

Ginseng, the Root of *Panax ginseng* C.A. Meyer, Protects Ethanol-Induced Gastric Damages in Rat through the Induction of Cytoprotective Heat-Shock Protein 27

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Abstract Ginseng, the root of *Panax ginseng* C.A. Meyer, has been reported to exert preventive effects on *Helicobacter pylori* infection [1]. Excessive ethanol gastropathy via anti-oxidative and anti-inflammatory actions. In this study, we investigated the protective effects of ginseng against ethanol-induced gastric damages in rat. To examine the preventive effect of ginseng, rats received two different ginseng extracts, A and B, 1 h prior to the administration of ethanol. Pretreatment of rats with ginseng extract A and B attenuated the ethanol-induced gastric lesions by 111 ± 48 and 142 ± 47 mm² compared to control group (164 ± 54 mm²). Significant induction of cytoprotective heat-shock proteins HSP27 and HSP70 was found in the ginseng-administrated rats, suggesting that the restoration of the proteins might contribute to prevention of ethanol-induced gastric injuries. It is, therefore, suggested that ginseng has a protective effect against ethanol-induced gastric damages by induction of heat-shock proteins and 27.

Keywords Alcohol-induced gastric damage · Ginseng · Cytoprotection · Heat-shock proteins

Introduction

Gastric ulcerative injuries are provoked by etiologic factors such as ethanol consumption, physiological stress,

nonsteroidal anti-inflammatory drugs (NSAIDs), and *Helicobacter pylori* infection [1]. Excessive ethanol ingestion leads to gastric lesions characterized by mucosa edema, subepithelial hemorrhages, cellular exfoliation and inflammatory cell infiltration. Consequently, ethanol abuse impairs the integrity of the gastric mucosa barrier and histology [2,3]. Pharmacotherapy against ethanol-induced gastric damages has primarily been focused on the enhancement of mucosal defense mechanisms, thereby accelerating healing and preventing relapses [4,5]. Enhancement of mucosa secretion, blood flow, cell regeneration, and the release of endogenous protective molecules such as prostaglandins and epidermal growth factors have been considered as treatment for ethanol-induced gastric mucosa damages.

Ginseng has been used for traditional herbal medicine for over 2,000 years in Asian countries including Korea, Japan, and China, and is believed to exert beneficial effects on health and to promote longevity. Recently, intensive biochemical and molecular studies have shown pharmacological effects of ginseng in the central nervous system and cardiovascular, endocrine, and immune systems as well as in cases of cancer [6,7]. These studies suggest that the preventive effects of ginseng are due to its anti-inflammatory, anti-oxidation, and cytoprotective activities [13,14]. Especially for alcohol-associated human diseases, ginseng has been shown to accelerate alcohol clearance in blood by increasing the activities of alcohol dehydrogenase and aldehyde dehydrogenase [4,5], attenuating plasma ethanol level [6], and reducing impairment of acquisition by ethanol intake [7]. However, the molecular mechanism of ginseng has not yet been fully elucidated.

In this study, we investigated the preventive effect of ginseng against ethanol-induced gastric damages in rat and attempted to elucidate its underlying molecular

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mechanisms. Our results demonstrated that pretreatment with absolute ethanol was orally given to the rats. The animals were sacrificed by anesthesia with excessive ether 1 h after lesions. Furthermore, COX-2 expression was significantly attenuated, and cytoprotective heat-shock proteins HSP27 and HSP70 were induced in rats who received ginseng extracts. These results suggested that ginseng has a cytoprotective effect against ethanol-induced gastric damages by induction of heat-shock proteins.

Methods

Reagents

Ginseng extracts were supplied by Ginseng Research Group, Korea Food Research Institute (Songnam, Korea). In brief, ginseng extract A was prepared from a 4-year-old *ginseng* C.A. Meyer (Keumsan, Choongchungnam-do, Korea). The aqueous extract of ginseng was prepared by decoction in a 20-fold amount of water at 120°C for 3 h and then filtered. The residue was further extracted with a 20-fold amount of 80% ethanol for 3 h, and the organic solvent was removed. The aqueous extract was combined with the ethanol extract. The mixture was concentrated under vacuum and lyophilized with a yield of about 58% of the original ginseng powder. For preparation of ginseng extract B, 2 g of ginseng powder was extracted twice with 50 ml of water-saturated butanol (BuOH) at 80°C for 1 h. The extract was filtered and evaporated under vacuum. The concentrate was extracted with 50 ml of ether, and the solvent was decanted. The residue was dried at 105°C for 30 min and cooled in a desiccator. The final products were used as crude saponin fraction (ginseng extract B) of white ginseng.

The ginseng extract A or B was suspended in 0.5% carboxymethylcellulose (CMC, Sigma, St. Louis, MO) and administered to rats at a concentration of 10 mg/kg body weight (BW).

Experimental design and treatment of animals

Sprague Dawley male rats (5 months old and 200–250 g BW) were purchased from Central Lab Animal (Seoul, Korea). Animals were housed in climate-controlled conditions (24 ± 1°C at 50% relative humidity) with 12 h light-dark cycle. The experimental protocol was approved by the Animal Care and Use Committee of Ajou University and complied with the international criteria for humane use of animals in research. All animals were fed standard chow and tap water ad libitum and acclimatized for 1 week before experiment. Rats were fasted for 24 h, and then 10 mg of ginseng extract A or B suspended in 0.5% CMC/kg was orally administered. One hour later, 1 ml of

The stomach was isolated quickly, opened along the greater curvature, rinsed in ice-cold saline and flattened on a glass slide. Macroscopic gastric injuries were photographed with a digital camera, and gastric lesion area (%) was measured. The isolated stomach tissues were further used for Western blot analysis and immunohistochemical evaluation. For microscopic histopathological evaluation, the stomach was fixed in 10% formalin solution and embedded in paraffin, and the standard hematoxylin-eosin stain was performed. The slides were assessed according to the following criteria of O'Brien: degree of mucosa edema, subepithelial hemorrhages, cellular exfoliation and inflammatory cell infiltration.

For immunohistochemical analysis, 10% buffered formalin-fixed, paraffin-embedded sections were deparaffinized (pH 7.6) with 5% urea in an 850-W microwave oven for 5 min, followed by two more treatments of 5 min each. Then, the sections were stained with Histostain-Plus kit (Zymed Laboratories, San Francisco, CA) according to the manufacturer's instructions. Primary antibodies recognizing COX-2 (C-20) and iNOS (C-11) were purchased from Santa Cruz Biotechnology (California), and antibody against nitrotyrosine (HM11) was obtained from Zymed. The sections were counterstained with hematoxylin.

TUNEL assay

Genomic DNA damages by ethanol exposure were analyzed by TUNEL assay using a frozen section of rat stomach, and the assay was performed with Fluorescent-FragEL DNA fragment detection kit (Oncogene, Boston, MA) according to the manufacturer's instructions. Fluorescein-labeled cells by TdT enzyme were visualized

Western blot analysis

Proteins isolated from gastric mucosa of rats or AGS cells were electrophoresed on 12% SDS-PAGE gels, and

transferred to PVDF membranes by using a semidry transfer system (Hoeffer Phamacia Biotech, San Francisco, CA). Membranes were blocked by 5% nonfat dry milk for 1 h and immunoprobed with specific antibodies. Antibodies used in this study were purchased from Santa Cruz Biotechnology [HSP27 (C-20), HSP70 (W27), HSP90 (F-8)] or Calbiochem (α -tubulin, DM1A).

Cell culture and cell viability assay

Human gastric epithelial AGS cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY), containing 10% fetal bovine serum and 100 units/ml penicillin, in a humidified 5% CO₂ atmosphere. The cells were exposed to various concentrations of ethanol for the indicated time and used for cell viability assay and Western blot analysis. To evaluate the effect of ethanol on viability of gastric epithelial AGS cells, the cells were exposed to various concentrations of ethanol for 16 h, harvested, and stained with trypan blue. Survival (%) was calculated by the following formula: no. of viable cells (dye-excluding cells)/no. of total cells \times 100.

Results

Pretreatment of rats with ginseng extracts prevents ethanol-induced gastric lesions

To test the protective effect of ginseng extracts on ethanol-induced gastric injuries, ginseng extract A or B at 10 mg/kg body weight was orally administered to rats 1 h before the intake of absolute ethanol. The administration of absolute ethanol induced severe hemorrhagic necrotic lesions of 164.0 \pm 54.6 mm² in the gastric mucosa (Fig. 1A and B, left panel) of control rats. Furthermore, Fig. 1B indicated that rats given absolute ethanol showed apparent changes such as formation of multifocal and diffuse epithelial erosion by exfoliation of the superficial gastric epithelium, extensive hemorrhage in mucosa, and significant edema with inflammatory cell infiltration into the mucosa and submucosa. However, rats pretreated with ginseng extract A or B showed reduction in gross gastric lesions of 111.0 \pm 45.0 and 142.0 \pm 47.0 mm², respectively (Fig. 1A and B, left panel), demonstrating that the severity of microscopic gastric injuries by ethanol intake was attenuated in the ginseng-treated rats. These findings indicate that pretreatment of rats with ginseng extracts prevents ethanol-induced gastric damages, macroscopically and microscopically.

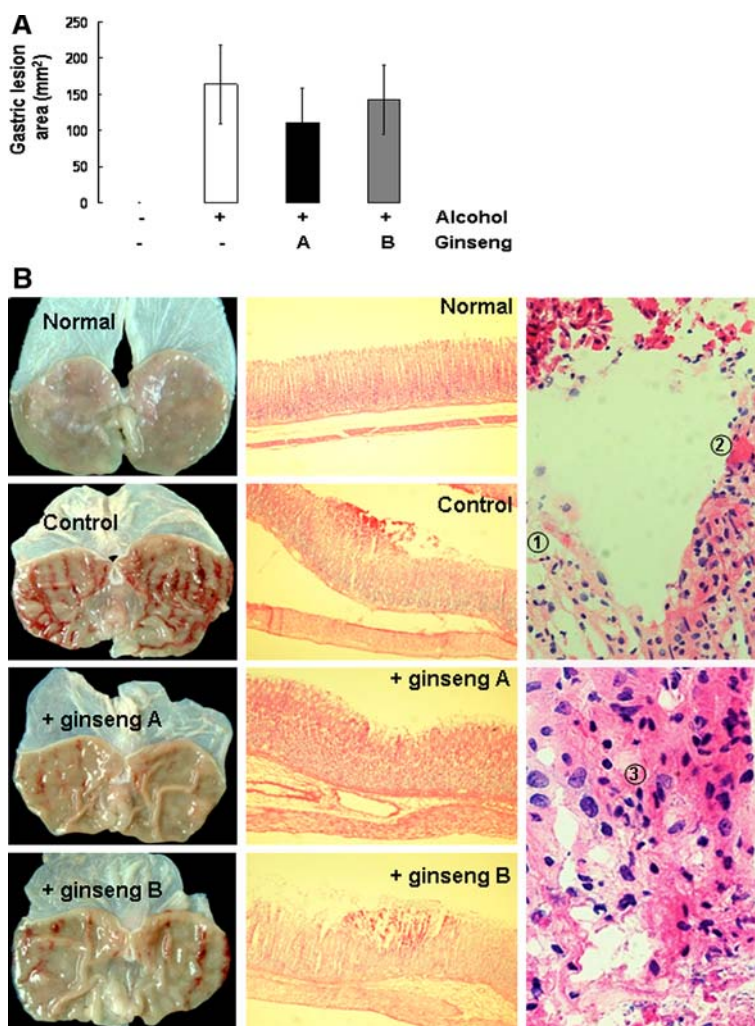
COX-2, iNOS, and nitrotyrosine are down-regulated in rats that received ginseng extracts. COX-2, iNOS, and nitrotyrosine have been considered as key mediators involved in the process of inflammation and oxidative stress [18, 19]. Therefore, they are immunohistochemically evaluated by using antibodies recognizing them. Figure 2A showed that strong immunoreactivity of COX-2 was observed in the damaged gastric mucosa of ethanol-treated rats compared to normal rats. COX-2 was predominantly located in the cytoplasm of mucosa neck cells and parietal cells. However, the expression of COX-2 was significantly reduced in rats pretreated with ginseng extract A or B. As seen in Fig. 2B, the ginseng-treated group showed low immunoreactivity against iNOS, while moderate reactivity was observed in the ethanol-treated control group. Nitrotyrosine, an indicator of the production of NO in cell damage and inflammation processes, was found to be widely expressed, ranging from the superficial gastric epithelium to the base of the gastric gland of the control group (Fig. 2C). However, the intensity was slightly reduced in the mucosa of ginseng-treated rats. This result indicated that ethanol in the stomach imposed a burden of oxidative stress on the gastric mucosa, resulting in acute gastric lesion that could be prevented by ginseng pretreatment.

Administration of ginseng extracts rescues gastric mucosa cells from ethanol-induced cell death and strongly induces the expression of cytoprotective

heat-shock proteins HSP27 and HSP70

TUNEL assay was performed to examine cell death accompanied by genomic DNA fragmentation. As shown in Fig. 3, many FITC-positive cells were found in the mucosa of rats treated with ethanol; their location corresponded to that of gastric erosive lesion in the stomach, demonstrating that gastric lesions induced by ethanol exposure were accompanied by cell death with genomic DNA damages. Ginseng pretreatment significantly rescued the gastric mucosa cells from ethanol-induced cell death. Interestingly, as shown in Fig. 4, cytoprotective heat-shock proteins HSP70 and HSP27 were overexpressed in the ginseng-pretreated rats in contrast to low expression in the control rats. The intensity of HSP27 and HSP70 in the ginseng extract A-treated group showed 2.4- and 2.8-fold increase compared to that of control group (Fig. 4B). In the ginseng extract B-treated group that received ginseng extract B, the increase in HSP27 was 2.1-fold and 4.25-fold for HSP70. HSP90 and α -tubulin did not display apparent differences between the groups. These data suggest that overexpression of HSP27

Fig. 1 Preventive effect of ginseng extracts against ethanol-induced gastric injury in rats. **(A)** Gross lesion area. Rats were pretreated orally with ginseng extract A or B at 10 mg/kg body weight prior to exposure to absolute ethanol. After 1 h of ethanol exposure, stomachs were isolated from the rats, and the area of gastric gross lesions was measured by using transparent 1 mm² grids. Lesion area (mm²) is presented as mean ± SD. **(B)** Histopathology. The left panel shows macroscopic gross findings and the right panels represent microscopic histological appearances with hematoxylin and eosin stain. The gastric mucosa of rats treated with absolute ethanol shows severe erosive hemorrhagic lesions, accompanied by disruption and exfoliation of the superficial gastric mucosa (1), extensive hemorrhage (2), and inflammatory-cell infiltration (3) under high magnification (× 100)



and HSP70 may play a cytoprotective role in ethanol-induced injuries of gastric mucosa.

HSP 27 expression confers a cytoprotective action against ethanol-provoked cell death in vitro

As shown in Fig.3, treatment of rats with ethanol induced HSP27 expression by 38% compared with control. Significant cell death in the gastric mucosa. We first examined the effect of ethanol on viability of gastric epithelial cells in cell culture system and found that ethanol exposure remarkably reduced cell viability in a dose-dependent manner (Fig.5). Ethanol in concentrations greater than 16% showed significant cytotoxic effects on gastric epithelial cells, evidenced by marked attenuation of AGS cell viability (0% cell viability by 16% ethanol and 0.77% cell viability by 32% ethanol). On the other hand, the lower-than-2% ethanol showed no effect on cell survival.

Next, we examined the possibility of whether restoration of HSP27 expression by ginseng, as shown in Fig. 4, could confer a cytoprotective role in ethanol-induced cell death. Thus, to evaluate the relationship between HSP27 expression and cell death, we checked the cell viability after ethanol treatment in the presence or absence of siRNA specific to HSP27. Human gastric epithelial AGS cells abundantly expressed endogenous HSP27. Incubation of AGS cells with siRNA_{HSP27} (10 μM) for 24 h attenuated HSP27 expression and cell viability to 79.49% (Fig.6). On the other hand, down-regulation of HSP27 by siRNA_{HSP27} significantly exposed cells to ethanol-induced cell death, evidenced by 45.84% reduction in cell viability. However, siRNA_{HSP27} itself did not alter the cell viability (98.7%). These results indicate that HSP27 confers cytoprotection in ethanol-induced cell death.

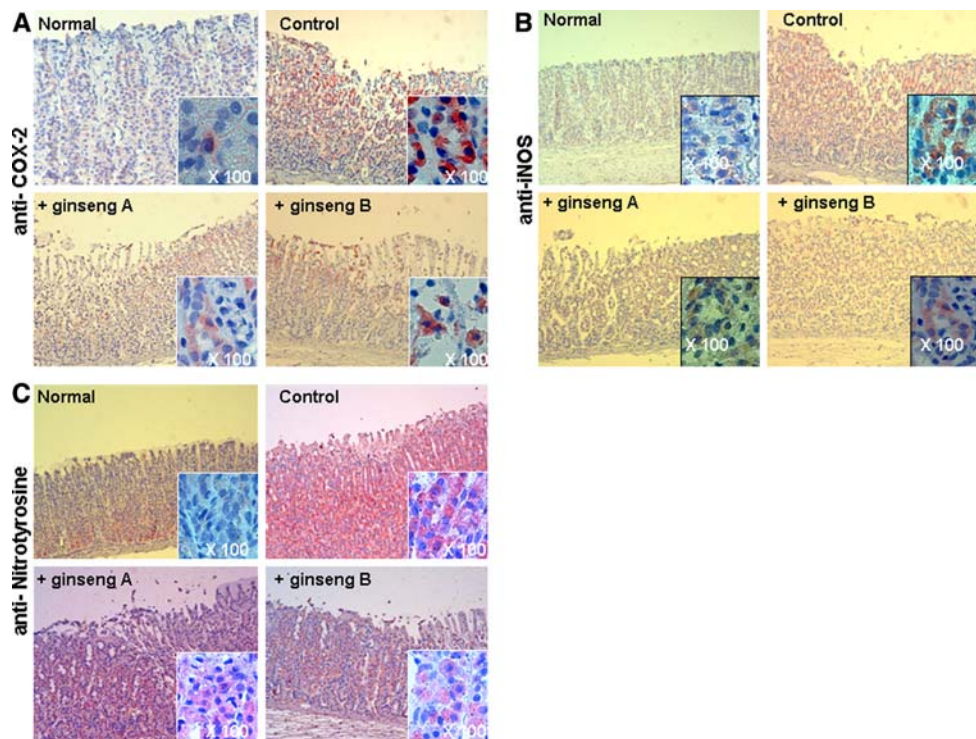
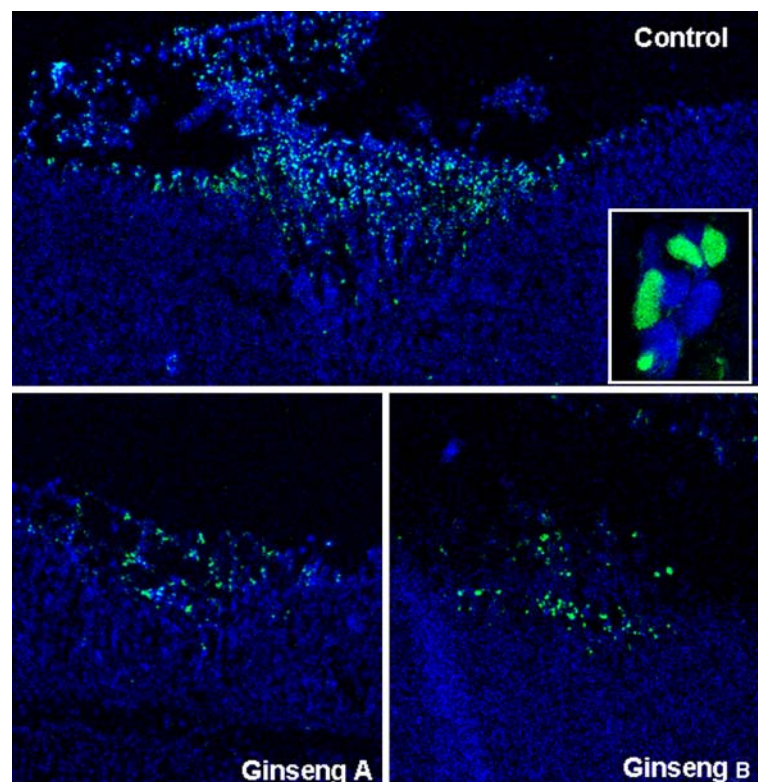


Fig. 2 Paraffin section of stomachs was immunohistochemically stained using specific antibodies recognizing COX-2 (A), iNOS (B), and nitrotyrosine (C). The figures represent immunoreactivity of each to ethanol treatment. The inserted photographs were taken under high magnification ($\times 100$)

Fig. 3 Attenuation of ethanol-induced cell death with ginseng pretreatment. To evaluate cell death with genomic DNA breakage by ethanol treatment, TUNEL assay was performed. Frozen sections of rat stomach were permeabilized with 1% Triton X-100. Cells having the 3'-OH ends of DNA fragment were labeled with fluorescein by TdT enzyme and visualized by photography ($\times 40$ magnification). The inserted photograph was taken under high magnification ($\times 100$). Figures are representative of each group: control, rats treated with absolute ethanol; ginseng A, rats administered orally with ginseng extract A at 10 mg/kg body weight 1 h prior to ethanol treatment; and ginseng B, rats administered orally with ginseng extract B at 10 mg/kg body weight 1 h prior to ethanol treatment



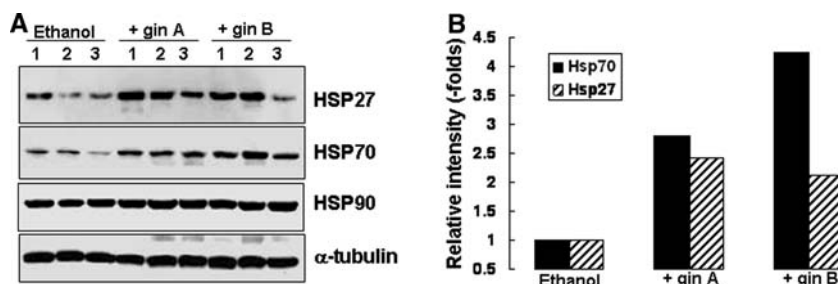


Fig. 4 Overexpression of cytoprotective heat-shock proteins, HSP70 and HSP27, by ginseng pretreatment. Western blot analysis was performed with gastric mucosa of rats. Proteins extracted from gastric mucosa were electrophoresed, transferred to PVDF membranes and probed with antibodies recognizing HSP27, 70, 90 and α-tubulin (A). Amount of protein expression was measured by densitometry and presented as average of relative intensity compared to that of the control group administered with ethanol (B).

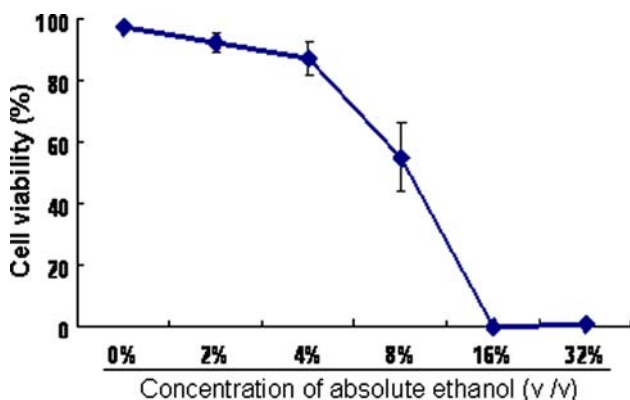


Fig. 5 Effect of ethanol treatment on viability of gastric epithelial AGS cells. Human gastric epithelial AGS cells were exposed to various concentrations of ethanol (0, 2, 4, 8, 16, 32%, v/v) for 16 h, and cell viability was measured by trypan blue exclusion assay. Survival (%) was calculated by the following formula: no. of viable cells (dye-excluded cells)/no. of total cells 100. The graph shows a mean of three independent experiments

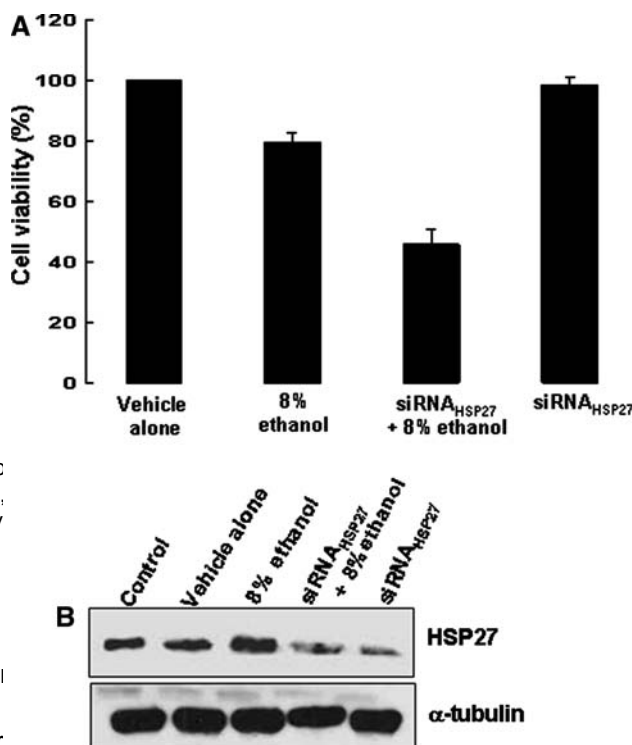


Fig. 6 Enhancement of ethanol-induced cell death by down-regulation of heat-shock protein 27A. Cell viability assay. AGS cells were transfected with siRNA_{HSP27} for 24 h and then exposed to 8% ethanol for 16 h. After ethanol treatment, cell viability was determined. The graph is a representative of three independent experiments. After measurement of cell viability, the cells were used for Western blot of HSP27

has commonly been considered for its toxic effects on liver and pancreas [20, 21]. However, relatively little attention has been paid to its actions within the stomach. Nevertheless, studies demonstrated that alcohol abuse associated with development of gastric diseases such as gastritis, gastric peptic ulcer disease, and even gastric cancer [22]. Alcohol stimulates acute gastritis as a result of direct mucosal damages that are further aggravated by other important risk factors such as nonsteroidal anti-inflammatory drugs (NSAIDs), acid, *Helicobacter pylori* infection, and physiological stress [23]. Despite widespread interest in alcohol-induced gastrointestinal diseases, the effects of acute and chronic exposure of the stomach to alcohol remain to be fully elucidated. Animal models serve as an essential tool for analyzing molecular mechanisms involved in alcohol-induced gastric diseases. Intragastric application of ethanol in animals causes acute gastric mucosal injuries characterized by linear hemorrhages and mucosal erythema and edema, scattered petechiae and

erosive changes [23]. Furthermore, ethanol-induced gastric damages have been shown to be associated with signaling molecules that are involved in the process of inflammation, apoptosis as well as oxidative stress. Activation of inflammation-associated transcriptional factors such as NF-κB, AP-1, and C/EBPβ and expression of its target genes such as COX-2, iNOS, and several pro-inflammatory cytokines have also been proposed to be responsible for alcohol-induced cell injuries [23, 24].

Notable increase in lipid peroxidation (LPO), MDA, and demonstrated significant induction of cytoprotective heat-MPO and depletion of glutathione (GSH) have also been reported in gastric mucosa exposed to ethanol [25, 26]. In the present study, we also confirmed the induction of COX-2 and iNOS after ethanol treatment of gastric mucosa in rats. HSP27 and HSP70. Down-regulation of HSP27 by siRNA_{HSP27} also resulted in a significant increase in ethanol-induced cell death. These findings suggest that ginseng has a cytoprotective effect on ethanol-induced gastric damages through induction of heat-shock proteins.

The focus on pharmacotherapy would be the enhancement of mucosal defense mechanisms to accelerate healing and prevent relapses. Mucoprotective agents such as prostaglandin, glutathione, ornoprostil, sucralfate, and misoprostol have been found to exert protective effects on ethanol-induced gastric damages [27]. In addition to these compounds, natural extracts such as green tea extracts, wogonin, and curcumin showed a potential protective effect against alcoholic diseases [28, 30]. Our present findings suggest that ginseng, the root of *Panax ginseng* C.A. Meyer, could be a potential protective source against ethanol-induced gastric damages. Pretreatment of animals with ginseng extracts significantly ameliorated pathological changes in gastric mucosa induced by ethanol and remarkably induced cytoprotective heat-shock proteins HSP 27 and HSP70.

Heat-shock proteins (HSPs) are crucial for the maintenance of cell integrity during both normal cell growth and pathophysiological conditions [1]. By controlling binding and release, HSPs function mainly as molecular chaperones that participate in the folding and assembly of nascent and unfolding proteins and facilitate protein transport to sub-cellular compartments. These HSPs are classified into four major families according to their biological activities and apparent molecular weights: HSP90, HSP70, HSP60, and small HSPs including HSP27. While HSP60, HSP70, and HSP90 are constitutively expressed, HSP70 and HSP27 are induced by various conditions, including heat, oxidative stress, or drug exposure [2]. The type of HSP induced and its level of expression can determine the fate of a cell in response to stress or stimulus. HSPs may also play a cytoprotective role in gastrointestinal tissue. For example, guinea-pig gastric mucosal cells in which HSPs were induced by heat-shock treatment showed considerable resistance against injury induced by 7.5% ethanol [3]. In addition, oral administration of geranylgeranylacetone (GGA), an anti-ulcer drug, rapidly induced HSP60, heat-shock cognate protein 70, HSC70, and HSP90 in rat gastric mucosal cells, and these induced HSPs contributed to the suppression of ulcer formation induced by water-immersion restraint stress [4]. Taken together, our findings suggest that induction of HSPs by ginseng treatment may positively contribute to gastric mucosal defense and participate in cytoprotection.

In conclusion, our results demonstrated that pretreatment of rats with ginseng prevented ethanol-induced gastric lesions, and rats that received ginseng extract

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