of BGU and catalase significantly inhibited lipid peroxidation induced by mepirizole. Morphological studies showed that mepirizole treatment causes duodenal injury and its effect is prevented by BGU.

Conclusion : These results indicate that BGU exerts a protective effect against cell injury in vitro and in vivo through antioxidant action. The present study suggests that BGU may play a therapeutic role in the treatment of human gastrointestinal diseases mediated by ROS.

Key Words: *Gamibaegi-eum*, Caco-2 cell, antioxidant, gastrointestinal disease.

Introduction

Reactive oxygen species (ROS) are unstable chemical entities that contain an unpaired electron in their outer orbital and are generally very reactive[1]. ROS participate in oxidation/reduction reactions with neighboring compounds in order to regain thermodynamic and electrochemical stability. Oxygen free radicals are continually produced in the mitochondrial electron transport chain of respiring cells as a consequence of the incomplete reduction of molecular oxygen[2]. The ROS formed in this process include superoxide anion(O_2-), hydrogen peroxide, and hydroxyl radical(-OH). The production of ROS exceeding the normal level of cellular cytoprotective

Received 13 December 2004; received in revised from 20 October 2004; accepted 25 October 2004
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Table 6. Prescription of *Gamibaegi-eum.*

Herbal name	Scientific name		Weight
original prescription Pericarpium(原方)	Chen Pi(陳皮)	*Aurantii Nobilis*	8g
	Mu Xiang(木香)	*Helenii Radix*	8g
	Zhi Ke(枳殼)	*Ponciri Fructus*	8g
	Hou Po(厚朴)	*Magnoliae Cortex*	8g
	Ze Xie(澤瀉)	*Alismatis Rhizoma*	8g
	Wu Yao(烏藥)	*Linderae Radix*	8g
	Huo Xiang(藿香)	*Agastachis Herba*	8g
	Xiang Fu Zi(香附子)	*Cyperi Rhizoma*	8g
additional drugs(加味)	Shan Zha(山査)	*Crataegi Fructus*	8g
	Bo He(薄荷)	*Menthae Herba*	8g
	Jue Ming Zi(決明子)	*Cassiae Semen*	8g
Total amount			88g

mechanisms can be highly toxic to cells[3]. ROS-induced cytotoxicity occurs by the oxidation of constituent proteins, carbohydrates, lipids and nucleic acids, which leads to impairing cellular function and kills the cell.

ROS are induced by gastrointestinal injury in various pathological conditions such as ischemia-reperfusion injury[4], certain types of drug-induced gastroenteropathy[5], necrotising enterocolitis[6], experimental colitis[7], and inflammatory bowel diseases[8]; Therefore, agents that efficiently scavenge ROS may protect the gastrointestinal damage induced by noxious chemicals[9].

Gamibaegi-eum (加味排氣飲)(BGU) which is originally prescribed by Shen Jinao (沈金鰲), a physician (1717-1776) of the Qing Dynasty and an author of "Shen' s Work on the importance of Life Preservation (沈氏尊生書)"[10], enhances the function of the stomach, resolves phlegm, checks upward adverse flow qi, air, and treats symptoms such as nausea, vomiting, and abdominal pain[11]. Thus, I believe that BGU is a very suitable and effective prescription for various gastrointestinal diseases among other treatments.

BGU is also generally used for an administration of medicine; nevertheless, this treatment has never been verified for medical effectiveness in terms of clinical experiments.

This study was undertaken to determine whether BGU protects against ROS-induced cell death *in vitro* and *in vivo* . I examined the effect of BGU treatment *in vitro* on cell viability in the human-derived cultured intestinal epithelial cell line Caco-2 as a model, which has been extensively employed in studies to characterize intestinal transport function[12] and that of BGU treatment *in vivo* on mepirizole-induced intestinal ulcers in rabbits. Mepirizole, a nonsteroidal antiinflammatory drug, is known to be a duodenal ulcerogenic[13], and its effect is mediated by generation of ROS[14].

Materials and Methods

1. BGU extract preparation (Table 1)

BGU (352g of crushed crude drugs) was extracted with 3000ml distilled water at 100 for 2 hr and the total extract was evaporated under reduced pressure to give 46g. The dried extract was dissolved in Hank' s

balanced salt solution (HBSS, Sigma Co. USA) just before use.

1) *In vitro* studies

(1) Culture of Caco-2 cells

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained by serial passages in 75cm2 culture flasks (Costar, Cambridge, MA). The cells were grown in Dulbecco' s modified Eagle' s medium/Ham' s F12 (DMEM/F12, Sigma Chemical Co.) containing 10% fetal bovine serum at 37oC in 95% air/5% CO2 incubator. When the cultures reached confluence, a subculture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were grown on 24-well tissue culture plates in DMEM/F12 medium containing 10% fetal bovine serum. All experiments started 3-4 days after plating when a confluent monolayer culture was achieved. Cells were treated with hydrogen peroxide (H_2O_2) in HBSS without serum in the presence or absence of BGU.

(2) Measurement of cell death

Cells were grown to confluence in 24-well dishes, incubated in the HBSS containing H_2O_2 for 120 min at 37℃ in 95% air/5% CO_2, and then harvested using 0.025% trypsin. Cells were incubated with 4% trypan blue solution. Cells failing to exclude the dye were considered nonviable, and the data are expressed as percentage of nonviable cells.

(3) Measurement of DNA single-strand breaks

The DNA strand break was measured by the DNA precipitation assay[15]. Confluent cells grown in 24-wells were labelled in the presence of 0.25 uCi/ml [^{3}H]methylthymidine for 24 hr. The cells were thoroughly washed with HBSS, and treated with H_2O_2 in the presence or absence of BGU. After treatment, the cells were washed with HBSS and lysed in an effendorf tube with 0.5ml of lysis buffer (10mM Tris/HCl, 10mM EDTA, 50mM NaOH, 2% SDS, pH 12.4), followed by addition of 0.5ml of 0.12M KCl. The lysate was incubated for 10 min at 65℃, followed by a 5 min cooling-and-precipitation period on ice. A DNA-protein K-SDS precipitate was formed under these conditions, from which low-molecular-mass Caco-2 DNA was released. This DNA was recovered in the supernatant from a 10 min centrifugation at 200g, 10℃, and transferred into a liquid scintillation vial containing 1ml of 200mM HCl. The precipitation pellet (intact double-stranded DNA) was solubilized in 1ml of water at 65℃. The tube was rinsed with 1ml of water, and 8ml of scintillation fluid was added to each vial. The amount of double-stranded DNA remaining was calculated for each sample by dividing the d.p.m. value of the pellet by the total d.p.m. value of the pellet plus supernatant and multiplying by 100. The extent of DNA damage was expressed as the ratio of single stranded DNA to total stranded DNA (double stranded + single stranded).

(4) Measurement of ATP content

ATP levels in Caco-2 cells were measured by a luciferin-luciferase assay. After an exposure to oxidant stress, cells were solubilized with 500ul of 0.5% Triton X-100 and acidified with 100ul of 0.6M perchloric acid and placed on ice. The cell suspension was then diluted with 10mM potassium phosphate buffer containing 4mM $MgSO_4$(pH 7.4) and 100ul of 20mg/ml luciferin-luciferase was added to 10ul of diluted sample. Light emission was recorded at 20 s with a luminometer (MicroLumat LB96P, Berthold, Germany). Protein content was determined on an aliquot of cell suspension.

(5) Measurement of lipid peroxidation

Lipid peroxidation was estimated by measuring the

renal cortical content of malondialdehyde (MDA) according to the method of Uchiyama and Mihar[16]. Cells were homogenized in ice-cold 1.15% KCl (5% wt/vol). A 0.5ml aliquat of homogenate was mixed with 3ml of 1% phosphoric acid and 1ml of 0.6% thiobarbituric acid. The mixture was heated for 45 min on a boiling water bath. After addition of 4ml of n-butanol the contents were vigorously vortexed and centrifuged at 2,000g for 20 min. The absorbance of the upper, organic layer was measured at 535 and 520nm with a diode array spectrophotometer (Hewlett Packard, 8452A), and compared with freshly prepared malondialdehyde tetraethylacetal standards. MDA values were expressed as pmoles per mg protein. Protein was measured by the method of Bradford[17].

(6) Measurement of ROS scavenging activity

① Superoxide scavenging activity

Dihydroethidium (DHE) enters the cell and can be oxidized by ROS including superoxide and/or hydroxyl radical to yield fluorescent ethidium(Eth). Eth binds to DNA(Eth-DNA), further amplifying its fluorescence. Thus, increases in DHE oxidation to Eth-DNA(i.e., increases in Eth-DNA fluorescence) are suggestive of superoxide generation[18]. To determine if BGU has the superoxide scavenging activity, cells were incubated with 2uM DHE in the presence or absence of 0.05% BGU and measured changes in chemiluminescence at excitation 475nm and emission 610nm with using a chemiluminescence analyzer (Biolumet LB 9505, Berthold, Germany).

② H_2O_2 scavenging activity

Measurement using fluorescence dye: The ability of BGU to scavenge H_2O_2 was estimated by measuring the effect of BGU on the intracellular generation of H_2O_2 induced by antimycin A, an inhibitor of mitochondrial electron transport, in cells. Alterations in the intracellular generation of H_2O_2 were measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA)[19]. The nonfluorescent ester penetrates into the cells and is hydrolyzed to DCFH by the cellular esterases. The probe (DCFH) is rapidly oxidized to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF) in the presence of cellular peroxidase and ROS such as hydrogen peroxide or fatty acid peroxides. Cells were cultured in 35-mm tissue culture petri dishes. The culture medium was removed, and the cells were collected from flasks using trypsin-EDTA solution. The cells were washed twice with DMEM/F12, and were suspended in glucose-free HBSS for fluorescence analysis. The reaction was carried out in a fluorescent cuvette. The cells were preincubated for 10 min at 37℃ in fluorescent cuvette containing 3ml of glucose-free HBSS with 20uM DCFH-DA (from a stock solution of 20mM DCFH-DA in ethanol) in the presence or absence of 0.005% BGU. After the preincubation, the cells were treated with antimycin A and incubated up to 60 min during which the fluorescent intensity was monitored on a spectrofluorometer (SPEX1681, SPEX Co., USA) with excitation wave length of 485nm and emission wave length of 530nm. The net increase in DCF fluorescence (arbitrary units) was calculated by taking the difference between the values before and after addition of antimycin A.

Measurement using chemiluminescence: H_2O_2 concentration was measured by changes in chemiluminescence according to the methods of Mueller. The assay is based on the oxidation of luminol by sodium hypochlorite (NaOCl). Luminol is oxidized by NaOCl to diazaquinone in a two-electron oxidation, which is further specifically converted by H_2O_2 to an excited aminophthalate via an α-hydroxy-hydroperoxide. The short luminescence signal of this reaction has a maximum wavelength at 431nm; it is linearly dependent on H_2O_2 down to the 10^{-9}M range. A 100ul of 50uM NaOCl was added to tube containing

100ul of 50uM luminol and various concentrations of H_2O_2 in the presence or absence of various concentrations of BGU. The addition of NaOCl caused a rapid increase in chemiluminescence, and changes in chemiluminescence were measured for 60 sec.

2) *in vivo* studies

(1) Induction of mepirizole-induced ulcers

New Zealand white male rabbits weighing 2-3kg were used. Animals were fasted for 24 hr prior to experiments but had free access to drinking water. Stomach and duodenal ulcers were induced by oral administration of mepirizole at a dose of 200mg/kg body weight. Mepirizole was suspended in 1ml of 0.5% carboxymethylcellulose solution. In other experiments, animals were pretreated with BGU(0.1%/kg, orally) and catalase(800Units/kg, i.v.) 2 hr before administration of mepirizole. Control groups received an equivalent volume of the vehicle alone.

(2) Assessment of ulcers

After sacrificing the animals, a segment of the stomach and duodenum was removed and opened with a longitudinal incision. The ulcer index was determined by calculating the area (square millimeters) of the ulcerative and erosive lesions measured with a dissecting microscope at a magnification of x10.

(3) Measurement of lipid peroxidation

The stomach and duodenal mucosa were scraped off using two glass slides. Lipid peroxidation in the mucosal tissues was measured as described above according to the method of Uchiyama and Mihara[16].

(4) Morphological analysis

Duodenal tissue was removed 24 hr after administration of mepirizole, fixed in formaldehyde and embedded in paraffin. Thin sections were processed and stained with hematoxylin and eosin.

(5) Chemicals

[^{3}H]methylthymidine were purchased from Amersham International (Amersham, UK). Hydrogen peroxide(H_2O_2), deferoxamine, mepirizole, luminol, and catalase were purchased from Sigma Chemical(St. Louis, MO, USA). Dihydroethidium and 2',7'-dichlorofluorescin diacetate were purchased from Molecular Probe (USA).

N,N'-diphenyl-p-phenylenediamine (DPPD) was obtained from Aldrich Chemical (Milwaukee WI, USA). All other chemicals were of the highest commercial grade available.

(6) Statistical analysis

The data are expressed as mean $\pm$SE and the difference between two groups was evaluated using Student's *t*-test. A probability level of 0.05 was used to establish significance.

Results

1) *in vitro* studies

(1) Time course of cell death in Caco-2 cells subjected to H_2O_2

In order to determine the time course of H_2O_2-induced cell injury, Caco-2 cells were exposed to 0.5mM H_2O_2, and cell viability was determined at various time points (0-180 min). The significant loss of cell viability determined by trypan blue exclusion was present 30 min after exposure of cells to H_2O_2, with irreversible cell injury increasing up to 180 min (Fig. 1). However, cell death was significantly prevented by addition of 0.1% BGU.

When cells were exposed to various concentrations (0.05-0.5 mM) of H_2O_2 for 120 min, cell death was increased in a dose-dependent manner. The significant

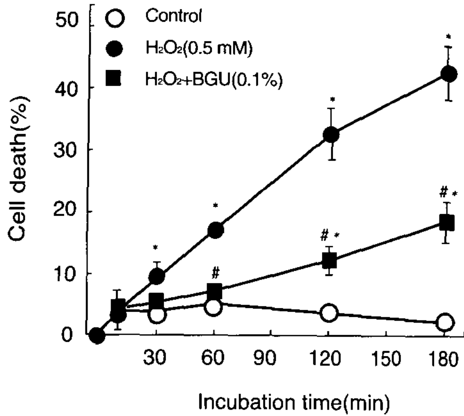

Fig. 1. Time course of H_2O_2 effect on cell death in Caco-2 cells. Cells were incubated for various times in medium containing 0.5mM H_2O_2 in the presence or absence of 0.1% *Gamibaegi-eum* (BGU). Cell death was measured by a trypan blue exclusion assay. Data are mean±SE of four experiments. *$p<0.05$ compared with control, #$p<0.05$ compared with H_2O_2 alone.

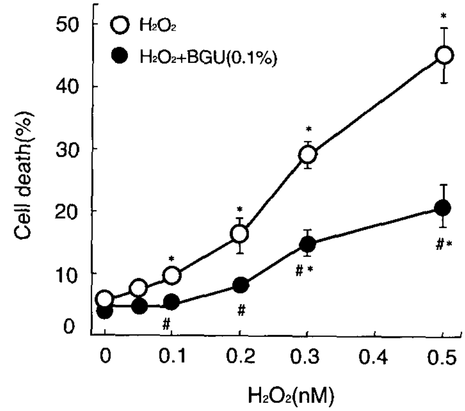

Fig. 2. Dose-dependency of H_2O_2 effect on cell death in Caco-2 cells. Cells were incubated for 120 min in medium containing various concentrations of H_2O_2 in the presence or absence of 0.1% Gamibaegi-eum(BGU). Cell death was measured by a trypan blue exclusion assay. Data are mean±SE of four experiments. *$p<0.05$ compared with the absence of H_2O_2, #$p<0.05$ compared with H_2O_2 alone.

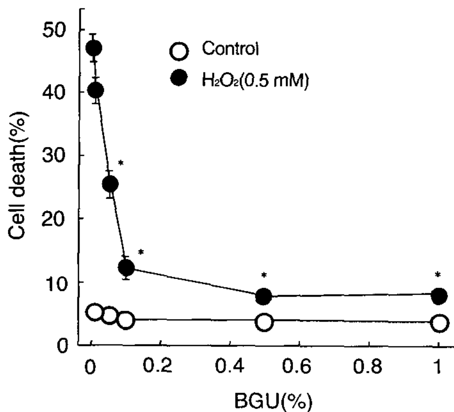

Fig. 3. Dose-dependency of *Gamibaegi-eum* (BGU) effect on H_2O_2-induced cell death in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5mM H_2O_2 in the presence or absence of various concentrations of BGU. Cell death was measured by a trypan blue exclusion assay. Data are mean ± SE of five experiments. *$p<0.05$ compared with H_2O_2 alone.

loss of cell viability was observed at 0.1mM H_2O_2 (9.38 ±1.03 vs. 5.37±0.66% in control). Such changes were significantly prevented by addition of 0.1% BGU (Fig. 2).

(2) Dose-dependency of protective effect of BGU against H_2O_2-induced cell death in Caco-2 cells

In order to determine dose-dependency of the protective effect of BGU, cells were treated with 0.5mM H_2O_2 in the presence of various concentrations of BGU. As shown in Fig. 3, BGU prevented H_2O_2-induced cell death in dose-dependent fashion and a significant protection was present at 0.05% (25.38± 2.06 vs. 46.94±2.08% in H_2O_2 alone). When BGU concentrations were increased up to 0.5 and 1%, cell death was decreased 7.98±1.38 and 8.46±1.4%, respectively, which were not different from the control.

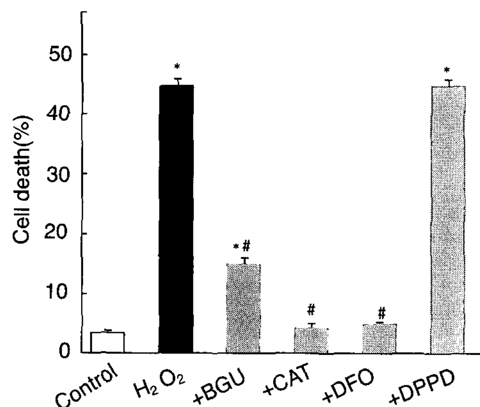

Fig. 4. Effects of Gamibaegi-eum (BGU), hydrogen peroxide scavenger, and antioxidants on H_2O_2-induced cell death in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5mM H_2O_2 in the presence or absence of 0.1% BGU, 500units/ml catalase (CAT), 5mM deferoxamine (DFO), and 0.01mM N,N' -diphenylphenylene diamine (DPPD). Cell death was measured by a trypan blue exclusion assay. Data are mean±SE of five experiments. $*p<0.05$ compared with the control; $\#p<0.05$ compared with H_2O_2 alone.

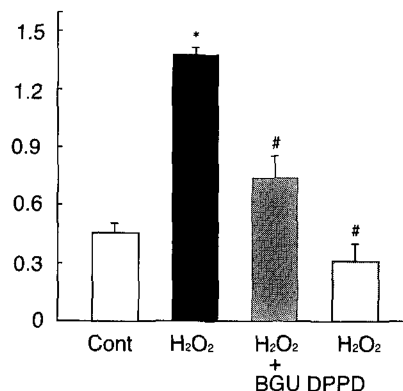

Fig. 5. Effect of *Gamibaegi-eum* (BGU) and antioxidant on H_2O_2-induced lipid peroxidation in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5mM H_2O_2 in the presence or absence of 0.1% BGU and 0.01mM N,N' -diphenylphenylene diamine (DPPD), and then lipid peroxidation was measured. Data are mean±SE of five experiments. $*p<0.05$ compared with the control(cont); $\#p<0.05$ compared with H_2O_2 alone.

However, BGU did not exert any effect in normal cells untreated with H_2O_2.

Effect of other well-known antioxidants on H_2O_2-induced cell death were examined to compare with that of BGU. The results are summarized in Fig. 4. Catalase (200units/ml), the hydrogen peroxide scavenger enzyme, prevented completely cell death induced by 0.5mM H_2O_2. Similar results were observed with deferoxamine (5mM), the iron chelator, suggesting involvement of an iron-dependent mechanism in H_2O_2-induced cell death. By contrast, a potent antioxidant DPPD at 20uM did not affect the loss of cell viability induced by H_2O_2, indicating that H_2O_2-induced cell death is not associated with lipid peroxidation.

(3) Effects of BGU and antioxidant on H_2O_2-induced lipid peroxidation in Caco-2 cells

The failure of the DPPD effect on H_2O_2-induced cell death may be due to that it does not block H_2O_2-induced lipid peroxidation in Caco-2 cells. To test this possibility, we examined if H_2O_2 induces lipid peroxidation and the effect was altered by BGU and DPPD. Exposure of cells to 0.5mM H_2O_2 caused an increase in lipid peroxidation, which could be prevented by 0.1% BGU and 20uM DPPD (Fig. 5).

(4) Effect of BGU on H_2O_2-induced DNA damage in Caco-2 cells

In order to examine if BGU exerts the beneficial effect against DNA damage induced by oxidants, DNA

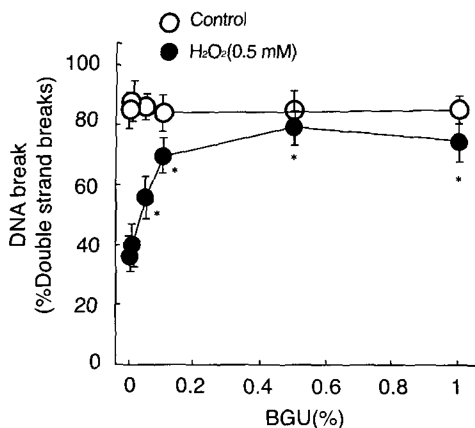

Fig. 6. Dose-dependency of Gamibaegi-eum (BGU) effect on H_2O_2-induced DNA damage in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5mM H_2O_2 in the presence or absence of various concentrations of BGU, and then DNA damage was measured. Data are mean ± SE of four experiments. *$p<0.05$ compared with H_2O_2 alone.

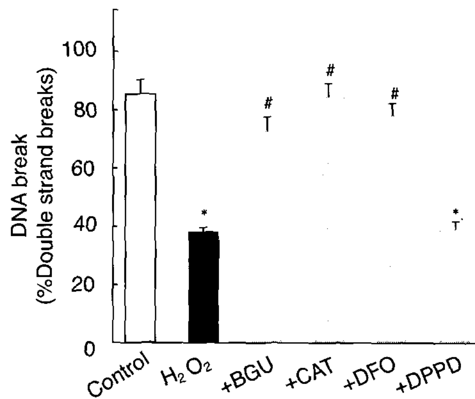

Fig. 7. Effects of Gamibaegi-eum (BGU), hydrogen peroxide scavenger, and antioxidants on H_2O_2-induced DNA damage in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5mM H_2O_2 in the presence or absence of 0.1% BGU, 500units/ml catalase (CAT), 5mM deferoxamine (DFO), and 0.01mM N,N'-diphenylphenylene diamine (DPPD). Data are mean ± SE of five experiments. *$p<0.05$ compared with the control; #$p<0.05$ compared with H_2O_2alone.

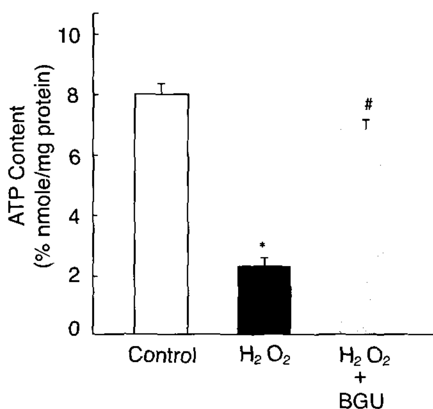

Fig. 8. Effects of Gamibaegi-eum (BGU), hydrogen peroxide scavenger, and antioxidants on H_2O_2-induced ATP depletion in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5mM H_2O_2 in the presence or absence of 0.1% BGU. Data are mean ± SE of four experiments. *$p<0.05$ compared with the control; #$p<0.05$ compared with H_2O_2 alone.

breaks were measured in cells treated with H_2O_2 in the presence of various concentrations of BGU. As shown in Fig. 6, exposure of cells to 0.5mM H_2O_2 resulted in a significant increase in DNA damage as evidenced by a decrease in double stranded DNA (36.93±5.96 vs. 85.07±6.03% in the control). These changes were prevented by BGU and its effect was dose-dependent. Double stranded DNA was increased from 36.93±5.96% to 55.9±7.02, 69.94±5.88, 79.98±5.93, and 75.39±6.34% by addition of 0.05, 0.1, 0.5, and 1% BGU, respectively. At concentrations higher than 0.1%, thus, BGU prevented completely DNA damage induced by 0.5mM H_2O_2.

As expected, H_2O_2-induced DNA damage was prevented by catalase. Similar results were observed with deferoxamine. However, DPPD did not affect the DNA damage (Fig. 7). Such results are consistent with

those in cell death.

(5) Effect of BGU on H_2O_2-induced ATP deletion in Caco-2 cells

It has been known that oxidants result in decreases in cell ATP content which may lead to cell death[20]. Thus, BGU exerts the protective effect against H_2O_2-induced cell death by preventing ATP depletion. To test the possibility, cellular ATP content was measured in Caco-2 cells exposed to 0.5mM H_2O_2 in the presence or absence of 0.1% BGU. As shown in Fig. 8, H_2O_2 decreased ATP content, which was restored by BGU and was not significantly different from the control.

(6) BGU radical scavenging activity

The ability of BGU to scavenge ROS was assessed using *in vitro* systems to generate specific ROS in the fluorescence probes. Cells were treated with xanthine oxidase (0.02U/ml)/xanthine(0.4mM) to generate superoxide anion in the presence or absence of DHE. Addition of xanthine oxidase/xanthine resulted in an increase in Eth fluorecence progressively over 15 min, suggesting generation of superoxide anion. However, addition of 0.05% BGU inhibited a time-dependent increase in Eth fluorescence(Fig. 9). These findings suggest that BGU scavenges directly superoxide radicals.

In order to determine the ability of BGU to inhibit generation of H_2O_2, caco-2 cells were treated with antimycin A, a site III inhibitor, in the presence or absence of BGU. Antimycin A produced an increase in DCF fluorescence, which was known to be dependent on generation of H_2O_2[19,21]. Antimycin A-dependent DCF fluorescence was decreased by addition of 0.005% BGU(Fig. 10), suggesting that BGU inhibits H_2O_2 generation.

The direct effect of BGU to scavenge H_2O_2 was

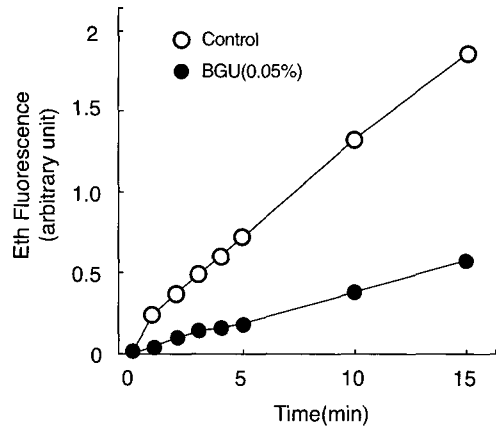

Fig. 9. Effect of Gamibaegi-eum (BGU) on Eth-fluorescence produced by xanthine oxidase /xanthine in Caco-2 cells. Cells were exposed to xanthine oxidase (0.02 U/ml)/xanthine (0.4mM) in the presence or absence of 0.05% BGU and measured changes in fluorescence.

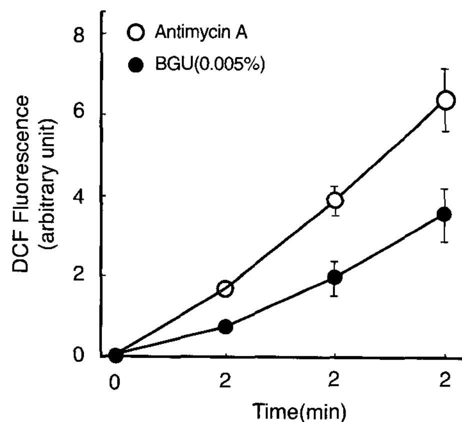

Fig. 10. Time course of the generation of reactive oxygen species in Caco-2 cells during exposure to antimycin A(AA). Cells were treated with 20uM AA in the presence or absence of 0.005% Gamibaegi-eum (BGU) and changes in DCF fluorescence were measured. Data are mean ± SE of three experiments.

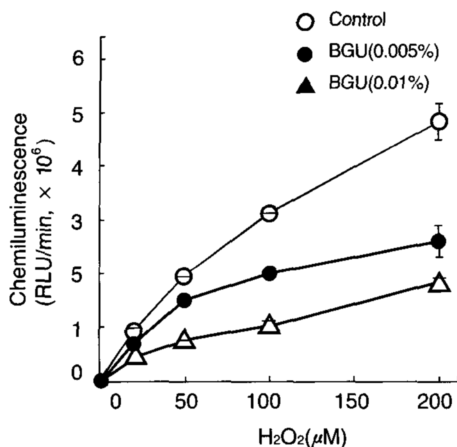

Fig. 11. H_2O_2 scavenging effect of Gamibaegi-eum (BGU). A 100ul of 50uM NaOCl was added to tube containing 100ul of 50uM luminol and various concentrations of H_2O_2 in the presence or absence of BGU. Chemiluminescence was measured at 431nm for 60 sec. Data are mean ±SE of four experiments.

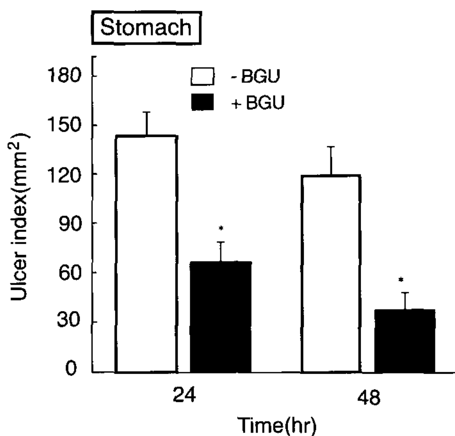

Fig. 12. Effect of Gamibaegi-eum (BGU) on ulcer index in the stomach after the oral administration of mepirizole at a dose of 200mg/kg body weight. Mepirizole was suspended in 1ml of 0.5% carboxymethylcellulose solution. In BGU pretreatment groups, animals received the oral administration of BGU at a dose of 0.1%/kg body weight 2 hr before administration of mepirizole. Data are mean ± SE of five animals in each group. *$p<0.05$ compared with the absence of BGU pretreatment (-BGU).

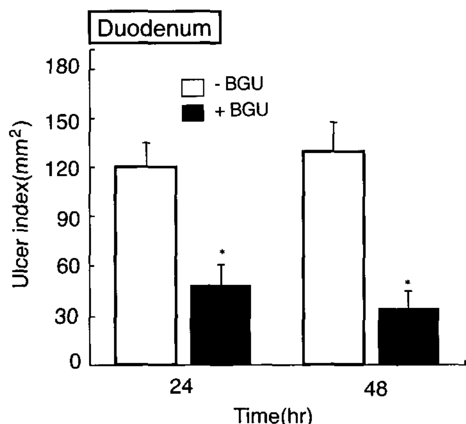

Fig. 13. Effect of *Gamibaegi-eum* (BGU) on ulcer index in the duodenum after the oral administration of mepirizole at a dose of 200mg/kg body weight. Mepirizole was suspended in 1ml of 0.5% carboxymethylcellulose solution. In BGU pretreatment groups, animals received the oral administration of BGU at a dose of 0.1%/kg body weight 2 hr before administration of mepirizole. Data are mean ±SE of five animals in each group. *$p<0.05$ compared with the absence of BGU pretreatment (-BGU).

examined using H_2O_2-induced changes in chemiluminescence. The addition of NaOCl in a medium containing luminol and various concentrations of H_2O_2 caused a dose-dependent increase in chemiluminescence, and this increase was inhibited by addition of BGU (Fig. 11). BGU inhibited the intensity of chemiluminescence in a dose-dependent fashion. These data provide an evidence supporting a direct H_2O_2 scavenging effect of BGU.

2) *in vivo* studies

(1) Effects of BGU and catalase on mepirizole-induced ulcers

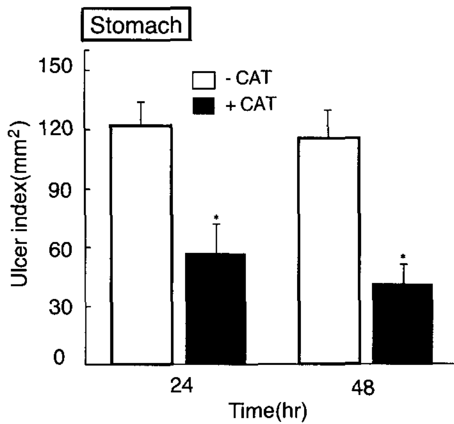

Fig. 14. Effect of catalase (CAT) on ulcer index in the stomach after the oral administration of mepirizole at a dose of 200mg/kg body weight. Mepirizole was suspended in 1ml of 0.5% carboxymethylcellulose solution. In catalase pretreatment groups, animals received the intravenous administration of CAT at a dose of 800Units/kg body weight 2 hr before administration of mepirizole. Data are mean $\pm$ SE of five animals in each group. $^*p<0.05$ compared with the absence of CAT pretreatment (-CAT).

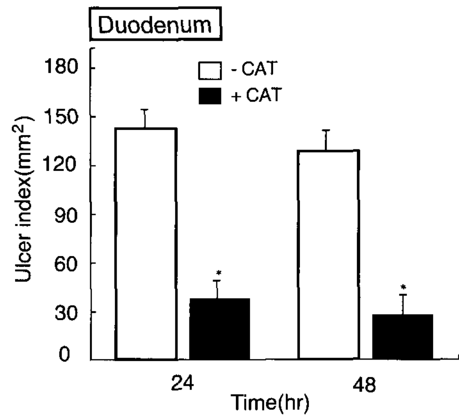

Fig. 15. Effect of catalase (CAT) on ulcer index in the duodenum after the oral administration of mepirizole at a dose of 200mg/kg body weight. Mepirizole was suspended in 1 ml of 0.5% carboxymethylcellulose solution. In CAT pretreatment groups, animals received the intravenous administration of CAT at a dose of 800Units/kg body weight 2 hr before administration of mepirizole. Data are mean $\pm$ SE of five animals in each group. $^*p<0.05$ compared with the absence of CAT pretreatment (-CAT).

Animals were sacrificed 24 and 48 hr after administration of 200mg/kg mepirizole. The ulcer index was determined in stomach and duodenum. Simple or multiple ulcer lesions were developed 24 hr after oral administration of mepirizole and remained unchanged even after 48 hr. The ulcer index in the stomach was 143 ± 15.2 and $120\pm18.3mm^2$ 24 and 48 hr after administration of mepirizole alone, respectively. However, ptretreatment with BGU significantly prevented the formation of ulcers (Fig. 12). Similar ulceration in the duodenum was present, showing ulcer index of 120 ± 15.0 and $130\pm19.1mm^2$, 24 and 48 hr after administration of mepirizole alone, respectively. Pretreatment of BGU also exerted a significant protective effect against the duodenal ulcers induced by mepirizole (Fig. 13).

In order to determine whether mepirizole induces ulcers via generation of ROS, animals were pretreated with catalase, a hydrogen peroxide scavenger. Similarly to BGU, catalase also significantly inhibited formation of ulcers induced by mepirizole in stomach and duodenum. The ulcer index in the stomach was $122\pm$ 12.0 and $116\pm14.3mm^2$ 24 and 48 hr after administra - tion of mepirizole alone, respectively, which was signi ficantly prevented by pretreatment of catalase (57 ± 15.1 and $42\pm9.7mm^2$) (Fig. 14). In duodenum, the ulcer index was 143 ± 12.9 and $129\pm14.2mm^2$ 24 and 48 hr after administration of mepirizole alone, respectively, and the value in animals pretreated with catalase was 38 ± 10.7 and $29\pm11.0mm^2$(Fig. 15).

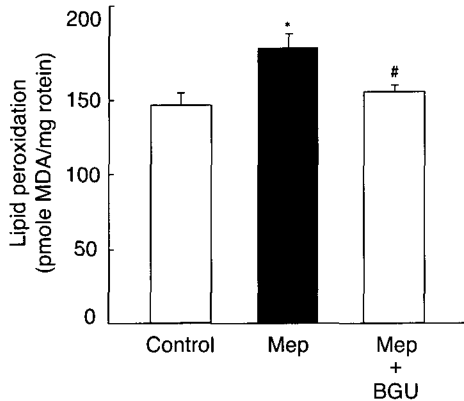

Fig. 16. Effect of Gamibaegi-eum (BGU) on lipid peroxidation in the mucosa of duodenum after the oral administration of mepirizole at a dose of 200mg/kg body weight. Mepirizole was suspended in 1ml of 0.5% carboxymethylcellulose solution. In BGU pretreatment groups, animals received the oral administration of BGU at a dose of 0.1%/kg body weight 2 hr before administration of mepirizole. The duodenal tissues were obtained 24 hr after administration of mepirizole. Data are mean ±SE of five animals in each group. *p<0.05 compared with control; #p<0.05 compared with mepirizole (Mep) alone.

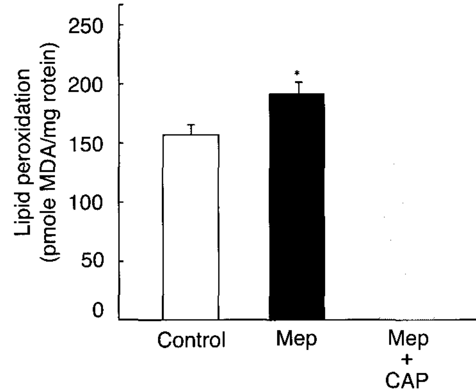

Fig. 17. Effect of catalase (CAT) on lipid peroxidation in the mucosa of duodenum after the oral administration of mepirizole at a dose of 200mg/kg body weight. Mepirizole was suspended in 1ml of 0.5% carboxymethylcellulose solution. In CAT pretreatment groups, animals received the intravenous administration of CAT at a dose of 800Units/kg body weight 2 hr before administration of mepirizole. The duodenal tissues were obtained 24 hr after administration of mepirizole. Data are mean ±SE of five animals in each group. *p<0.05 compared with control; #p<0.05 compared with mepirizole (Mep) alone.

To further confirm the role of ROS in formation of ulcers induced by mepirizole, lipid peroxidation was determined in animals treated with mepirizole for 24 hr with or without pretreatment of BGU. When animals were treated with mepirizole alone, lipid peroxidation in duodenal mucosa increased from 145.37±8.93pmole MDA/mg protein to 185.28±9.04pmole MDA/mg protein. Such an increase in lipid peroxidation was prevented by BGU (155.92±5.67pmole MDA/mg protein) (Fig. 16). Similar protective effects were obtained with pretreatment of catalase (Fig. 17).

The formation of duodenal ulcers induced by mepirizole was evaluated by histological findings. As shown in Fig. (18B), in the duodenum necrosis and detachment of villi and necrosis of goblet cells and Brunner' s glands in mucosa and submucosa were apparent as compared with the normal control animals (18A). But when animals were treated with mepirizole after pretreatment of BGU, detachment of villi was ameliorated (18C).

Discussion

A growing body of evidence suggests that ROS should be implicated in the pathogenesis of stress- and chemically-induced gastrointestinal injury[22]. A potent antioxidant may serve as a possible preventive intervention for gastrointestinal injury. In recent times therefore, the search for natural antioxidants and other preparations of plant origin to achieve this objective has been intensified.

Medical herbs continue to play an important therapeutic role in the treatment of human ailments. In fact, plant-derived drugs exert the intensive influence on the practice of Western medicine. Approximately 120 drugs are obtained from plants, a large number of therapeutic activities are mediated by these drugs, and a host of the drugs currently in use are still obtained from plants in which they are synthesized. Examples include steroids, cardiotonic glycosides, anticholinergics, analgesics, antimalarials and anticancer agents[23].

In terms of oriental medicine, gastrointestinal diseases generally results from the reversed flow and stagnation of qi, retention of food, and hyperactive liver-qi attacking the stomach. In order to cure this diseases, a medical treatment should have the effects such as invigorating the spleen for eliminating dampness, strengthening the stomach to promote digestion, promoting circulation of qi to alleviate stagnation in the middle-jiao, and regulating the liver-qi.

As I mentioned before, *Gamibaegi-eum* (加味排氣飮)(BGU) has the effects which strengthen the function of the stomach, resolve phlegm, check upward adverse flow qi, air which leads to relieve such symptoms as nausea, vomiting, fullness and pain in epigastrium. BGU is generally used for the treatment of patients with anorexia, dyspepsia, diarrhea, abdominal pain due to hypo-function of the spleen and stomach with obstruction in the channels[11].

There are several components in BGU, which enhance its effectiveness for gastrointestinal disease. Each component contributes to the optimized effect of BGU as following. *Aurantii Nobilis Pericarpium*(陳皮) and *Alismatis Rhizoma*(澤瀉) have been used to promote circulation of qi and digestion, to remove dampness as well as reduce phlegm. *Helenii Radix*(木香) and *Ponciri Fructus*(枳殼) have been used to promote flow of qi, to relieve pain, to warm the middle-jiao as well as to restore normal function of the stomach. Menthae Herba (薄荷) has been used to dispel wind, heat and eruption. *Crataegi Fructus* (山査) has been used to remove food stagnancy and blood stasis. *Cyperi Rhizoma* (香附子) has been used to smooth the liver, to regulate the circulation of qi, to normalize menstruation and to relieve pain. *Agastachis Herba* (藿香), an aromatic material, has been used to disperse dampness as a stomachic, antiemetic, and diaphoretic. *Linderae Radix* (烏藥) has been used to promote circulation of qi, to decrease pain, to dispel cold and to warm kidney. *Magnoliae Cortex* (厚朴) has been used as an agent to promote circulation of qi and remove dampness, and to relieve asthma. *Cassiae Semen* (決明子) has been used to remove heat from the liver and to improve acuity of vision, and to relieve constipation as laxative[24].

When I performed the medical experiment, I have added three components: *Crataegi Fructus* (山査), *Menthae Herba* (薄荷) and *Cassiae Semen* (決明子). Because these components resolve the hyperactive liver-qi attacking the stomach, which cause dizziness, chest discomfort, irritability, epigastric distention and pain, anorexia, nausea, vomiting, acid regurgitation.

The present study was undertaken to determine whether BGU exerts protective effect against oxidant-induced cell death in human intestinal cells using Caco-

2 cells. In this study, H_2O_2 increased cell death in a time- and dose-dependent manner in Caco-2 cells as measured by a trypan blue exclusion assay (Figs. 1 and 2). BGU prevented H_2O_2-cell death in a dose-dependent manner (Fig. 3). Cell death induced by 0.5mM H_2O_2 was completely prevented by 500units/ml catalase. This effect was expected because catalase is a scavenger enzyme of hydrogen peroxide (Fig. 4).

Iron appears to be the critical in the cytotoxic effect of H_2O_2 in Caco-2 cells as the iron chelator deferoxamine was markedly protective (Fig. 4), indicating that H_2O_2-induced cell killing is resulted from an iron-dependent mechanism. The source of iron, how it becomes biologically available, and the mechanism of action of deferoxamine on this system remain unknown. Gannon et al[25] have presented evidence that, in oxygen radical mediated cell death, the source of iron is the target cell itself. They demonstrated that stimulated neutrophils were cytotoxic for endothelial cells in an iron dependent manner. Pretreatment of the neutrophils with deferoxamine did not protect against the cytotoxicity. However, pretreatment of the endothelial cells with deferoxamine was significantly protective in a time and dose dependent manner. Whether BGU exerts the protective effect against H_2O_2-induced cell death by an action mechanism similar to deferoxamine remains to be defined.

Iron chelators have been shown to be protective in several *in vivo* models of tissue injury[26]. Although the role of iron is not completely understood[27], the protective effect of iron chelators has been generally taken as evidence for the participation of hydroxyl radical in cell injury, because a trace metal such as iron appears to be critical for the generation of hydroxyl radical from H_2O_2 via metal-catalyzed Haber-Weiss reaction[28]. Thus, if BGU scavenges hydroxyl radicals, it could prevent H_2O_2-induced cell killing. Interestingly, a potent antioxidant DPPD did not affect H_2O_2-induced cell death (Fig. 4).

Although lipid peroxidation of cell membrane has been considered to be an evidence for oxidant-induced cell injury[28], the role that lipid peroxidation plays as a critical event in the pathogenesis of oxidant-induced cell injury has not been clearly established[29,30]. Lipid peroxidation can be a result or an epiphenomenon of cell death rather than a cause of cell injury[28]. To determine whether H_2O_2 leads to cell death via a lipid peroxidation-dependent mechanism, Caco-2 cells were treated with H_2O_2 in the presence of antioxidants. DPPD have been reported to effectively prevent oxidant-induced cell injury in renal proximal tubular cells[31] and renal cortical slices[32]. If H_2O_2-induced cell injury was caused by lipid peroxidation, both the cell death and lipid peroxidation should be prevented by DPPD. In the present study, however, despite the fact that H_2O_2-induced lipid peroxidation was completely blocked by DPPD(Fig. 5), the cell death was not prevented (Fig. 4). These results suggest that H_2O_2-induced cell death is not mediated by lipid peroxidation in Caco-2 cells. H_2O_2-induced lipid peroxidation may be a result of the cell injury rather than a mechanism by which the cell death is induced[28]. The results of the present study showed that BGU inhibits H_2O_2-induced lipid peroxidation; therefore, its protective effect against H_2O_2-induced cell death may be attributed to a mechanism other rather than antioxidant action.

DNA is an important cellular and molecular target of oxidant stress. Oxidant stress results in DNA damage by induction of single-strand breaks, by base modification, or by the induction of apoptosis[28,33,34]. However, whether DNA damage leads to cell killing is controversial. various investigators reported that DNA damage plays a central role in cell death[28,33,35], whereas DNA damage is not the primary mediators of cell death following oxidative stress in renal epithelial cells[36] and

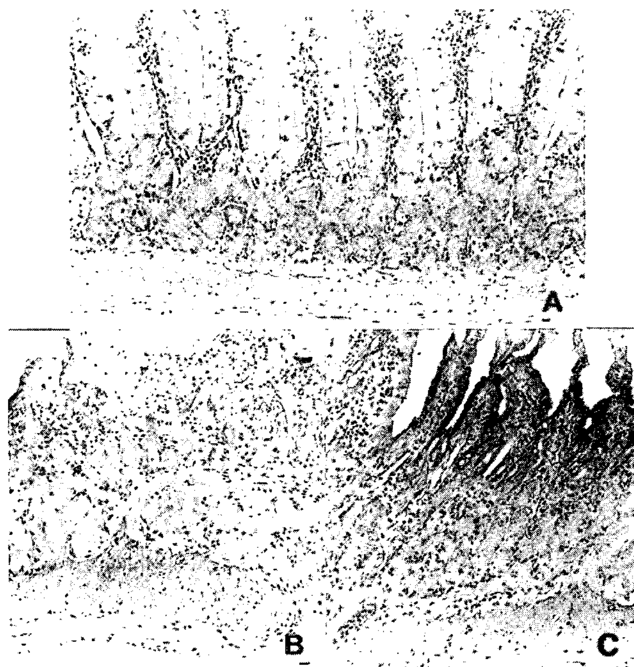

Fig. 18. Effect of Gamibaegi-eum (BGU) on morphological changes in the duodenum after the oral administration of mepirizole at a dose of 200mg/kg body weight. Mepirizole was suspended in 1ml of 0.5% carboxymethylcellulose solution. In BGU pretreatment groups, animals received the oral administration of BGU at a dose of 0.1%/kg body weight 2 hr before administration of mepirizole. The duodenal tissues were obtained 24 hr after administration of mepirizole.

A, control; B, mepirizole treatment; C, mepirizole treatment after BGU pretreatment. Hematoxylin-eosin staining. x66.

hepatocytes[37]. The present study demonstrated that BGU, catalase, and deferoxamine at concentrations that effectively decrease H_2O_2-induced cell death prevented H_2O_2-induced DNA damage (Figs. 6, 7). Similarly to cell death, however, DPPD did not prevent H_2O_2-induced DNA damage. This may suggest that DNA damage is linked to cell death in Caco-2 cells.

It has been demonstrated that ATP levels decline and ATP metabolites are lost from cells as an early response to oxidant injury[38]. Such changes may lead to cell death. In the present study, H_2O_2 decreased significantly ATP levels, which was prevented by addition of BGU (Fig. 8).

In order to determine whether BGU treatment *in vivo* exerts protective effect against the intestinal injury induced by ROS, we employed a nonsteroid antiinflammatory drug mepirizole. This drug has been known to induce duodenal ulcers through generation of ROS[14]. The present study showed in rabbits that the oral administration of mepirizole at a dose of 200 mg/kg produced deep perforated ulcers in the stomach and the proximal duodenum as evidenced by ulcer index(Figs. 12, 13) and morphological evaluation (Figs.18), similar to previous studies[13,14]. Such changes were significantly prevented by oral pretreatment of BGU at a dose of

0.1%/kg. The protective effect of BGU was supported by morphological studies.

Since mepirizole has been reported to induce duodenal ulcers via generation of ROS[14], the effect of catalase, a hydrogen peroxide scavenger, was determined. When inflammatory cells such as polymorphonuclear leukocytes and macrophages were activated, these cells release enzymatically synthesized superoxide and its dismutation product H_2O_2 into the surrounding medium. Thus, the hydrogen peroxide is considered as normal physiological products with widespread occurrence in both the interior and exterior milieu of the cell[28]. In the present study, mepirizole-induced ulcers were prevented by pretreatment of catalase (Figs. 14, 15). These results suggest that generation of hydrogen peroxide may play an important role in formation of ulcers induced by mepirizole, a result consistent with reports in rats by Iinuma et al[14]. They reported that catalase decreased to almost normal control levels the ulcer index in animals treated with mepirizole.

Mepirizole treatment *in vivo* induced lipid peroxidation, which was prevented by BGU (Fig. 16), similarly to catalase (Fig. 17). These findings suggest that ROS generation is involved in mepirizole-induced ulcers and the protective effect of BGU may be the result of its scavenging activity of H_2O_2. In fact, BGU inhibited generation of superoxide and H_2O_2 and also scavenged directly H_2O_2 in *in vitro* systems (Figs. 9-11).

The present study demonstrated that BGU exerts a beneficial effect against H_2O_2-induced cell injury in intestinal cells. The underlying mechanism of the BGU protective effect is not clear from the results of the present study. In the present study, the effect of BGU mimics that of catalase and deferoxamine rather than DPPD. Therefore, BGU may act as a H_2O_2 scavenger and/or as an iron chelator. Although the precise mechanism remains to be explored, the results of the present study provide extensive information on the underlying mechanism of ROS-induced cell death, and suggest that BGU may be useful in treatment and prevention of gastrointestinal injuries mediated by ROS.

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