Gastroprotective Effects of Grape Seed Proanthocyanidin Extracts against Nonsteroid Anti-Inflammatory Drug-Induced Gastric Injury in Rats

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INTRODUCTION

Nonsteroid anti-inflammatory drugs (NSAIDs) have been frequently prescribed worldwide because of their powerful analgesic, anti-inflammatory, and antipyretic actions. If NSAIDs are persistently administered, however, edema of the gastric mucosa, erythema, erosion, submucosal hemorrhage, and ulceration occur at a frequency of 10% to 25%. In particular, in 0.5% to 1% of patients who take NSAIDs chronically, complications such as ulcer bleeding or perforations occurred. To prevent complications, various methods such as the use of selective cyclooxygenase (COX-2) inhibitors and a concomitant use of a gastric acid suppressive agent or misoprostol have been recommended. At present, among the drugs used to suppress NSAID-induced gastric mucosal injury, misoprostol has been studied extensively, and it is effective in reducing the risks of developing NSAID-induced gastric ulceration by 51% to 75%. However, compared with conventional types of NSAIDs, the effects of selective COX-2 inhibitors in preventing upper gastrointestinal complications are not satisfactory (relative risk, 0.8; 95% confidence interval, 0.6 to 1.1). The mechanisms by which NSAIDs trigger the occurrence of gastric mucosal injury are mainly divided into local stimulation and systemic action. Of these, the deficiency of prostaglandin due to the inhibition of COX reduces the blood flow to the gastric mucosa, which leads to hemodynamic derangement of the microcirculation. Additionally, this also leads to a decreased secretion of mucus, thereby lowering the defense function against stimulation, which is considered to be an important mecha-

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nism. According to a study in COX-1 knockout rats, however, even when the synthesis of prostaglandin was suppressed, spontaneous gastric ulcers did not occur. In cases where the local stimulation was additionally applied by the oral administration of indomethacin, damage of the gastric mucosa occurred. This suggests that NSAID-induced damage of the gastric mucosa cannot be solely explained by the suppression of the prostaglandin synthesis. An NSAID-induced decrease in the blood flow to the gastric mucosa induces vascular endothelial damage prior to the suppression of COX function by NSAIDs, where neutrophils may play a key role. Oral administration of NSAIDs leads to the increased expression of intercellular adhesion molecule-1 in the vascular endothelial cells of the gastric mucosa. As a result, a massive amount of neutrophils are attached to the vascular endothelial cells, which may be associated with inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1. As described here, the reactive oxygen species, which are synthesized in the increased numbers of neutrophils, are involved in the oxidation of key biomolecules such as lipid, protein and DNA. This eventually leads to a damage of the vascular endothelial cells. As a result, the blood flow to the gastric mucosa is decreased, which leads to hemodynamic derangement of the microcirculation. Additionally, NSAIDs directly act on the gastric mucosa and terminate the oxidative phosphorylation. Thus, they induce a loss of the transfer of electron in mitochondrial membranes, and thereby release cytochrome c. As described here, the released cytochrome c produces active oxygen radicals, which leads to the activation of proteases, such as caspase-9 and caspase-3, thus contributing to apoptosis.

Oxidative stress is a key mechanism in NSAID-induced gastric mucosal injury. If the oxidative stress of the gastric mucosa could be suppressed by powerful anti-oxidants, NSAID-induced gastric mucosal damage would be markedly suppressed. In recent years, it has been reported that various antioxidants such as vitamin C, sunflower seed oil, and grape seed extract, prevent damage to the gastric mucosa.

Grape seed proanthocyanidin extracts (GSPEs; Hanlim Pharm. Co., Ltd., Seoul, Korea), which was used in the current study, is a commercially available product of the substances that were extracted from European red grape seed, Vitis vinifera. It is a proanthocyanidolic oligomer that is chemically composed of a mixture of pycnogenol and flavonoid, with a structure of flavane-3-ol and the condensed polymers, including procyanidol dimer, trimer, and oligomer. It is a polyphenolic compound whose major constituent is a bioflavonoid, proanthocyanidin, which plays a role as a scavenger of free oxygen radicals, and performs powerful antioxidative functions with a magnitude that is ten times higher than that of vitamin E or vitamin C. At now, it is used to improve numbness or symptoms of pain associated with the impaired functions of the venous and lymphatic systems. Furthermore, it is also used as an adjuvant in controlling lymphatic edema from breast cancer treatment and to treat hemodynamic derangements occurring in the retina or choroidal membrane.

The aim of this study was to determine whether GSPE could prevent NSAIDs-induced gastric mucosal damage by using an experimental animal model of indomethacin-induced damage of the gastric mucosa in rats.

**MATERIALS AND METHODS**

1. **Experimental animals and treatments**

Sprague-Dawley male rats aged 7 weeks were purchased for the current experiment. The animals were housed in a standard sterile room at 20°C to 24°C, with 40% to 60% humidity, and with a 12-hour light/dark cycle. During all the experiments, the experimental animals were protected under the guidelines issued by the American Institute of Laboratory Animal Resources.

To induce damage to the gastric mucosa, indomethacin (100 mg/kg) was used. GSPE (100 and 300 mg/kg) was used to protect the gastric mucosa against the indomethacin-induced injury. The LD50 (lethal dose 50%) of GSPE was 3,100 mg/kg during oral administration in rats. A low dose of 100 mg/kg and a high dose of 300 mg/kg were determined. For a comparison of the protective effect of GSPE on the gastric mucosa, misoprostol (50 µg/kg) was used.

Based on the drugs that were administered to protect the gastric mucosa, the experimental animals were divided into five groups: 1) the normal control group [saline+0.5% carboxymethylcellulose]; 2) indomethacin group [saline+indomethacin]; 3) low-dose GSPE group [GSPE 100 mg/kg+indomethacin]; 4) high-dose GSPE group [GSPE 300 mg/kg+indomethacin]; and 5) misoprostol group [misoprostol 50 µg/kg+indomethacin]. Each group was composed of five animals. Access to food, except for water, was restricted 24 hours prior to the experiment.

During the experiment, saline, GSPE or misoprostol were orally administered to the experimental animals in each group once a day for 6 consecutive days. On the 5th and 6th day, in the four groups except for the normal control group, indomethacin 100 mg/kg was orally administered 1 hour after the gastroprotective agents were administered to cause the acute damage to the gastric mucosa. In the normal control group, the same volume of 0.5% carboxymethylcellulose was infused instead of indomethacin.

Following the initial infusion of gastroprotective agents and prior to the infusion of the final agent, the experimental animals were allowed free access to the standard diet, 5L79 (LabDiet, Framingham, MA, USA) and water. In the low-dose GSPE group and in the high-dose GSPE group, GSPE, which was suspended in saline, was infused at a concentration of 30 and 10 g/L and at a volume of 0.01 mL/mg. Misoprostol was dissolved in an ethanol buffer (20% ethanol, 104 mM NaHPO₄, and 16 mM NaOHPO₄) at a concentration of 5 mg/L, and it was infused at a volume of 0.01 mL/mg. All the drugs were administered via oral route.
On day 6, when the gastroprotective agents were finally administered to the experimental animals, access to food and water was restricted. And then, the experimental animals were sacrificed after 6 hours following the infusion of indomethacin and 0.5% carboxymethylcellulose (Fig. 1).

Blood samples were obtained through a cardiac puncture in a pyrogen-free polypropylene tube that was treated with 5 mg/mL ethylene-diamine-tetraacetic acid (EDTA). The samples were well mixed and centrifuged for 10 minutes at 4°C at 5,000 rpm. The plasma was collected in a 1.5 mL tube and preserved at a temperature of -70°C until further analysis.

2. Histopathologic assessment

Following the blood sampling, the abdomen was incised, and the stomach was dissected. The dissected stomach was incised along the great curvature, so that the mucosal side was placed superiorly. Half of the dissected tissue was fixed in 10% buffered formalin for histopathologic analysis. Paraffin-embedded blocks were then prepared. The remaining tissue was frozen in liquid nitrogen for a biochemical test.

For the histopathologic assessment of the damage to the gastric mucosa, a section of 0.5 cm in width was prepared homogeneously. In all the gastric tissue samples, the same section was cut at the same location. Tissue slides, which were prepared as described herein, were examined at a magnification of ×40 and ×100 under a light microscope. The number of gastric erosions and ulcers, and the area of the lesions were measured. The assessment of lesions was performed with reference to the histological characteristics of NSAID-induced gastritis, which were previously described by Chen et al.17 based on the following criteria: the intensity of the gastric mucosal damage was assessed based on four categories, such as normal, erosion restricted to the mucosa, ulcer invading the submucosa and ulcer invading the proper muscle layer. In each experimental animal, the most severe lesions were used for the assessment. The degree of inflammation occurring in the mucosa and submucosa was assessed based on such findings as edema, congestion and the hyperproliferation of the basal line and the degree of proliferation of smooth muscle, whose severity was classified as mild, moderate and severe for comparative analysis.

3. Biochemical test

1) The measurement of the concentration of glutathione (GSH) present in the gastric mucosa

The stomach tissue sample was mixed with 150 mM KCl and then homogenized. Following this, the concentration of GSH present in the gastric mucosa, indicating the decreased oxidative stress, was measured.

GSH in the gastric mucosa was measured using a GSH assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) based on the modified methods of Ellman.18 The homogenized gastric mucosal tissue was centrifuged at 2,000 g for 10 minutes. Subsequently, the 0.5 mL of the supernatant was harvested and treated with 0.3 mol/L Na₂HPO₄·2H₂O solution at a 2 mL volume. Then, this mixture was treated with 0.2 mL of dithiobisnitrobenzoate (0.4 mg/mL, 1% sodium citrate). The optical density was measured at a wavelength of 412 nm, and the concentration was calculated accordingly.

2) The measurement of prostaglandin E₂ (PGE₂) present in the gastric mucosa

The concentration of PGE₂ present in the gastric mucosa was measured using the methods of Batu and Erol.19 Gastric gland tissue was homogenized in 99.5% ethanol 2 mL containing 10 mM indomethacin, and this mixture was centrifuged at 10,000 g at a temperature of 4°C for 15 minutes. The supernatant was harvested and then preserved at -80°C. Following this, the measurements were recorded using a PGE₂ EIA Kit-Monoclonal (Cayman Chemical Company). Then, the optical density was measured at a wavelength of 412 nm, and the concentration
was calculated accordingly.

3) The measurement of proinflammatory cytokines

To evaluate the levels of the proinflammatory cytokines, the concentrations of TNF-α and IL-1β in the blood were measured. The plasma samples that were collected and preserved at -70°C were used for these measurements. The 50 μL of samples were mixed with TNF-α and IL-1β conjugates and the mixture was preserved at room temperature for 2 hours. Then, the 100 μL substrate solution was added. After 30 minutes, the optical density was measured at a wavelength of 450 nm, using an ELISA Kit-Rat TNF-α, IL-1β (R&D System Inc., Minneapolis, MN, USA).

4. Statistical analyses

Data are expressed as mean±SD. Groups of data were compared using Mann-Whitney U test and Kruskal-Wallis test. Differences were considered statistically significant at p<0.05.

| Table 1. Effects of GSPE on Indomethacin-Induced Gastric Damage |
|---------------------------------------------------------------|
| Group                                         | No. of erosions | Total area of erosions, mm² | No. of ulcers | Total area of ulcers, mm² |
|-----------------------------------------------|-----------------|-----------------------------|---------------|--------------------------|
| Control (n=5)                                  | 0.00±0.00       | 0.00±0.00                   | 0.00±0.00     | 0.00±0.00                |
| Indomethacin (n=5)                             | 4.00±1.05       | 1.86±0.77                   | 1.40±0.25     | 11.26±5.91               |
| GSPE 100 mg/kg/Indomethacin (n=5)              | 1.60±0.68       | 1.69±0.70                   | 0.40±0.25*    | 0.31±0.19*               |
| GSPE 300 mg/kg/Indomethacin (n=5)              | 1.00±0.00*      | 1.92±1.29                   | 0.20±0.20*    | 0.63±0.62*               |
| Misoprostol/Indomethacin (n=5)                 | 2.20±0.66       | 4.00±2.70                   | 0.20±0.20*    | 0.16±0.15*               |

Data are presented as mean±SD.

GSPE, grape seed proanthocyanidin extract.

*Significant difference (p<0.05) from the indomethacin group.

Fig. 2. Microscopic findings from the rat stomach (H&E stain, ×40). (A, B) Erosions induced by indomethacin show shedding of the superficial epithelium and thinning of the height of the epithelium. (C, D) Ulcers induced by indomethacin show sharply demarcated mucosal defects containing inflamed, necrotic debris on the base with exposure of the submucosal layer. (E, F) Ulcers penetrate through the muscularis and transmural inflammatory infiltration is seen.
RESULTS

1. The assessment of the damage to the gastric mucosa

1) The number and area of erosions and ulcers

In the normal control group, where only saline was infused, there was no damage to the gastric mucosa. In the indomethacin monotherapy group, there were multiple erosions and ulcers. In the groups that were pretreated with GSPE and misoprostol, as compared with the indomethacin group, the number and area of gastric ulcers were significantly decreased (p<0.05). However, there were no significant differences between the low-dose GSPE group, the high-dose GSPE group and the misoprostol group (Table 1, Fig. 2).

2) The assessment of the intensity of damage in the mucosa and submucosa

In the normal control group, there was no damage to the mucosa and submucosa. With regard to the intensity of maximal mucosal damage, in case of the indomethacin group, ulcers were observed in all the experimental animals. However, in the low-dose GSPE group, the high-dose GSPE group and the misoprostol group, ulcers were found at a frequency of 40%, 20%, and 20%, respectively (Fig. 3). With regard to the degree of inflammation in the mucosa and submucosa, a moderate degree of inflammation was observed at a frequency of 100% in the indomethacin group. However, in the low-dose GSPE group and the high-dose GSPE group, inflammation was observed at a frequency of 40%, while in the misoprostol group, it was observed at a frequency of 80% (Fig. 4).

2. Changes in the biochemical markers due to indomethacin in the gastric mucosa

The concentrations of PGE$_2$ in the gastric mucosa were found to be 197±24.8 pg/mL in the normal control group, 85.1±13.5 pg/mL in the indomethacin group, 88.9±24.0 pg/mL in the high-dose GSPE group, and 68.6±23.6 pg/mL in the misoprostol group, respectively. These results indicate that the concentration of PGE$_2$ was markedly decreased (p<0.05). But there was no significant difference among the indomethacin group, the GSPE group and the misoprostol group (Fig. 5).

Additionally, the concentrations of GSH measured in the gastric mucosa were found to be 0.14±0.01 μM in the normal control group, 0.11±0.02 μM in the indomethacin group, 0.13±0.01 μM in the high-dose GSPE group, and 0.09±0.02 μM in the misoprostol group, respectively. These results indicate that the concentration of GSH in the gastric mucosa was decreased in the indomethacin group and in the misoprostol group, compared with the normal control group. In the high-dose GSPE group, however, there was no significant difference compared with the normal control group (Fig. 6).

Histopathologic analysis revealed that there was no difference in mucosal injury based on the dose of GSPE administered. Therefore, the concentrations of PGE$_2$ and GSH were not measured in the low-dose GSPE group.

3. Changes in the proinflammatory cytokines

There was no significant difference in proinflammatory cytokine levels, TNF-α and IL-1β among the groups (Table 2).

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Fig. 3. Degree of maximal mucosal injury. Pretreatment with grape seed proanthocyanidin extract (GSPE) and misoprostol diminished the gastric mucosal injury caused by indomethacin.

*Significant difference (p<0.05) from the indomethacin group.

Fig. 4. The grade of submucosal inflammation. In comparison with the findings in the indomethacin group and the misoprostol group, the grade of submucosal inflammation in the grape seed proanthocyanidin extract (GSPE) group was less severe.

*Significant difference (p<0.05) from the indomethacin group.
DISCUSSION

In the current study, GSPE and misoprostol significantly reduced the area and number of gastric ulcers caused by indomethacin. Between the GSPE group and the misoprostol group, there was a similar profile of the gastroprotective effect. But there were no differences in these effects depending on the dose of GSPE. The total number and area of erosions seems to be more prominent in misoprostol group than GSPE groups, however, there are no statistically significant differences between them.

The degree of submucosal inflammation was relatively lower in GSPE group, compared with misoprostol group. In other words, in cases in which GSPE was concomitantly administered with indomethacin, there was a similar effect on suppressing the occurrence of gastric ulcers as compared with misoprostol. However, GSPE had an excellent effect of suppressing the inflammatory responses in the submucosal layer compared with misoprostol. Based on these results, misoprostol plays the primary role in suppressing the damage to the mucosal surface. However, once the damage has progressed on the mucosal surface, misoprostol may not be effective in suppressing the damage to the blood vessels and the tissues in the submucosal layer. Meanwhile, GSPE in the early stages suppresses the damage to the mucosal surface. The antioxidation effects of GSPE are seen in the blood vessels and tissue in the submucosal layer, which eventually suppresses the ischemia reperfusion injury caused by the inflammatory actions of the neutrophils and vascular local inflammation. This is assumed to suppress the progression of apoptosis and cellular damage. However, further study will be needed to elucidate this point.

According to a review of the literature, proanthocyanidin, one of the major constituents of GSPE, regulates the expression of such molecules as IL-6, TNF-α, adiponectin, and CRP. In an experimental animal model of alcohol-induced damage to the gastric mucosa, the activity of myeloperoxidase (MPO), a marker of mucosal injury and inflammation, was markedly suppressed by proanthocyanidin. The activity of superoxide dismutase (SOD), a defense mechanism against oxidative stress, was increased. According to Pohle et al., even in cases of gastric mucosal injury due to aspirin, the gastroprotective effect of vitamin C suppresses the activity of MPO, increases the activity of SOD and retains the activity of GSH peroxidase, which is down-regulated by aspirin. Also, according to Motawi et al. and El Eter et al., leptin and ghrelin also diminished the oxidative stress, and thereby reduced the consumption of GSH in the gastric mucosa. Thus, they produce antiulcer effects in the gastric mucosa. Meanwhile, in the current study, the concentration of GSH in the gastric mucosa was decreased in the indomethacin group and in the misoprostol group compared with the normal control group. In the GSPE group, however, it was not decreased. These
results were in agreement with the above reports. In addition, sunflower seed oil, which has an antioxidative effect,\(^5\) the extracts of the fruit of *Ficus glomerata*\(^6\) and DA-6034,\(^7\) one of the new synthetic derivatives of eupatilen that was extracted from a mugwort, have also been reported to have an antulcer effect. In the current study, however, there was a lack of the systemic inflammatory responses to NSAID-induced ulcers. An NSAID-associated increase in the concentration of local inflammatory mediators (proinflammatory cytokines), such as IL-1, TNF-\(\alpha\), IL-8, and IL-17,\(^8\) did not lead to the increase in concentration of systemic cytokines present in the blood. Accordingly, to make a diagnosis of NSAID-induced damage of the gastric mucosa using blood sampling without esophagogastroduodenoscopy for chronic NSAID users, a substantial effort may be made to identify a novel type of marker indicating inflammation of the gastric mucosa.\(^9\)

To prevent NSAID-induced gastric mucosal damage concomitant drugs, such as proton pump inhibitors and misoprostol, have been used. In addition, selective COX-2 inhibitors also have been used, but there is a limitation to their use due to cardiovascular side effects. Accordingly, at present, the administration of concomitant drugs is considered first. When NSAIDs are administered for more than 4 weeks on a regular basis, a persistent concomitant administration of proton pump inhibitors or misoprostol could prevent the occurrence of peptic ulcers (placebo-controlled group 47%, proton pump inhibitors 83%, and misoprostol 88%)\(^9\) and could also promote the healing of NSAID-induced gastric ulcers.\(^9\)

Recently, the defense mechanisms against NSAID-induced gastric mucosal damage have been of much interest. Of the defense mechanisms, heme oxygenase-1 (HO-1) has been reported to reduce oxidative stress. This effect has been examined in a substantial number of studies. HO has three subtypes, HO-1, HO-2, and HO-3, and it produces carbon monoxide, iron, and biliverdin by degrading heme. Of these, the expression of HO-1 is increased by oxidative stress, and it plays a role in protecting cells. Also, HO-1 is expressed in response to various stimulants, such as NSAIDs, ultraviolet rays, and inflammatory mediators including cytokines and heavy metals.\(^8\) But HO-1 expression can increasingly occur in the presence of ‘nonstressful’ stimulants (e.g., antioxidants) as well as the ‘stressful’ stimulants described earlier.\(^9\) As described herein, due to the presence of HO-1, which was induced, carbon monoxide dilates the blood vessels and suppresses the aggregation of platelets. Thus, it plays a role in increasing the blood flow in the gastric mucosa. Also, biliverdin, synthesized by HO-1, and its reduced form, bilirubin, play a role as powerful antioxidants, thereby suppressing cellular injury. The increase of HO-1 mRNA due to the presence of NSAIDs in the gastric mucosa did not have a significant antioxidant effect, as shown in the cardiovascular system. The reason is that the increased expression of HO-1 mRNA did not lead to that of HO-1 protein. Under the same conditions, following the addition of the antioxidant vitamin C, this plays a role as a ‘nonstressful’ stimulant. Thus, following the induction of protein transcription, the gastroprotective effect is observed.\(^10\) Also in the current experiment, it can also be inferred that GSPE acted on the expression of HO-1 protein as a ‘nonstressful’ stimulant, which led to the gastroprotective effect. Further studies are also warranted to measure the expression of HO-1 mRNA and that of HO-1 protein.

Our study has two substantial limitations. First of all, the apoptotic and vasodilator effect are also an important mechanisms of GSPE in preventing gastric mucosal damage in the literature, however, nitric oxide system and apoptosis were not evaluated in this study.\(^11\) Second, water was used as buffer in GSPE group and 20% alcohol in misoprostol group. This difference of buffers could make different effects on the induction induced gastric ulcer model in misoprostol and GSPE group.

In conclusion, GSPE had a similar antulcer effect as misoprostol. Apart from the effects of misoprostol due to the actions of prostaglandin, the antioxidative effects of GSPE may play a key role in diminishing the occurrence of gastric mucosal damage.

**CONFLICTS OF INTEREST**

No potential conflict of interest relevant to this article was reported.

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