

Ganoderma lucidum Pharmacopuncture for the Treatment of Acute Gastric Ulcers in Rats

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Key Words

acupoint injection, acute gastric ulcer, *Ganoderma lucidum*, pharmacopuncture

Abstract

Objectives: The gastric ulcer is a common disorder of the stomach and duodenum. The basic physiopathology of a gastric ulcer results from an imbalance between some endogenous aggressive and cytoprotective factors. This study examined whether *Ganoderma lucidum* pharmacopuncture (GLP) would provide protection against acute gastric ulcers in rats.

Methods: Sprague-Dawley rats were divided randomly into 4 groups of 8 rats each: normal, control, normal saline (NP) and GLP groups. The experimental acute gastric ulcer was induced by using an EtOH/HCl solution and the normal group received the same amount of normal saline instead of ethanol. The NP and the GLP groups were treated once with injections of saline and GLP, respectively. Two local acupoints were used: CV12 (中脘) which is the alarm point of the Stomach Meridian, and ST36 (足三里), which is the sea point of the Stomach Meridian. The stomachs from the rats in each group were collected and analyzed for gross appearance and histology. Also, immunohistochemistry staining for

BAX, Bcl-2 and TGF- β 1 was performed.

Results: Histological observations of the gastric lesions in the control group showed comparatively extensive damage of the gastric mucosa and necrotic lesions had penetrated deeply into the mucosa. The lesions were long, hemorrhagic, and confined to the glandular portions. The lesions were measured microscopically by using the clear depth of penetration into the gastric mucosal surface. The length and the width of the ulcer were measured and the inhibition percentage was calculated. Wound healing of the acute gastric ulcer was promoted by using GLP, and significant alterations of indices in gastric mucosa were observed. Such protection was shown by gross appearance, histology and immunohistochemistry staining for BAX, Bcl-2 and TGF- β 1.

Conclusion: These results suggest that GLP administered at CV12 and ST36 can provide significant protection to the gastric mucosa against an ethanol-induced acute gastric ulcer.

1. Introduction

Gastric disease is common, affecting millions of people yearly. Among these diseases, the gastric ulcer is defined as a disturbance of the integrity of the gastric

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mucosa that causes a local defect or excavation due to active inflammation [1]. Nowadays, aggressive and defensive factors are widely known to play a role in the development of a gastric ulcer. The aggressive factors are gastric acid, pepsin, bile reflux, non-steroidal anti-inflammatory drugs (NSAIDs), *Helicobacter pylori* bacteria and alcohol, while the defensive factors are mucosal blood flow, surface epithelial cells, prostaglandin, phospholipids or surfactants, mucus, bicarbonate secretion, gastric motility, mucosa impermeability against H⁺ ions, heat shock protein, and others [2-4].

Ganoderma lucidum (靈芝) is a polypore mushroom that grows on the lower trunks of deciduous trees [5]. This mushroom, as a traditional Oriental medicine, has been widely used as a tonic in promoting longevity and health for thousands of years in Asian countries including China, Japan and Korea. Many works have been carried out on the therapeutic potential of *Ganoderma lucidum* [6]. *Ganoderma lucidum* has been shown to have anti-tumor, anti-cancer, immunomodulatory and immunotherapeutic effects. It has also been found to inhibit platelet aggregation, and to lower blood pressure, cholesterol, and blood sugar [7].

Pharmacopuncture is an effective therapy in Oriental medicine. A solution extracted from medicinal herbs is injected into acupuncture points according to the condition of the patient [8]. The present study was undertaken to determine whether *Ganoderma lucidum* pharmacopuncture (GLP), which is actually used in clinics, protects against acute gastric ulcers in rats.

2. Materials and Methods

Ganoderma lucidum caps, 500 g, grown in South Korea were washed thoroughly with distilled water, cut into pieces, and submerged into 4 L of 25% alcohol for 10 hours at room temperature to be extracted. The alcohol extract was condensed to 500 mL by using a rotary evaporator, and impurities were removed by using a 0.22- μ m filter. The extract was sterilized and then stored at a temperature of 20°C. The extract was dissolved in ethanol before administration and diluted with 5% DW (dextrose water, JW Pharmaceutical) to keep the final concentration of *Ganoderma lucidum* at 10%.

Adult male Sprague-Dawley (SD) rats (weighing 220 – 240 g, 15-weeks old and housed five rats per cage) were purchased from SAM-Taco Co. They were provided with standard food and water ad libitum and were maintained in an animal house at a controlled temperature (22 \pm 2°C) with a 12-hours light/dark cycle. The rats were divided randomly into 4 groups of 8 rats each: normal, control, normal

saline (NP) and GLP groups. The study was approved by the Ethics Committee for Animal Experimentation, Dong-Eui University.

The rats were fasted for 24 hours, but were allowed free access to drinking water until 2 hours before the experiment. A gastric injury model based upon a modification of the method described by Mizui and Doteuchi [9] was induced by using an acidified ethanol solution (150 mM HCl/absolute ethanol) 40 : 60 v/v, (HCl/ethanol solution).

The normal group was orally administered saline. The control, NP and GLP groups were orally administered EtOH/HCl (5 mL/kg). Immediately after EtOH/HCl administration, the NP and the GLP group were treated once with injections of saline and GLP, respectively. Two local acupoints were used: CV12 (中脘), which belongs to the Controlling Vessel, and ST36 (足三里), which belongs to the Stomach Meridian. A pharmacopuncture needle (29 gauge \times 8 mm, 1 mL, disposal, insulin-injection syringe from HWAJIN Co. Busan, Korea) was used, and the amount of injection was 1 mL for each animal. One hour after this injection, the rats were sacrificed, and their stomachs were immediately excised.

The reactivity, the activity and the death of rats in each group were observed during the experiment. The animals were killed by cervical dislocation after administration of an overdose of ether at the end of the 2 week experiment. The abdomen was opened immediately. The whole stomach was cut and removed 1.5 cm away from the cardia and the pylorus. Dissection was done along the greater curvature for general specimen observation. The obviously-damaged gastric-mucosa specimen was rinsed in cold saline solution. Then, the specimen was placed in formaldehyde and glutaraldehyde, and stored in a liquid nitrogen solution for later observation.

The length and the width of the injured gastric mucosa region were measured with a vernier caliper. The gastric mucosa ulcer index (UI) was determined according to the Guth standard [10]: spot erosion was recorded as 1 point, erosion lengths < 1 mm were recorded as 2 points, erosion lengths from 1 to 2 mm were recorded as 3 points, those from 2 to 3 mm were recorded as 4 points, and those > 3 mm were recorded as 5 points; the score was doubled if the erosion width was > 1 mm.

Ulcers of the gastric mucosa appear as elongated bands of hemorrhagic lesions parallel to the long axis of the stomach. The gastric mucosa of each rat was examined in order to estimate damage. The length and the width of the ulcer (mm) were measured. The inhibition percentage (%) was calculated by using the following formula [11]:

$$\text{The inhibition percentage (\%)} = \frac{UA \text{ of Control} - UA \text{ of treated}}{UA \text{ of Control}} \times 100$$

Where UA = ulcer area

To investigate whether GLP affects the activity of radical scavenging enzymes, was measured the activity of superoxide dismutase (SOD) in the gastric mucosa to the method of McCord and Fridovich [12]. The standard assay was performed in 3 mL of 50 mM potassium phosphate buffer at pH 7.8 containing 0.1-mM EDTA in a cuvette thermostated at 25 °C. The reaction mixture contained 0.1-mM ferricytochrome c, 0.1-mM xanthine, and sufficient xanthine oxidase to produce a reduction rate of ferricytochrome c at 550 nm of 0.025 absorbance units per minutes. Tissue homogenate was mixed with the reaction mixture. A kinetic spectrophotometric analysis was started with the addition of xanthine oxidase at 550 nm. Under these conditions, the amount of SOD required to inhibit the reduction rate of cytochrome c by 50% was defined as 1 unit of activity. The results were expressed as units/mg of protein.

The opened stomachs were preserved in 10% buffered formalin overnight after they had been cut into small pieces. The tissues underwent automated tissue processing the next day. Next, the biopsy samples were embedded in paraffin wax, sectioned into 5- μ m thicknesses by using a microtome, and then stained with hematoxylin and eosin (H&E). The tissue sections were assessed for histopathological changes such as congestion, edema, hemorrhage and necrosis by using a light microscope.

Tissue section slides were heated at 60 °C for approximately 25 minutes in a hot oven. The tissue sections were deparaffinized in xylene and rehydrated with graded alcohol. An antigen retrieval process was performed in a 10 mM sodium citrate buffer. Immunohistochemical staining was conducted according to the manufacturer's protocol (Dakocytomation, USA). Briefly, endogenous peroxidase was blocked by using a peroxidase block (0.03% hydrogen peroxide containing sodium azide) for 5 minutes. Tissue sections were washed gently with a wash buffer and then incubated with Bcl-2-associated X protein (BAX) (1 : 200), Bcl-2 (1 : 200), and transforming growth factor- β 1 (TGF- β 1) (1 : 100) biotinylated primary antibodies for 15 minutes. The sections were rinsed gently with the wash buffer and placed in a buffer bath. The slides were then placed in a humidified chamber and incubated for 15 minutes. Then, the tissue sections were rinsed gently in the wash buffer and placed in buffer bath. Following washing and counterstaining with hematoxylin for 5 seconds, we added diaminobenzidine substrate – chromagen to the tissue sections and incubated them further for 5 minutes.

All photomicrographs were obtained using Infinity Capture imaging software (ver. 3, Lumenera, Canada) at 100 \times magnification. For statistical analysis, the densities of immunopositive cells were compared. The total immunopositive cells per field ($10^5 \mu\text{m}^2$) were counted in at least 15 randomly-chosen fields at 100 \times magnification.

All values are reported as means \pm standard errors (S.Es). The statistical significance of differences among groups were assessed with the one-way ANOVA (post-hoc analysis). A value of $P < 0.05$ was considered significant. SPSS 18.0 for Windows (SPSS Co., Chicago, IL, USA) was used for the statistical analyses.

3. Results

The areas of gastric ulcer formation were reduced in the GLP group compared with control group (Fig. 1). The GLP significantly ($P < 0.05$) suppressed the formation of the ulcers and the effects in NP group were not significant compared with GLP group (Fig. 2).

Histological observation of gastric lesions in control group showed comparatively extensive damage of the gastric mucosa, and necrotic lesions penetrated deeply into the mucosa. Also, extensive edema and leukocytes infiltrations of the submucosal layer appeared microscopically (Fig. 3B). The effect of NP was not significant (Fig. 3C). However, GLP group had good protection for the gastric mucosa from reduction or absence of ulcer area, submucosal edema, and leukocytes infiltration (Fig. 3D).

Ethanol was able to reduce SOD activity as shown in the control group compared with normal group. The treatment of GLP increased significantly ($P < 0.05$) the enzyme activity of SOD (Fig. 4).

BAX protein in the animals treated with EtOH/HCl was up-regulated. In contrast, the expression of BAX protein in the GLP group was down-regulated significantly ($P < 0.05$) (Fig. 5, 7). Bcl-2 protein in the control group underwent down-regulation compared with normal groups, but the treatment with GLP caused up-regulation of Bcl-2 protein (Fig. 6, 7).

TGF- β 1 immunoreactivities was weakly observed in normal group, control group showed slight increase compared with normal group. However, TGF- β 1 activities of NP and GLP group were dynamically increased. GLP group was more significant ($P < 0.05$) than Control group (Fig. 8, 9).

4. Discussion

Gastritis is an inflammation, irritation, or erosion that occurs when the endogenous defensive mechanisms of the mucosal barrier cannot properly protect the organ [13]. Usually, exposure to excess acid and pepsin causes insult to the gastrointestinal wall. Several factors, including Helicobacter pylori infection, increase the incidence of gastric ulcer disease. Some medications such as NSAIDs, potassium, and iron supplements, have been reported to have

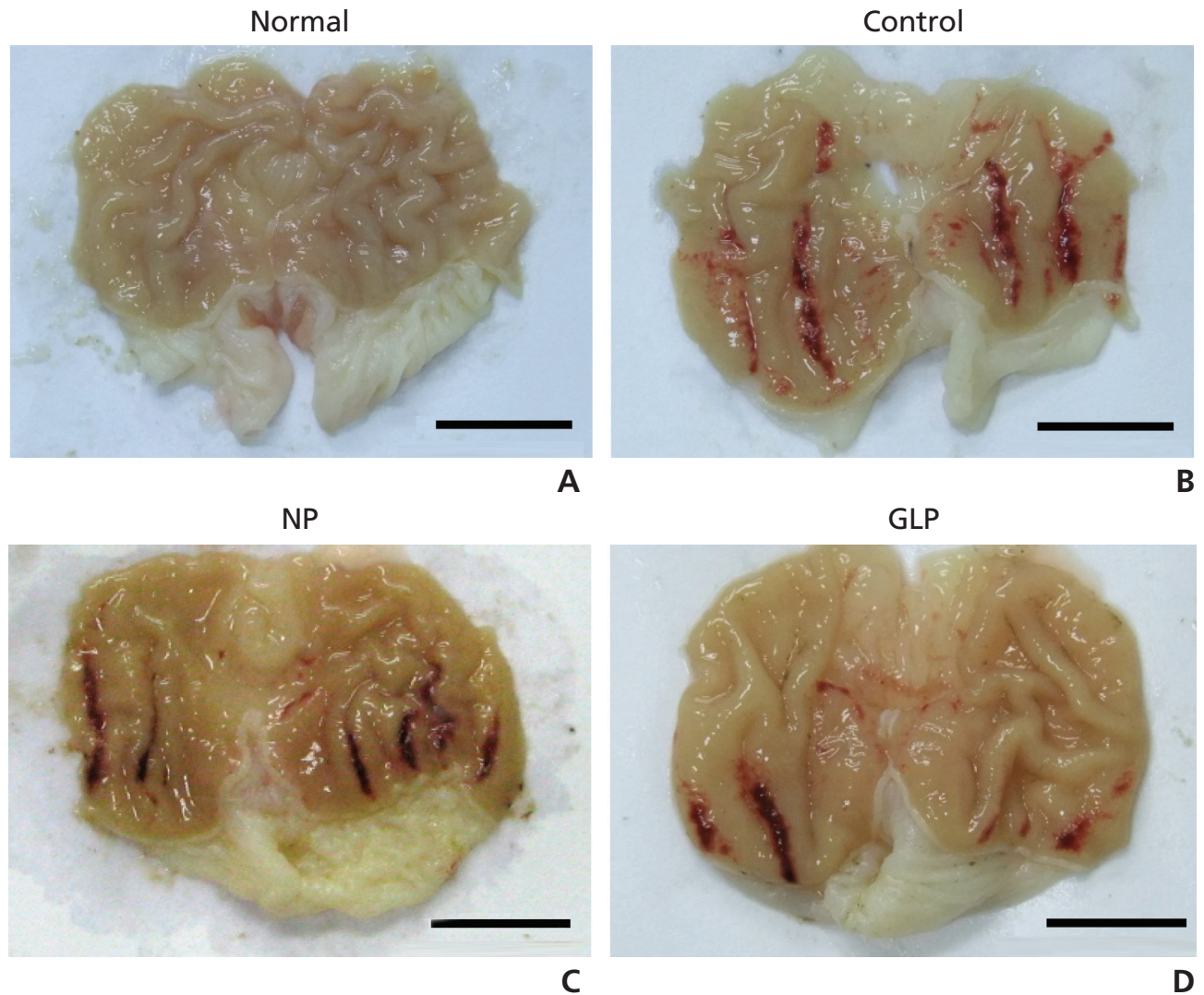


Figure 1 Gross appearance of an EtOH/HCl-induced acute gastric ulcer in a rat. Scale bar = 1 cm; NP, injection of saline; GLP, injection of *Ganoderma lucidum* pharmacopuncture.

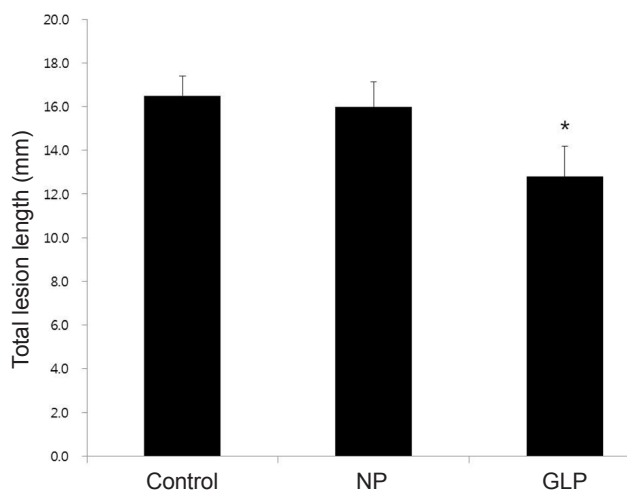


Figure 2 Total length of the lesion induced by using an EtOH/HCl solution. Mean \pm S.E. * $P < 0.05$ vs. Control; NP, injection of saline; GLP, injection of *Ganoderma lucidum* pharmacopuncture.

the potential for increasing the risk gastric ulcer disease [14]. Generally, an imbalance among aggressive chemical agents versus protective substances, such as SOD, catalase, and glutathione peroxidase, causes gastric mucosal lesions [15]. In experimental animals, the oral administration of ethanol rapidly induces gastric mucosal lesions, and these ethanol-induced lesions are commonly used to study both the pathogenesis of and the therapy for human ulcerative disease [16]. In this study also, the oral administration of ethanol was used for gastric-ulcer induction. Consequently, acute ethanol administration caused damage to the gastric mucosa in the control group, including the general view and microscopic structures, compared with the normal group.

Pharmacopuncture is a new form of therapy derived from combinations of two traditional therapeutic methods, herbal medicine and acupuncture therapy [8]. In general,

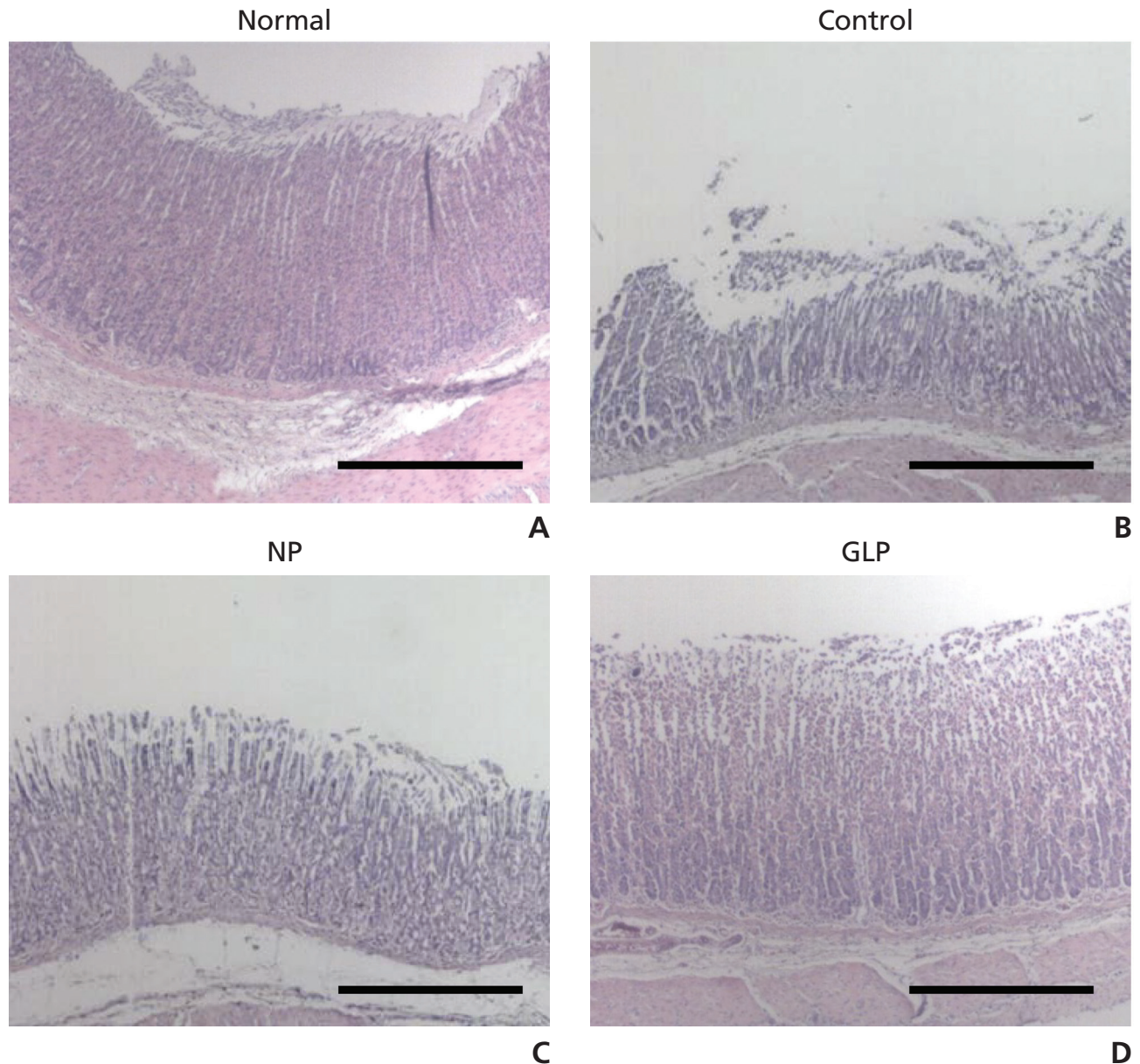


Figure 3 Light microscopic images of EtOH/HCl-induced acute ulcers in rats. Scale bar = 200 μ m. Magnification: 40 \times ; NP, injection of saline; GLP, injection of *Ganoderma lucidum* pharmacopuncture.

pharmacopuncture treatment is performed by injecting small amounts of extracted medicinal materials at acupoints or in affected areas in order to obtain the combined efficacies of acupuncture and herbs. Although the first primitive trials with bee venom were recorded in old medical books from the Han Dynasty of China, acupuncture with injection started in the early 1950s in China and was referred to a aqua-puncture. Recently, pharmacopuncture therapy in Korea has developed into quite a unique and systematic framework for the diagnosis and the treatment of various diseases [17].

Ganoderma lucidum is a well-known medicinal mush-

room, particularly in Eastern Asia. The pharmacological activities of *Ganoderma lucidum*, especially its intrinsic immunomodulating and anti-tumor properties, have been well documented [6]. Several studies have demonstrated that various extracts of *Ganoderma lucidum* interfere with cell-cycle progression, induce apoptosis, and act as anti-cancer agents by suppressing angiogenesis in human cancer cells [18]. However, the biological and the pharmacological effects of GLP are still poorly defined. In particular, there are no reports of the gastroprotective effect of GLP against ethanol-induced gastric mucosal lesions. Because ethanol-induced gastric mucosal lesions

are mediated by the generation of free radicals, we hypothesized that GLP might inhibit ethanol-induced gastric mucosal lesions through a protective effect that might directly involve its antioxidant property. Therefore, this study was designed to investigate the gastroprotective effect of GLP by measuring the amount of lipid peroxidation and by comparing the activities of the SOD enzyme.

In this study, gastric mucosal lesions in rats were induced by using EtOH/HCl for an acute injury model. Animals were treated with GLP or NP and were kept under observation for 1 hour, during which time they remained alive. Gastric lesions were evaluated macroscopically by using the depth of penetration into the gastric mucosal surface. The rats treated with the GLP had significantly reduced areas of gastric ulcer formation when compared with the

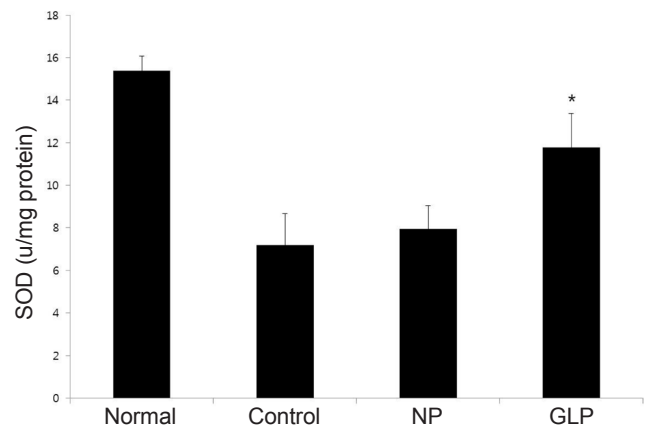


Figure 4 Changes of SOD activity after acute gastric injury induced by EtOH/HCl. Mean \pm S.E. * $P < 0.05$ vs. Control; NP, injection of saline; GLP, injection of *Ganoderma lucidum* pharmacopuncture.

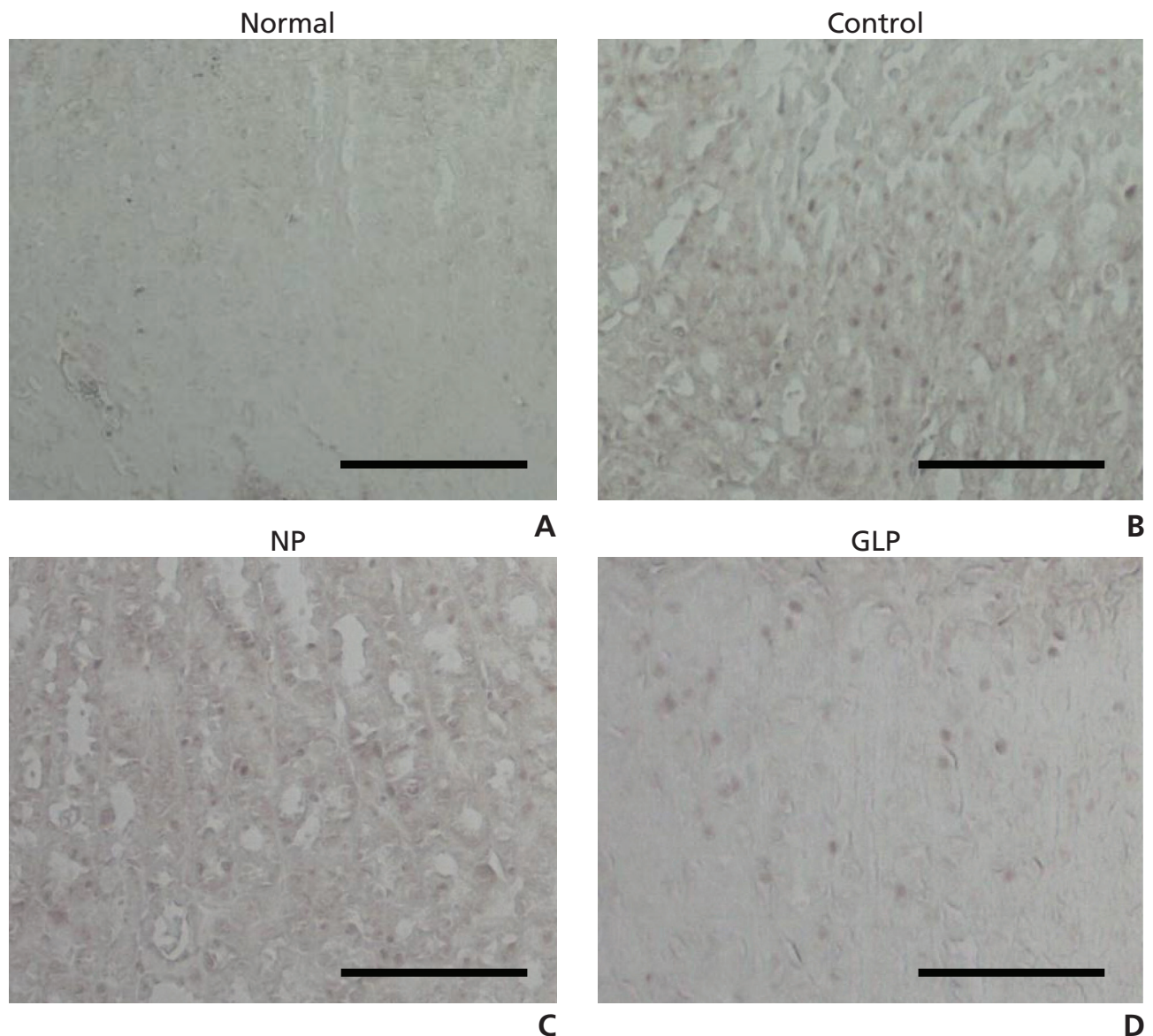


Figure 5 Immunohistochemical staining of BAX proteins in EtOH/HCl-induced acute gastric ulcers in rats. Scale bar = 100 μ m. Magnification: 100 \times ; NP, injection of saline; GLP, injection of *Ganoderma lucidum* pharmacopuncture.

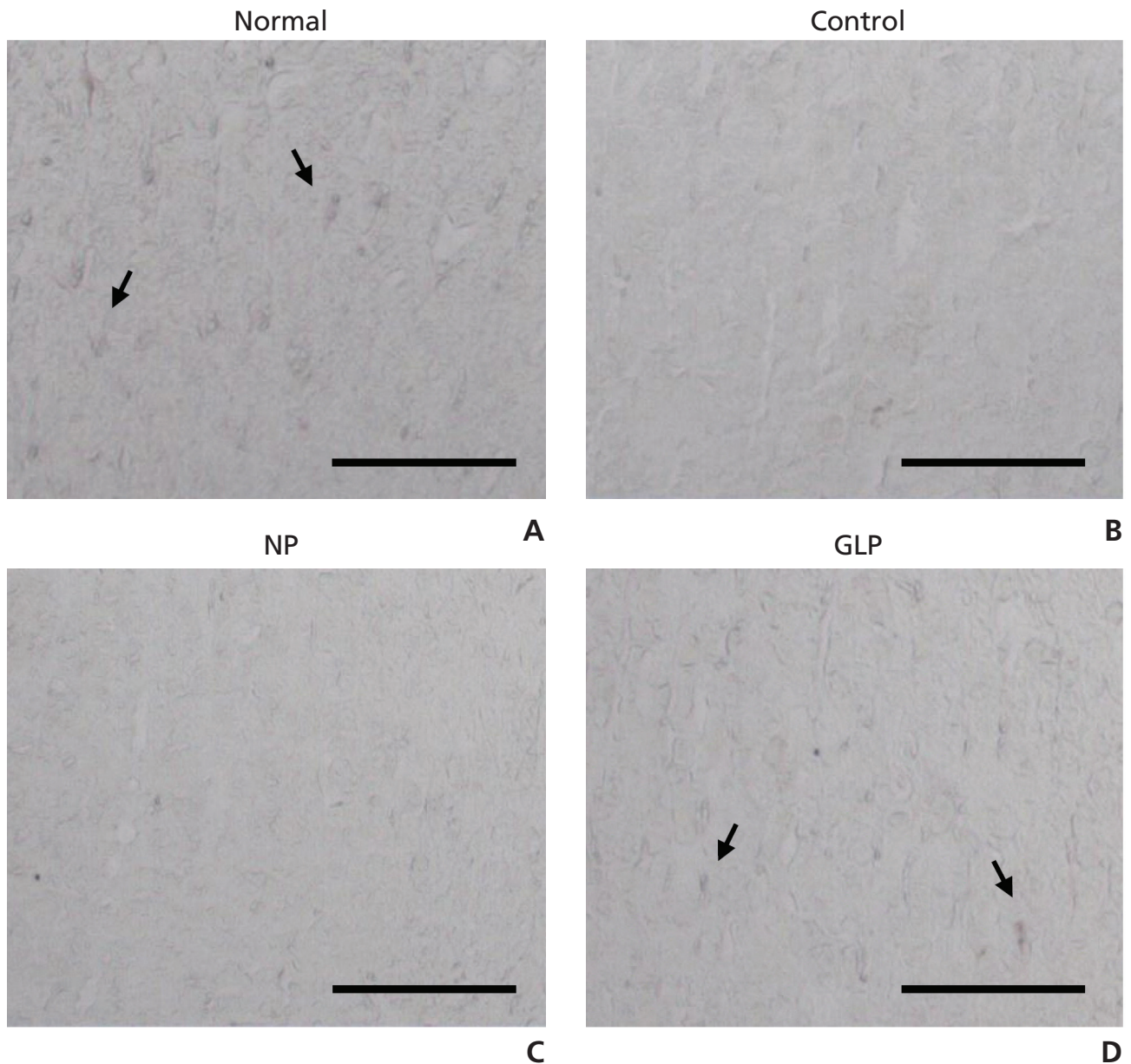


Figure 6 Immunohistochemical staining of Bcl-2 proteins in EtOH/HCl-induced acute gastric ulcers in rats. Scale bar = 100 μ m. Magnification: 100 \times ; NP, injection of saline; GLP, injection of *Ganoderma lucidum* pharmacopuncture.

ulcer control group (Fig. 1).

To confirm the effect of GLP against ethanol-induced acute gastric ulcers, gastric ulcer lesions were measured microscopically by using the clear depth of penetration into the gastric mucosal surface in all experimental groups except the normal group (Fig. 3). The length and the width of the ulcer were measured, and the inhibition percentage was calculated. We hypothesized that GLP could inhibit ethanol-induced gastric mucosal lesions, and that such protective effects might directly involve its antioxidant property. We investigated the effect of GLP on the activities of the radical scavenging enzymes, SOD, in the gastric

mucosa. As shown in Fig. 4, the control group had significantly reduced SOD activities in comparison to the normal group, but the activities of these enzymes were significantly increased in the GLP group compared to those in the control group.

In the present study, to investigate the role of GLP in mitochondrial regulation in ethanol-induced acute gastric ulcer, we carried out immunohistochemical analyses for BAX and Bcl-2. BAX immunoreactivities in the GLP group were down-regulated significantly compared with those in the control group, and Bcl-2 immunoreactivities in the GLP group were up-regulated compared with those in the

control group. The NP group also showed a reduced expression of BAX and an enhanced expression of Bcl-2, but the effects of NP were not significant (Fig. 6, 7). Therefore, GLP can be assumed to suppress apoptosis by regulating the mitochondrial-damage-mediated endogenous pathway, which might be one of the important mechanisms for preventing gastric ulcer disease.

Cells of the digestive tract are well known to have a rapid turnover rate, which makes the gastrointestinal mucosa one of the most rapidly-proliferating tissues in the body [19]. The gastrointestinal mucosa has a remarkable ability to repair damage. Both the intensive proliferation rate and the remarkable ability to repair damage are supported by the coordinated actions of a variety of growth factors, one of which is transforming growth factor- β 1 (TGF- β 1) [20]. In this study, to investigate the effects of GLP on the ability to repair gastric ulcer damage, we carried out an

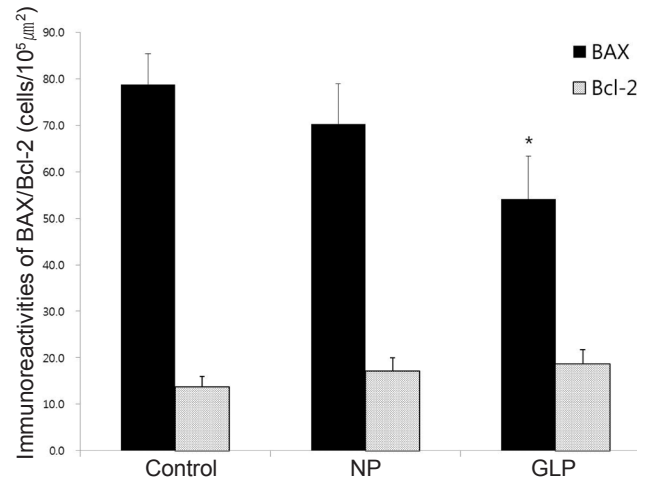


Figure 7 Immunoreactivities of BAX/Bcl-2 proteins in EtOH/HCl-induced acute gastric ulcers in rats. Mean \pm S.E. * $P < 0.05$ vs. Control; NP, injection of saline; GLP, injection of *Ganoderma lucidum* pharmacopuncture.

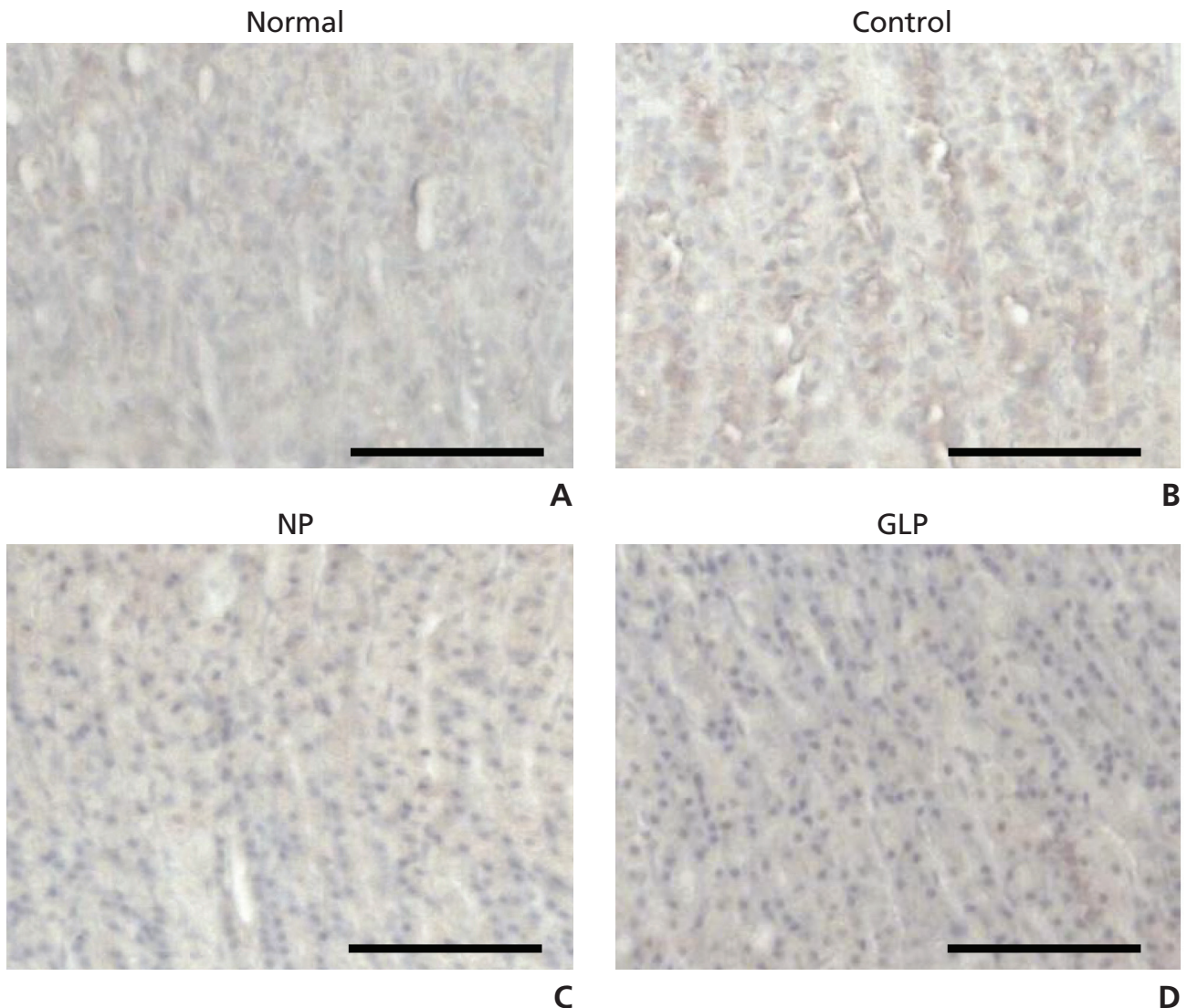


Figure 8 Immunohistochemical staining of TGF- β 1 proteins in EtOH/HCl-induced acute gastric ulcers in rats. Scale bar = 100 μ m. Magnification: 100 \times ; NP, injection of saline; GLP, injection of *Ganoderma lucidum* pharmacopuncture.

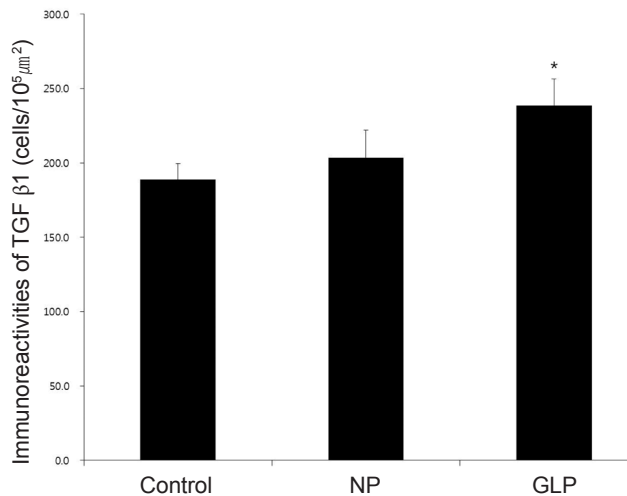


Figure 9 Immunoactivities of TGF-β1 proteins in EtOH/HCl-induced acute gastric ulcers in rats. Mean ± S.E. **P* < 0.05 vs. Control; NP, injection of saline; GLP, injection of *Ganoderma lucidum* pharmacopuncture.

immunohistochemical analysis of TGF-β1 in rats with ethanol-induced acute gastric ulcers (Fig. 8, 9). The level of TGF-β1 was significantly ameliorated in the control group, but it was significantly increased in the GLP group (Fig. 8, 9). This means that GLP stimulates TGF-β1 up-regulation, which induces angiogenesis through the induction of vascular endothelial growth factor to promote recovery in the injured mucosa of the gastric ulcer.

5. Conclusion

This study revealed that GLP can significantly protect the gastric mucosa against an ethanol-induced gastric ulcer. Such protection was shown by gross appearance, histology, and immunohistochemistry staining for BAX, Bcl-2 and TGF-β1.

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Conflict of interest

The authors declare that there are no conflict of interest.

References

1. Arakawa T, Watanabe T, Tanigawa T, Tominaga K, Fujiwara Y, Morimoto K. Quality of ulcer healing in gastrointestinal tract: its pathophysiology and clinical relevance. *World J Gastroenterol.* 2012;18(35):4811-22.
2. Vakil N. Treatment of helicobacter pylori infection. *Am J Ther.* 1998;5(3):197-201.
3. Spirt MJ. Stress-related mucosal disease: risk factors and prophylactic therapy. *Clin Ther.* 2004;26(2):197-213.
4. Monnig AA, Prittie JE. A review of stress-related mucosal disease. *J Vet Emerg Crit Care (San Antonio).* 2011;21(5):484-95.
5. Batra P, Sharma AK, Khajuria R. Probing lingzhi or reishi medicinal mushroom *ganoderma lucidum* (higher Basidiomycetes): a bitter mushroom with amazing health benefits. *Int J Med Mushrooms.* 2013;15(2):127-43.
6. Haniadka R, Popouri S, Palatty PL, Arora R, Baliga MS. Medicinal plants as antiemetics in the treatment of cancer: a review. *Integr Cancer Ther.* 2012;11(1):18-28.
7. Weng CJ, Yen GC. The in vitro and in vivo experimental evidences disclose the chemopreventive effects of *ganoderma lucidum* on cancer invasion and metastasis. *Clin Exp Metastasis.* 2010;27(5):361-9.
8. Kim J, Kang DI. A descriptive statistical approach to the korean pharmacopuncture therapy. *J Acupunct Meridian Stud.* 2010;3(3):141-9.
9. Mizui T, Doteuchi M. Effect of polyamines on acidified ethanol-induced gastric lesions in rats. *Jpn J Pharmacol.* 1983;33(5):939-45.
10. Owu DU, Nwokocha CR, Obembe AO, Essien AD, Ikpi DE, Osim EE. Effect of *gongronema latifolium* ethanol leaf extract on gastric acid secretion and cytoprotection in streptozotocin-induced diabetic rats. *West Indian Med J.* 2012;61(9):853-60.
11. Arumugam S, Selvaraj SV, Velayutham S, Natesan SK, Palaniswamy K. Evaluation of anti-ulcer activity of *samanea saman* (Jacq) merr bark on ethanol and stress induced gastric lesions in albino rats. *Indian J Pharmacol.* 2011;43(5):586-90.
12. McCord JM, Fridovich I. The biology and pathology of oxygen radicals. *Ann Intern Med.* 1978;89(1):122-7.
13. Anand BS, Graham DY. Ulcer and gastritis. *Endoscopy.* 1999;31(2):215-25.
14. Pezzi JS, Shiao YF. Helicobacter pylori and gastrointestinal disease. *Am Fam Physician.* 1995;52(6):1717-24.
15. Bandyopadhyay D, Biswas K, Bhattacharyya M, Reiter RJ, Banerjee RK. Gastric toxicity and mucosal ulceration induced by oxygen-derived reactive species: protection by melatonin. *Curr Mol Med.* 2001;1(4):501-13.
16. Wilson DE. Role of prostaglandins in gastroduodenal mucosal protection. *J Clin Gastroenterol.* 1991;13:S65-

71.

17. Yin C, Park HJ, Chae Y, Ha E, Park HK, Lee HS, *et al.* Korean acupuncture: the individualized and practical acupuncture. *Neurol Res.* 2007;29:S10-5.
18. Boh B, Berovic M, Zhang J, Zhi-Bin L. *Ganoderma lucidum* and its pharmaceutically active compounds. *Biotechnol Annu Rev.* 2007;13:265-301.
19. Konturek PC, Konturek SJ, Brzozowski T, Ernst H. Epidermal growth factor and transforming growth factor-alpha: role in protection and healing of gastric mucosal lesions. *Eur J Gastroenterol Hepatol.* 1995;7(10):933-7.
20. Milani S, Calabrò A. Role of growth factors and their receptors in gastric ulcer healing. *Microsc Res Tech.* 2001;53(5):360-71.