

## The Protective Effect of the Soybean Polyphenol Genistein against Stress-Induced Gastric Mucosal Lesions in Rats, and Its Hormonal Mechanisms

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**Summary** The present study investigated the effect of the soybean polyphenol genistein on the stomach using a water immersion restraint (WIR) stress model. Male Wistar rats were administered 50 or 100 mg/kg/d of genistein for 2 wk or were not given any drug. Rats were subjected to WIR stress for 4 h. At the end of the WIR period, rats were sacrificed. Subsequently, rats underwent measurement of the ratio of the mucosal hemorrhagic erosion area to the whole stomach body area, myeloperoxidase (MPO) activity, superoxide dismutase (SOD) activity, thiobarbituric acid reactive substances (TBARS) level, and proinflammatory cytokines (tumor necrosis factor (TNF)- $\alpha$  and cytokine-induced neutrophil chemoattractant (CINC)-1) levels in the gastric tissue. Furthermore, an isolated rat stomach infusion model was employed for the endocrinological investigation of the effect of genistein. The extracted stomach canal and the vascular system, which comprised the experimental model, were subjected to perfusion. After 20 min of perfusion, the perfusate from the portal vein was collected, and the concentrations of histamine, gastrin, and somatostatin in the perfusate were measured. Experiments demonstrated that genistein administration resulted in significant suppression of WIR stress-induced gastric mucosal injury and MPO activity. Further, genistein significantly elevated SOD activity and significantly suppressed the TBARS level, production of TNF- $\alpha$  and CINC-1, and secretion of gastrin, histamine, and somatostatin. These data suggest that genistein protected against gastric mucosal injury, likely via its ability to inhibit oxidation, inflammation, and secretion of gastrin and histamine.

**Key Words** genistein, water-immersion restraint stress, isolated stomach infusion model, gastric mucosal lesions, gastrin

Isoflavone is a flavonoid component of soybean seeds with three or more phenol hydroxyl residues and is therefore called a soybean polyphenol. Isoflavones contains three aglycones: genistein, daidzein, and glycitein. In particular, genistein exerts various effects, including estrogen-like activity (1), anti-oxidant effects (2), and an anti-cancer effect (3). In the gastrointestinal field, genistein has been reported to inhibit activity of *Helicobacter pylori*, a bacterium that plays a pathophysiologic role in gastritis and gastric ulcer formation (4).

Stress-induced gastric mucosal injury is a common clinical entity that requires prompt treatment. Various types of stress decrease blood flow and increase the infiltration of neutrophils into the gastric mucosa, thereby inducing gastric mucosal injury (5–8). The water immersion restraint (WIR) stress model is widely used as an experimental model of stress-induced acute gastric mucosal injury because this model induces reproducible gastric mucosal lesions without resorting to employing surgical or anesthetic procedures. Ischemia-reperfusion (I/R) injury is one of the important mecha-

nisms for the gastric mucosal lesions induced by WIR (8). WIR stress induces microcirculatory disturbances via induction of reactive oxygen species (ROS) generation in the mucosal tissue or neutrophils in the context of the ischemia-reperfusion phenomenon (9, 10). ROS and lipid peroxidation play a critical role in the pathogenesis of acute gastric mucosal injury in humans and in experimental animal models (9–11).

Cellular defense against oxidative stress is provided by enzymatic (superoxide dismutase, glutathione peroxidase, etc.) and non-enzymatic (flavonoids, vitamin C, etc.) free-radical scavenging systems. Superoxide dismutase (SOD, a scavenger of superoxide) activity reflects the intracellular anti-oxidant defense system of various tissues, including the gastric mucosa, and SOD administration significantly suppresses formation of WIR stress-induced gastric lesions (9, 12). Flavonoids also have anti-oxidant/scavenging properties (13).

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a proinflammatory cytokine that mediates ischemia-reperfusion induced gastric mucosal injury (14) and that strongly stimulates neutrophil adherence by inducing the synthesis and expression of adhesion molecules on endothelial cells and neutrophils (15). Hamaguchi et al.

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showed that treatment with a neutralizing antibody against TNF- $\alpha$  inhibits formation of gastric lesions and neutrophil accumulation induced by WIR stress (16). Further, the cytokine-induced neutrophil chemoattractant-1 (CINC-1) has a potent neutrophil chemotactic activity in rats, similar to the effect of IL-8 in human neutrophils. Yagihashi et al. reported that pretreatment with anti-CINC-1 antibody significantly reduced tissue myeloperoxidase activity and injury after small intestinal I/R treatment in rats (17).

Many studies have described the protective effects of flavonoids on stress-induced gastric mucosal injury (18, 19). However, the effects of genistein on gastric mucosal injury have not been investigated. Therefore, the aim of the present study was to identify the role of genistein in WIR stress-induced gastric mucosal injury in rats. For this purpose, changes in tissue SOD activity and the thiobarbituric acid reactive substance (TBARS, an indicator of lipid peroxidation) level, which reflects the anti-oxidative properties, and proinflammatory cytokines (TNF- $\alpha$  and CINC-1) levels were determined. Further, the concentrations of representative gastrointestinal hormones, including serum gastrin, histamine, and somatostatin, were measured as markers of the risk of gastric ulceration in an isolated stomach infusion model.

## MATERIALS AND METHODS

**Chemicals.** Genistein was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Since genistein does not dissolve in water, a solution was prepared in 2% methylcellulose Na, sonicated for 10 min and mixed thoroughly. Genistein was administered at doses of 50 or 100 mg/kg/d.

**Animals and diets.** All experiments were performed in accordance with the "Guidelines for Animal Experimentation" approved by the Nihon University School of Medicine. Seven-week-old male Wistar rats (Charles River Japan Inc., Tokyo, Japan) were housed under a 12- and 12-h light/dark cycle at a temperature of 23°C and 50% humidity and were fed with commercially available chow (Oriental Yeast Co., Ltd., Tokyo, Japan). The normal and the control group ( $n=8$ ) received 2% methylcellulose Na alone, while the two experimental groups ( $n=8$ ) received 50 or 100 mg/kg/d of genistein (1 mL aliquots twice daily at 7:00 and 17:00), respectively, by direct stomach intubation (KN-348  $\phi$  1.2×L 80 mm, Natsume Ltd., Tokyo, Japan) for 2 wk. At the end of the treatment period, there was no significant difference in body weight when comparing the four groups. Rats were maintained in the fasting condition for 24 h before all experiments.

**Gastric mucosal injury and measurement of the Lesion Index.** The animals were randomly assigned to one of four groups: (1) the normal group (normal), which did not undergo WIR stress; (2) the control group (control), in which animals underwent WIR stress; (3) the genistein-treated (50 mg/kg/d) group (50 mg genistein), in which animals underwent WIR stress; and (4) the genistein-treated (100 mg/kg/d) group (100 mg

genistein), in which animals underwent WIR stress. The WIR stress-induced gastric mucosal injury model was prepared according to the method reported by Takagi and Okabe (20). Briefly, rats were immobilized in a restraint cage and were immersed in water up to the level of the xiphoid process in a temperature-controlled water tank (23°C) for 4 h. The rats were then immediately anesthetized with pentobarbital sodium, and the stomach was isolated by laparotomy. After dissection along the greater curvature, the extent and areas of the gastric mucosal lesions were assessed. The areas of hemorrhagic erosion in the isolated gastric body were measured after imaging with a digital camera (FINE-CAM, Kyocera Ltd., Tokyo, Japan). The ratio of areas of hemorrhagic erosion to gastric body mucosa was calculated with image analysis software, Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, USA) and was designated as the Lesion Index. From the same rats, gastric mucosal specimens were collected and homogenized for biochemical assays.

**MPO measurement.** The resected gastric mucosal specimens were homogenized in 25-fold volumes of 0.5% hexadecyltrimethylammonium bromide (HTAB) dissolved in 50 mM potassium phosphate buffer (pH 6.0) in a homogenizer. The homogenates were sonicated five times at 20 kHz for 30 s on ice, and freezing and thawing (at 37°C) were conducted sequentially and repeated three times. The homogenate was then centrifuged at 40,000× $g$  at 25°C for 15 min for extraction. Total protein in the supernatant was measured by the method of Lowry et al. (21). MPO activity was measured by the *o*-dianisidine method (22). MPO activity was measured spectrophotometrically: 0.1 mL of supernatant was combined with 2.9 mL of 50 mM potassium phosphate buffer (pH 6.0), containing 0.16 mg/mL *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The mixture was incubated for 15 min, and the optical density (OD) was then measured at a wavelength of 460 nm.

**Tissue homogenization for TBARS and SOD.** To the tissues (100 mg) were added a 10-fold volume of 1.15% KCl and they were homogenized with Polytron under N<sub>2</sub> stream at 4°C. The homogenates were sonicated two times at 20 kHz for 30 s on ice and then centrifuged at 1,700× $g$  for 15 min at 4°C. Total protein in the supernatant was measured by the method of Lowry et al. (21).

**Lipid peroxidation measurement.** The concentration of thiobarbituric acid-reactive substances (TBARS), an index of lipid peroxidation, was measured in the supernatant using the method of Ohkawa et al. (23). The level of TBARS in the mucosal homogenate was expressed as nmol of malondialdehyde per mg of tissue protein using 1,1,3,3-tetramethoxypropane as the standard.

**SOD measurement.** SOD activity was measured by the nitrite method as modified by Oyanagui (24). The supernatant was incubated with KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> (pH 8.0) containing hypoxanthine and hydroxylamine at pH 8.0, 37°C, for 10 min. Then, the reaction mixture

was added to the buffer containing xanthine oxidase and was incubated at pH 8.0 and 37°C for 30 min. Finally, color developer was added, and the mixture was incubated at room temperature for 30 min. OD was subsequently measured at a wavelength of 550 nm.

**Proinflammatory cytokines measurement.** The tissues (400 mg) were supplemented with 1 mL of saline solution, and homogenized four times each for 15 s. Then, 2 mL of saline solution was added to the homogenates and mixed well. The homogenates were centrifuged at  $12,500 \times g$  for 30 min at 4°C. Total protein in the supernatant was measured by the method of Lowry et al. (21). The concentration of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in the supernatant was determined using a rat TNF- $\alpha$  enzyme-linked immunosorbent assay (ELISA) kit (Bio Source International, Inc., CA, USA). The concentration of cytokine-induced neutrophil chemoattractant-1 (CINC-1) was determined using a rat GRO/CINC-1 ELISA kit (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan). These assays were performed according to the manufacturer's instructions.

**Preparation of the isolated stomach infusion model and measurement of gastrointestinal hormones.** The concentration of histamine, gastrin, and somatostatin in the perfusate was measured by radioimmunoassay (RIA) methods, using the isolated stomach infusion technique (18) (Fig. 1). The animals were randomly assigned to one of three groups: (1) the control group; (2) genistein-treated (50 mg/kg/d) group; or (3) the genistein-treated (100 mg/kg/d) group. Briefly, after rats were anesthetized with pentobarbital sodium, a portion of the gastroduodenal canal, together with vascular tissue, was extracted. The celiac artery and the portal vein were cannulated with fine polyethylene tubes (KN-392 outer diameter: 0.80 mm, Natsume Ltd.), while the other arteries and veins and pancreatic tissue were ligated and excised. Polyethylene tubes (KN-392 outer diameter: 1.90 mm, Natsume Ltd.) were also inserted into the cardiac region and the pyloric portion of the stomach, which were then ligated and fixed. The extracted stomach was bathed in Krebs Ringer-bicarbonate buffer (KRBB) solution with 4% dextran and

44 mM glucose and was aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The solution was adjusted to pH 7.4, and the temperature was maintained at 37°C.

To counteract the effects of gastric acid, the extracted stomach canal was perfused with NaOH (adjusted to pH 7.4 with 1% HEPES) at a rate of 2 mL/min. For perfusion of the vascular system, 4% dextran and 44 mM of glucose was added to the KRBB solution, and the solution was aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> to adjust the pH to 7.4. Next, the celiac artery was perfused with a solution containing 2 mg/mL of bovine serum albumin (Sigma Chemical Co., St. Louis, USA) at a rate of 2 mL/min. The temperature of both solutions was maintained at 37°C. After 20 min of perfusion, the perfusate draining from the portal vein was sampled every 2 min, fractionated, and immediately frozen at -70°C.

**Radioimmunoassay.** Gastrin was measured using a Gastrin RIA kit (Dinabott, Tokyo, Japan). Histamine was measured using a Histamine kit, EIKEN (Eiken Chemical, Tokyo, Japan). An RIA system for somatostatin was established using antiserum against somatostatin obtained from a rabbit immunized with synthetic somatostatin conjugated to bovine serum albumin. <sup>125</sup>I-Tyr<sup>1</sup>-somatostatin was purchased from NEN Life Science Products (Boston, MA, USA).

**Statistical analysis.** Results were expressed as means  $\pm$  SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test. Statistical significance was established at the  $p < 0.05$  level. All analyses were performed using the SPSS version 11.0 statistical software package (SPSS® Inc., USA).

## RESULTS

Genistein treatment significantly attenuated gastric mucosal injury (Fig. 2).

The gastric mucosal MPO activity and TBARS were significantly increased in the control group when compared with the normal group. The increase in gastric mucosal MPO activity and TBARS were significantly attenuated in the 50 mg and 100 mg genistein groups

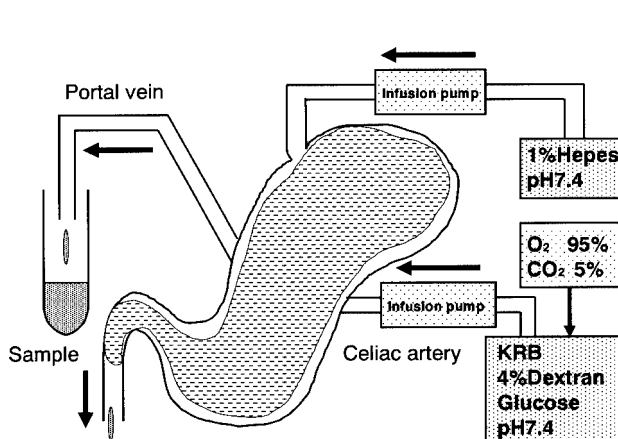


Fig. 1. Isolated rat stomach infusion model. KRBB, Krebs-Ringer-bicarbonate; HEPES, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid).

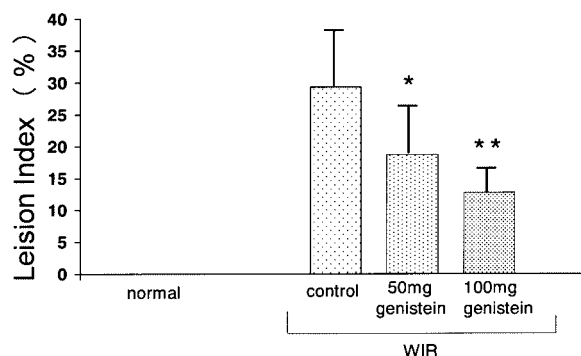


Fig. 2. Protective effects of genistein on gastric mucosal lesions induced by water immersion restraint stress. The Lesion Index was determined as the ratio of the area of mucosal hemorrhagic erosion to the stomach body area. The data are shown as mean  $\pm$  SD of 8 rats (\* $p = 0.018$ , \*\* $p = 0.001$  vs. control. ANOVA;  $p = 0.001$ ).

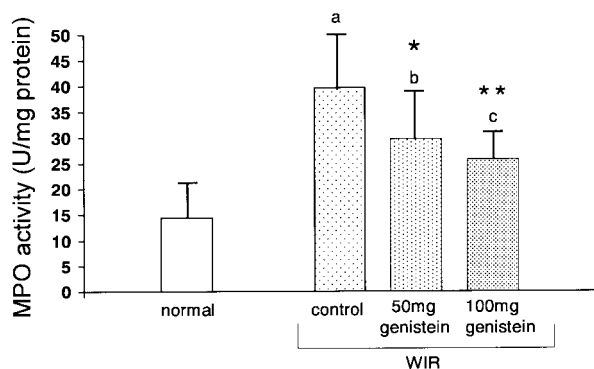


Fig. 3. Gastric mucosal MPO activity in normal rats without stress (normal), in control rats with WIR stress (control), in 50 mg/kg/d genistein-treated rats with WIR stress (50 mg genistein) and in 100 mg/kg/d genistein-treated rats with WIR stress (100 mg genistein). The data are shown as mean $\pm$ SD of 8 rats (<sup>a</sup> $p=0.000$ , <sup>b</sup> $p=0.015$ , <sup>c</sup> $p=0.003$  vs. normal. \* $p=0.02$ , \*\* $p=0.004$  vs. control. ANOVA;  $p=0.000$ ).

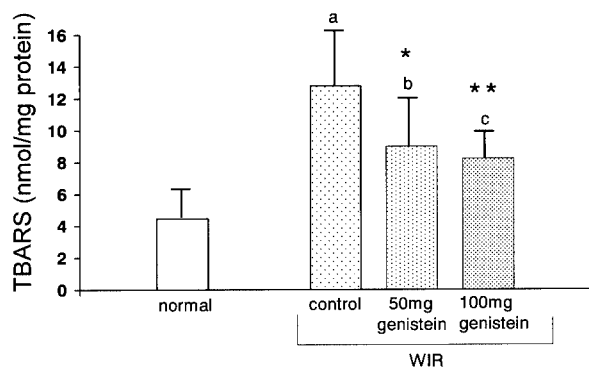


Fig. 4. Gastric mucosal TBARS level in normal rats without stress (normal), in control rats with WIR stress (control), in 50 mg/kg/d genistein-treated rats with WIR stress (50 mg genistein) and in 100 mg/kg/d genistein-treated rats with WIR stress (100 mg genistein). The data are shown as mean $\pm$ SD of 8 rats (<sup>a</sup> $p=0.000$ , <sup>b</sup> $p=0.002$ , <sup>c</sup> $p=0.001$  vs. normal. \* $p=0.002$ , \*\* $p=0.000$  vs. control. ANOVA;  $p=0.000$ ).

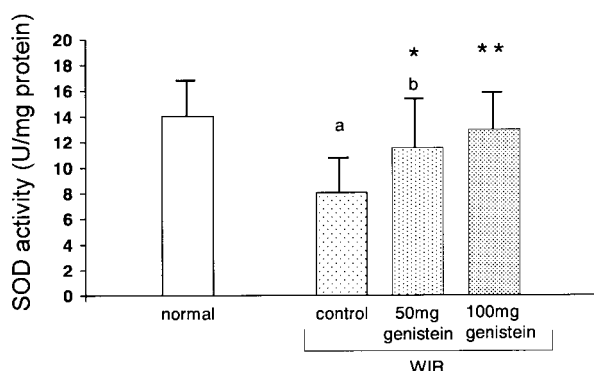


Fig. 5. Gastric mucosal SOD activity in normal rats without stress (normal), in control rats with WIR stress (control), in 50 mg/kg/d genistein-treated rats with WIR stress (50 mg genistein) and in 100 mg/kg/d genistein-treated rats with WIR stress (100 mg genistein). The data are shown as mean $\pm$ SD of 8 rats (<sup>a</sup> $p=0.000$ , <sup>b</sup> $p=0.004$  vs. normal. \* $p=0.045$ , \*\* $p=0.003$  vs. control. ANOVA;  $p=0.000$ ).

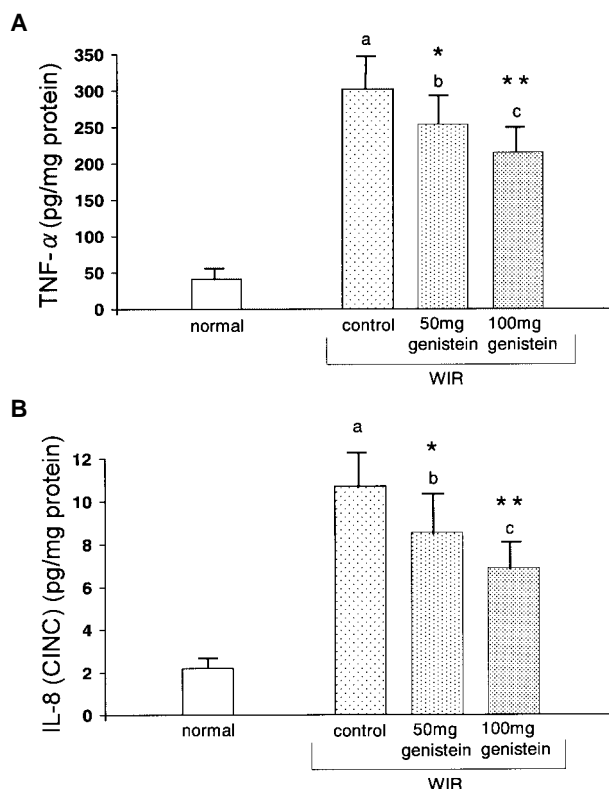


Fig. 6. Gastric mucosal TNF- $\alpha$  (A) and CINC-1 (B) concentrations in normal rats without stress (normal), in control rats with WIR stress (control), in 50 mg/kg/d genistein-treated rats with WIR stress (50 mg genistein) and in 100 mg/kg/d genistein-treated rats with WIR stress (100 mg genistein). The data are shown as mean $\pm$ SD of 8 rats ((A) <sup>a,b,c</sup> $p=0.000$  vs. normal. \* $p=0.007$ , \*\* $p=0.001$  vs. control. (B) <sup>a,b,c</sup> $p=0.000$  vs. normal. \* $p=0.015$ , \*\* $p=0.001$  vs. control. ANOVA; (A, B)  $p=0.000$ ).

when compared with the control group (Figs. 3 and 4). The gastric mucosal SOD activity was significantly attenuated in the control group when compared with the normal group. By contrast, the gastric mucosal SOD activity was significantly increased in the 50 mg and 100 mg genistein groups when compared with the control group (Fig. 5).

The gastric mucosal TNF- $\alpha$  and CINC-1 concentrations were significantly increased in the control group when compared with the normal group. The increase in gastric mucosal TNF- $\alpha$  and CINC-1 concentrations was significantly attenuated in the 50 mg and 100 mg genistein groups when compared with the control group (Fig. 6a and 6b).

Genistein treatment significantly inhibited gastrin and histamine secretion (Fig. 7a and 7b). Somatostatin secretion was significantly lower in the 100 mg genistein group than in the control group (Fig. 7c).

## DISCUSSION

The present study demonstrated that genistein administration significantly suppressed WIR stress-induced gastric mucosal injury. WIR stress-induced gastric mucosal injury may be mediated by a variety of

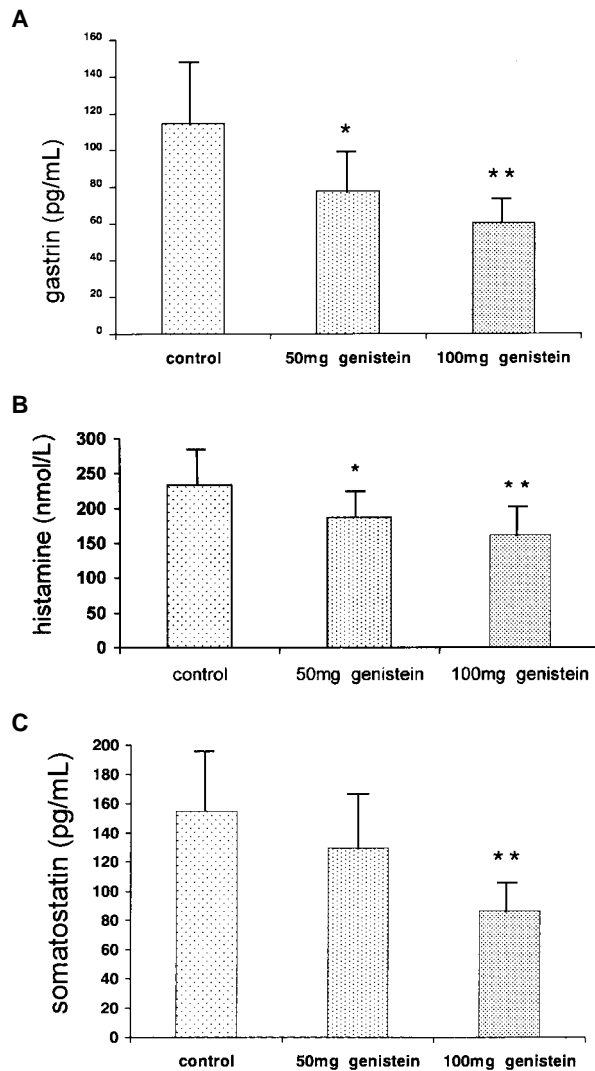


Fig. 7. Effects of genistein on secretion of gastrin (A), histamine (B), and somatostatin (C) in perfusate. The data are shown as mean  $\pm$  SD of 8 rats ((A) <sup>a</sup> $p=0.014$ , <sup>b</sup> $p=0.003$  vs. control. (B) <sup>a</sup> $p=0.029$ , <sup>b</sup> $p=0.002$  vs. control. (C) <sup>b</sup> $p=0.015$  vs. control. ANOVA; (A)  $p=0.000$ , (B)  $p=0.002$ , (C)  $p=0.033$ ).

mechanisms (6, 8–10, 14). Microcirculation disturbances may contribute to gastric mucosal injury because of the secondary production of ROS in the mucosal tissue or neutrophils. ROS, such as superoxide anion residual ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $HO^\bullet$ ), are cytotoxic agents that induce lipid peroxidation and other cellular oxidative stress by cross-linking proteins, lipids, and nucleic acids, subsequently leading to cellular dysfunction, damage, and eventually death (9–11). Meanwhile, the integrity of the gastric mucosa is dependent on a variety of factors, including mucus-alkaline secretion, and the activity of anti-oxidizing enzymes, such as SOD and glutathione peroxidase, that protect the stomach against exogenous and endogenous irritants (25, 26). SOD is one of the primary anti-oxidative enzymes in the gastric mucosa, and studies have demonstrated that SOD administration significantly suppressed WIR stress-induced gastric mucosal injury (9, 12). In the present study, the finding

that SOD activity was significantly decreased in the control group when compared with the normal group is probably the result of the inactivation by ROS produced by WIR.

The present study demonstrated that genistein treatment resulted in an increase in gastric mucosal SOD activity in the WIR stress model. Since it has been reported that antioxidant-treatment prevents the loss of SOD activity via scavenging of excess ROS (27), our data suggest that genistein may act as an anti-oxidant in gastric mucosa, thereby maintaining SOD activity in the present experimental conditions. Indeed, our results are consistent with those reported by Choi et al. that genistein increased the activity of SOD in a lipopolysaccharide-stimulated macrophage cell line (28). Moreover, the present study demonstrated that genistein treatment resulted in a decrease in gastric mucosal TBARS, which is considered a good indicator of lipid peroxidation, in the WIR stress model. Since genistein contains a phenol hydroxyl residue and has a potent radical scavenging effect (2, 28, 29), data from the present experiments indicate that genistein may act as an antioxidant in the gastric mucosa, thereby scavenging ROS in the gastric mucosa.

Xanthine oxidase (XO), which generates  $O_2$  and  $H_2O_2$ , promotes adherence of neutrophils to endothelial cells via a mechanism involving  $H_2O_2$  (30). Further, XO-activated neutrophils play critical roles in the development of the gastric mucosal injury induced by stress (7, 9, 16). Esplugues and Whittle reported that local intra-arterial infusion of XO in rats induces gastric mucosal injury and that this injury is inhibited by concurrent infusion of SOD (31). Furthermore, this is a significant finding, as neutrophils utilize MPO to infiltrate tissues and provoke local inflammation. The present study demonstrated that genistein suppressed gastric mucosal MPO activity in the WIR stress model, suggesting that genistein inhibits ROS generation and scavenges ROS in gastric mucosa and thereby inhibits neutrophil adherence in the gastric mucosa.

TNF- $\alpha$  is a major proinflammatory cytokine and plays an important role in the development of acute inflammation, including neutrophil infiltration of gastric mucosa. Furthermore, TNF- $\alpha$  stimulates transcription factors, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B), induces the synthesis of various inflammatory cytokines, including IL-1, -6, and -8, and promotes expression of IL-6 and -8 receptors. In fact, WIR-induced gastric inflammation is accompanied by an increase in the expression of several proinflammatory cytokines, including TNF- $\alpha$ , that promote permeability of blood vessels to neutrophils (14–16). In the present study, genistein administration resulted in decreased TNF- $\alpha$  and CINC-1 concentrations in the gastric mucosa of WIR-stressed rats. Previous reports have already demonstrated that genistein inhibits TNF- $\alpha$  production in monocytic macrophage cell lines in vitro (32) and in lipopolysaccharide-stimulated-monocytes and macrophages in vivo (33). Further, genistein inhibits tyrosine kinase, which participates in local inflamma-

tory reactions (34), and NF- $\kappa$ B activity (28, 35). These findings indicate that genistein likely inhibits neutrophil infiltration into the gastric mucosa by suppressing TNF- $\alpha$  production from monocytes and macrophages and by inhibiting CINC-1 production.

In addition, the present study investigated the direct influence of genistein on gastrointestinal hormones that are fundamentally associated with peptic ulcer formation. An isolated rat stomach infusion model was employed to evaluate the effects of genistein on the isolated stomach canal, thereby preserving intact interactions among G cells, D cells, enterochromaffin-like (ECL) cells, and parietal cells, without systemic-interference from the hormonal or nervous systems. In this study, genistein administration resulted in significant inhibition of the secretion of gastrin, histamine, and somatostatin. While changes in gastric acid secretion are a major influence in the hormonal dynamics in the stomach, it is difficult to measure gastric acid secretion using the stomach infusion model. However, flavonoids inhibit gastric acid secretion (36, 37), and our data indicate that genistein inhibits gastric acid secretion in the gastric mucosa. Indeed, previous studies used the isolated stomach infusion model to demonstrate that flavonoids, such as catechin and proanthocyanidin, inhibit histidine carboxylase (HDC) activity (18, 19, 38), resulting in decreased synthesis and release of histamine (18, 19), which is consistent with results from the present study. Based on these findings, we speculate that genistein may first inhibit gastrin secretion from G cells, leading to a decrease in HDC activity and a subsequent reduction in histamine synthesis in ECL cells. Genistein may also reduce somatostatin release by decreasing the amount of gastrin available to bind to receptors on D cells. Since the reported mechanisms of WIR stress-induced lesions include an increased capacity for gastric acid production (6), the present data suggest that the inhibitory effect of genistein on gastric mucosal lesions may be mediated by its inhibitory effect on acid secretion via G cells.

In conclusion, genistein treatment attenuated stress-induced gastric mucosal injury, likely via its abilities to inhibit oxidation, inflammation and acid secretion. However, the association between antioxidant properties and the antisecretory activity of genistein remains unclear. Further study of the mechanisms of action of genistein, including its effects on G cells, would be of benefit. This is the first report to demonstrate that genistein had protective effects against gastric mucosal injury in rats, and these data provide new insight into the effects of genistein on gastric mucosal integrity.

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