Original Article
Glycyrrhiza polysaccharide protects GES-1 cells from ethyl alcohol induced injury through Akt-Mdm2-p53 signal pathway

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Abstract: Ethyl alcohol (EtOH) induced gastric mucosal damages are frequently occurring and refractory disease clinically, including gastric ulcer, gastritis, stressful gastric mucosa lesion, etc. With cytotoxic effect, EtOH in high concentration can induce oxidative stress and attach essential components of cell, resulting in increasing apoptosis and necrosis. *GlycyrrhizauralensisFisch* has been known as "the king of herbs" due to its widely medicinal and officinal properties for thousands of years. *Glycyrrhiza* polysaccharide (GPS), one of the main active ingredients of glycyrrhiza is attributed to many healing properties of the herb. The study applied GES-1 cells as model cell to investigate the effects of GPS pretreatment on EtOH induced injury. Cells were divided into control, EtOH (0.8 M), GPS1 (100 μg/mL) + EtOH and GPS2 (400 μg/mL) + EtOH group. The influences of GPS pretreatment on cell viability in EtOH induced injury were detected through RTCA and MTT assay. Apoptosis in different experimental conditions was analyzed by FCM. Expression levels of apoptosis related genes Bax and Bcl-2 were detected by real-time quantitive PCR (RT-qPCR) and Western blot. Activation status of Akt-Mdm2-p53 pathway was observed via western blot. The study discovered the protective effect of GPS pretreatment on EtOH induced damage to GES-1 cells including improving cell viability and proliferation and reducing apoptosis rate, and we proved that inhibition of Akt phosphorylation substantially decreased the effect of GPS on apoptosis. GPS triggered the repair processed of GES-1 cells in EtOH induced injury through regulating the expression Akt-Mdm2-p53 pathway.

Keywords: *Glycyrrhiza* polysaccharide, ethyl alcohol, GES-1 cells, Bax, Bcl-2, Akt-Mdm2-p53 pathway

Introduction

Alcoholic gastric mucosal damages, such as gastric ulcer and gastrorrhagia, are mainly caused by heavy drinking, clinically appearing as abdominal pain, emesis, heartburn, etc. Though gastroscopy, universal congestion, edema, erosion and hemorrhage are usually observed in gastric mucosa of alcoholic gastric mucosal patients [1-3]. Rangaraj et al previously found that when Wistar rats were given by gavage with 40% EtOH, exfoliation of their superficial gastric mucosa and degeneration as well as necrosis of their upper half of gland in gastric fundus would be showed up after 30 min. Copious quantities of alcohol affects synthesis of oligosaccharide in mucoprotein, and induces oxygen radicals to cause mucosa lipid peroxidation, resulting in apoptosis of gastric mucosa cells or further caricinogenesis [4].

Apoptosis, also named programmed cell death, is a protective cell-suicide process regulated by gene coding through inducing intrinsic death program of organisms [5-7]. However, EtOH induced apoptosis of gastric mucosa cells, apart from direct chemical burns, is considered as one of the primary physiopathologic mechanisms of gastric mucosal injury [2, 3]. Several lines of evidence proved the important role of Akt-Mdm2-p53 in regulation of cell apoptosis and proliferation. Akt, a protein kinase of serine/threonine related to several signal pathways, is able to promote cell cycle progress, cell proliferation and cell survival [8, 9]. p53, a cancer suppressor gene, can promote apoptosis

and retard cell growth [10]. Mdm2 is an oncoprotein which can combine with p53 to inhibit its function [11]. The interaction effect of Akt and p53 plays a significant part to determine cell survival or death [12-14].

GPS, one of main active ingredients isolated from a characteristic Chinese medicine *glycyrrhizauralensis*Fisch (*G. uralensis*), is a water-soluble polysaccharide with gycosidic units which composed of alpha (1-4) linked D-glucana [15, 16]. According to previous studies, with strong immune and antioxidant activities, GPS was widely applied in anti-tumor, immunoregulation, anti-aging, etc. and suitable for people of all generations [17-19]. The study applied GES-1 cells as model cell to investigate the effects of GPS pretreatment on EtOH induced injury, and try to prove the essential role of Akt-Mdm2-p53 pathway in the research.

Materials and methods

Cell culture

GES-1 cells were obtained from Genome ditech Co. Ltd., Shanghai, China), and cultured in Dulbecco's modified eagle medium (DMEM) containing 10% Fetal Bovine Serum (FBS). Cells were then incubated in 5% CO_2 incubator at 37°C overnight.

RTCA

Cells were seeded into 16-well E-plates and put in detector for collecting parameters of medium. Cells in logarithmic phase were collected, seeded into 16-well E-plates and then treated with respectively 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M EtOH (Shenzhen Hongsheng Chemical Technology Co. Ltd., Guangdong, China) and 100 μg/mL and 400 μg/mL GPS (GanzhouHuahan Biotechnology Co. Ldt., Jiangxi, China) to compare with a normal control group. Cell index (CI) in each group at time point of 2 h, 6 h and 12 h was respectively detected.

Cell grouping and treatment

Cells were randomly divided into four groups: control group, EtOH model group, GPS1 + EtOH group and GPS2 + EtOH group. 0.8 M EtOH was added into EtOH model group to construct injury model in the study. Cells in GPS1 + EtOH

group and GPS2 + EtOH group were pretreated with respective 100 μg/mL and 400 μg/mL GPS for 6 h, then were treated with 0.8 M EtOH for 12 h.

CCK-8 assay

Cell viability of SH-CY5Y cells in each group was detected through using CCK8 kit (Shanghai Beyotime Biotechnology Co. Ltd., Shanghai, China). Cells were grouped, and seeded into 96-well plates at amount of 100 μL per well, and then incubated at 37°C in 5% CO_2 incubator for 4 h. After added with 10 μL CCK reagent to each well, cells were putted into 5% CO_2 incubator at 37°C for 1-4 h. Optical density (OD) value of each group was observed at 450 nm by a spectrophotometer (Sigma-Aldrich Co. LLC., USA).

FCM

Cells in logarithmic phase were collected and seeded into 6-well plates. Cells were digested by EDTA free trypsin (Shanghai Beyotime Biotechnology Co. Ltd., Shanghai, China), stained with Annexin V-FITC and propidiumiodide (Shanghai BestBio Science Co. Ltd., Shanghai, China), and incubated in dark place for 15 min at room temperature afterwards. Apoptosis rate of each group was detected by EPICS XL-MCL FCM (Beckman Coulter, Inc., USA) with excitation wavelength 488 nm and emission wavelength 530 nm.

RT-qPCR

Expression levels of Bax, Bcl-2, PCNA, DNMT1, DNMT3a, DNMT3b and MeCP2 mRNA were detected by means of RT-PCR. Cells were seeded into 6-well plates at a density of 2×10^7 cells/well, divided into 3 groups: control, Lido, and Lido + SB203580 group. Total RNA was extracted with Trizol (Thermo Fisher Scientific Inc., New York, USA) according to the manufacture's instruction. Concentration of extracted RNA was read through a UV spectrophotometer (Thermo Fisher Scientific Inc., New York, USA). cDNA was synthesized by reverse transcription. Reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) was applied as the internal control to monitor the efficiency of RT-PCR. All primers in this study were designed by Shanghai Sangon Biotech Co. Ltd. (Shanghai, China).
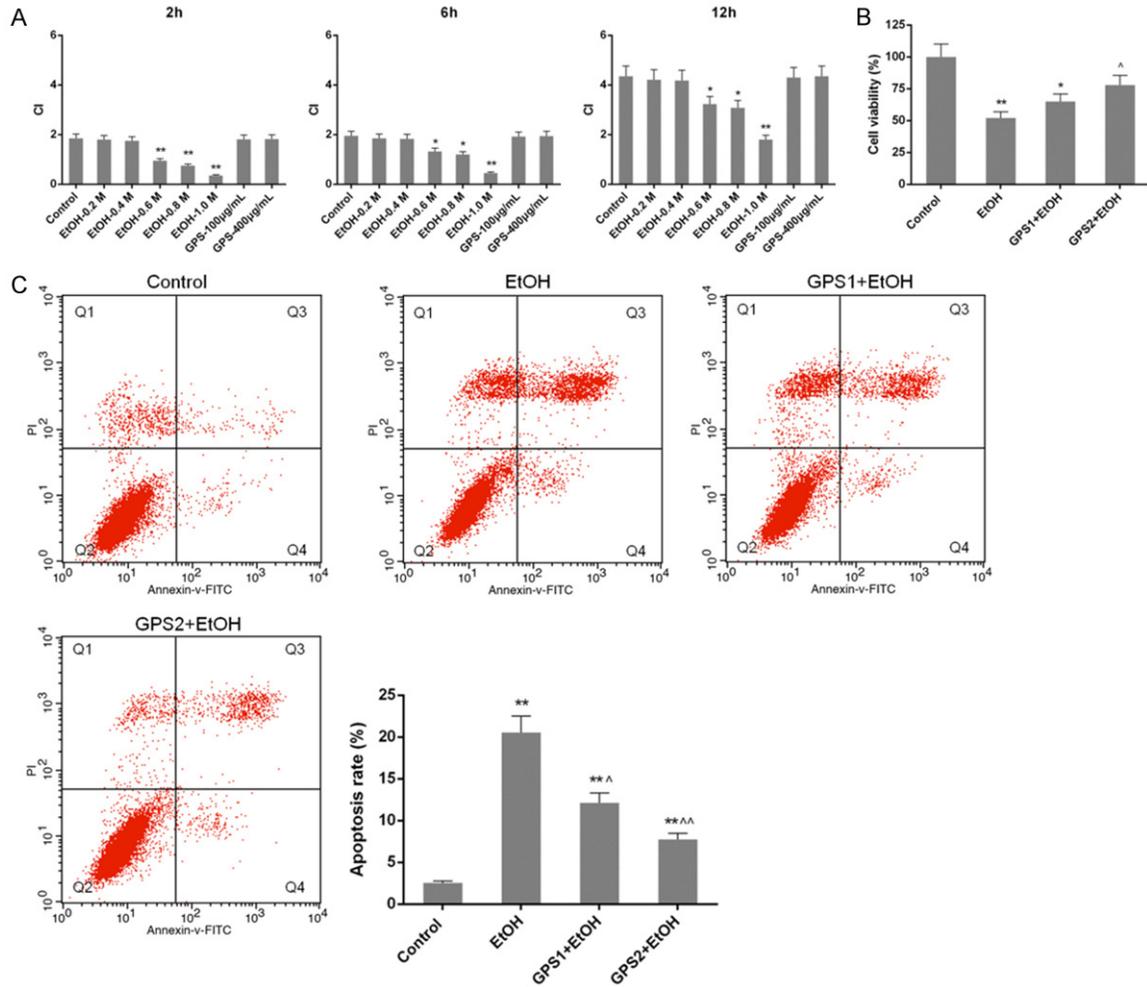
Figure 1. Observation of changes in cell proliferation and apoptosis under the different treatments of EtOH (0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M) and GPS (100 µg/mL and 400 µg/mL). A: High concentration of EtOH (0.6 M, 0.8 M and 1.0 M) apparently reduced viability of GES-1 cells. B: GPS pretreatment improved cell viability in EtOH (0.8 M) induced damages to GES-1 cells. C: GPS pretreatment reduced apoptosis rate in EtOH (0.8 M) induced damages to GES-1 cells. Data were presented as mean ± SD, n=3, $^*P<0.05$ and $^{**}P<0.01$ vs. control group, $^#P<0.05$ and $^{##}P<0.01$ vs. EtOH model group (0.8 M).

The specific primer sequences for each gene were listed as the follows: 5' GCCAGCAAACT-GGTGCTCAA 3' and 5' CCAACCACCCTGGTCTT-GGA 3' for Bax (product: 126 bp); 5' CACTGG-CCAGGGTCAGAGTT 3' and 5' TGGCCATAGACC-CTGTCAGC 3' for Bcl-2 (product: 85 bp) and 5' CGGGAAACTGTGGCGTGATG 3' and 5' ATGACC-TTGCCCACAGCCTT 3' for GAPDH (product: 87 bp). Each reaction was run in triplicate.

Western blot

Cells were seeded in 6-well plates at a density of 2×10⁷ cells/well, grouped into control, Lido, and Lido + SB203580 group. Cells were harvested and washed twice with PBS, protein

lysed in ice-cold radio immunoprecipitation assay buffer (Whiga Technology Co. Ltd., Guangdong, China) with freshly mixed 0.01% protease inhibitor phenylmethanesulfonyl fluoride (Beijing O'BioLab Technology Co. Ltd., Beijing, China), then incubated for 30 min on ice. Cell lysis was centrifuged at 10,000×g for 5 min at 4°C, collected supernatants containing 20-30 µg of protein were run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and electrophoretically transferred to a nitrocellulose membrane (Merk Millipore, Germany). Protein expression level of Bax, Bcl-2, PCNA, p-p38MARK, p38MARK, DNMT1, DNMT3a, DNMT3b and MeCP2 were detected.
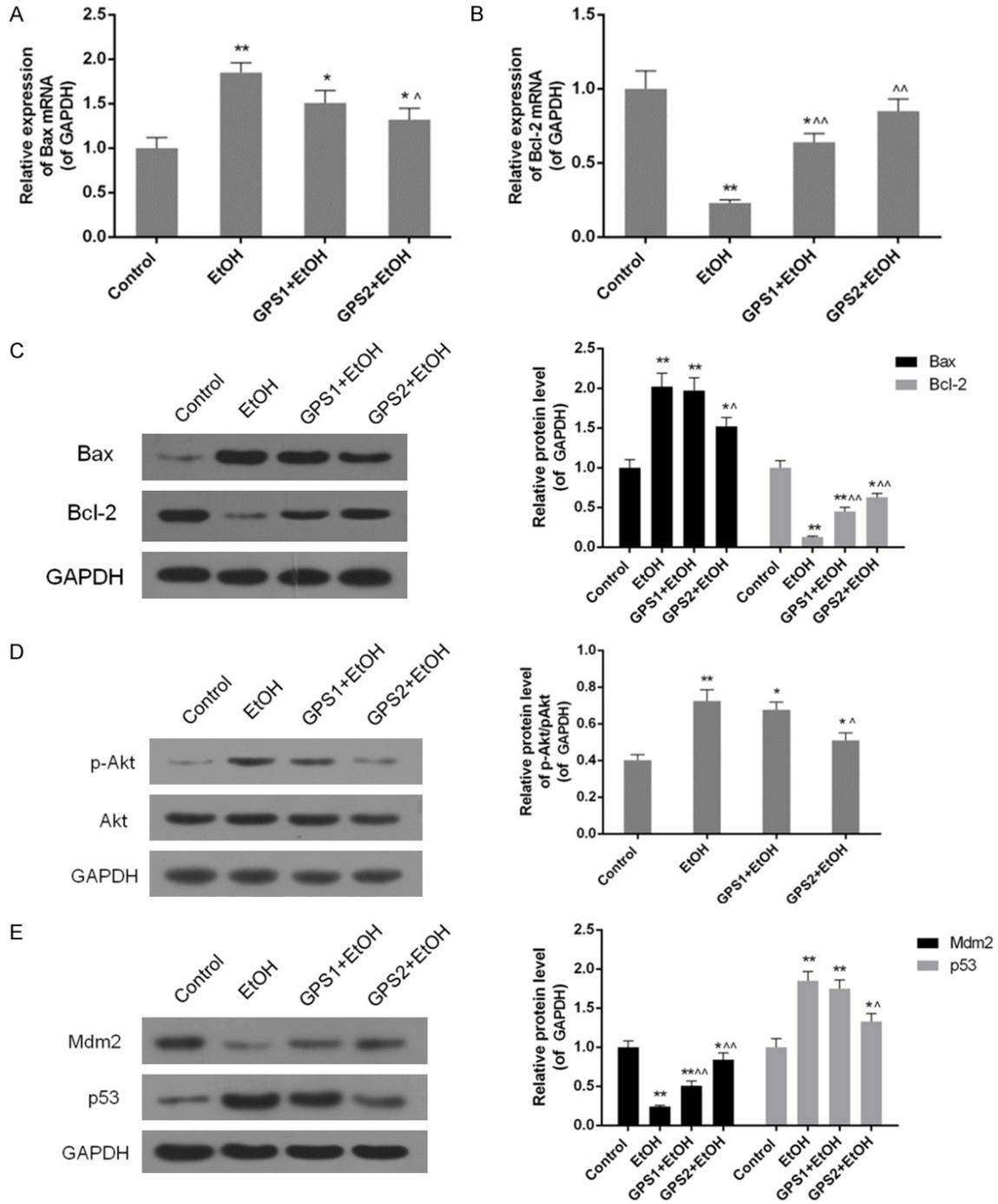
Figure 2. Expression levels of Bax, Bcl-2 and Akt-Mdm2-p53 pathway in control, EtOH (0.8 M), GPS1 (100 μg/mL) + EtOH (0.8 M), and GPS2 (400 μg/mL) + EtOH (0.8 M) group. A: GPS pretreatment down-regulated the expression of Bax mRNA in EtOH induced damages to GES-1 cells. B: GPS pretreatment up-regulated the expression of Bcl-2 mRNA in EtOH induced damages to GES-1 cells. C: GPS pretreatment decreased protein level of Bax and increased that of Bcl-2 in EtOH induced damages to GES-1 cells. D: GPS pretreatment inhibited phosphorylation of Akt in EtOH induced damages to GES-1 cells. E: GPS pretreatment increased protein level of Mdm2 and decreased that of p53 in EtOH induced damages to GES-1 cells. Data were presented as mean ± SD, n=3, $^*P<0.05$ and $^{**}P<0.01$ vs. control group, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. EtOH model group (0.8 M).

β-actin monoclonal antibody was used to estimate protein loading. Blots were visualized via an enhanced chemiluminescence (Thermo Fisher Scientific Inc., NY, USA).

Statistical analysis

Statistical analyses were performed using SPSS software, version 22.0 (SPSS, Inc., Chicago, IL, USA). Each experiment was repeated three times, with all the data presented as mean ± standard deviation. Differences among groups were evaluated through variance analysis and student's t-test. Statistical significance was defined as $P<0.05$, significant statistical difference was defined as $P<0.01$.

Results

EtOH in high concentration inhibited cell proliferation

The effects of EtOH and GPS on cell viability of GES-1 cells were respectively detected by RTCA. Compared to the control group, cell proliferation was markedly decreased when cells were treated with EtOH in high concentration (0.6 M, 0.8 M and 1.0 M) ($P<0.05$ or $P<0.01$). 100 μg/mL and 400 μg/mL of GPS and weak concentration of EtOH (0.2 M and 0.4 M) were found no apparent influence on GES-1 cells (**Figure 1A**). 0.8 M of EtOH was selected to construct EtOH injury model in the study.

GPS pretreatment improved cell viability in EtOH induced injury

MTT assay observed that incubation under the condition of 0.8 M EtOH for 12 h obviously inhibited cell proliferation of GES-1 cells in comparison with normal cells ($P<0.01$). With pretreatment of 100 μg/mL and 400 μg/mL GPS, cell viability was substantially enhanced. Particularly, in GPS2 + EtOH group, cell viability was increased to 78.52 ± 7.51% from 52.10 ± 4.95% in EtOH model group ($P<0.05$) (**Figure 1B**).

GPS pretreatment reduced apoptosis rate in EtOH induced injury

By the means of FCM, we detected distinct increase of apoptosis rate from 2.54 ± 0.24% in control group to 20.56 ± 1.95% in EtOH group ($P<0.01$). GPS pretreatment not only improved cell proliferation, was also found to mitigate the increase of apoptosis rate induced by EtOH ($P<0.05$ or $P<0.01$). In GPS1 + EtOH and GPS2 + EtOH group, apoptosis rates were respectively reduced to 12.12 + 1.20% and 7.73 + 0.74% (**Figure 1C**).

Effects of GPS pretreatment on expression of apoptosis related genes in EtOH induced injury

mRNA and protein levels of Bax and Bcl-2 were investigated respectively through RT-qPCR and western blot. In EtOH model group, the expression of pro-apoptotic gene Bax was significantly up-regulated while the expression of anti-apoptotic gene Bcl-2 was obviously down-regulated in comparison with normal GES-1 cells ($P<0.01$). Under the condition of pretreatment with GPS, gene products of Bax were decreasingly expressed when those of Bcl-2 were increasingly expressed in EtOH induced injury. The effect of GPS was in a concentration-dependent manner. Except Bax in GPS1 + EtOH group, there were significant differences in expression of Bax and Bcl-2 between GPS pretreated groups and EtOH model group ($P<0.05$ or $P<0.01$) (**Figure 2A-C**).

Effects of GPS pretreatment on expression of Akt-Mdm2-p53 related proteins in EtOH induced injury

Protein levels of p-Akt, Akt, Mdm2 and p53 in control, EtOH, GPS1 + EtOH and GPS2 + EtOH group were measured through western blot. Incubation with 0.8 M EtOH for 12 h was detected to promote phosphorylation of Akt, resulting in remarkable increase of p-Akt/Akt level in EtOH group compared to normal cells ($P<0.01$). In addition, EtOH obviously decreased the expression of Mdm2 but increased p53 level ($P<0.01$). In the study, we found GPS pretreatment evidently mitigate the changes by EtOH in expression of these proteins induced by EtOH. With GPS pretreatment, the ratio of P-Akt and Akt was decreased, Mdm2 was up-regulated whereas p53 was down-regulated. The effects were in a concentration-dependent manner. The differences between GPS pretreated cells and EtOH model cells were significant ($P<0.05$ or $P<0.01$) (**Figure 2D**, **2E**).

Inhibition of Akt phosphorylation by LY294002 weakened the protective effect of GPS pretreatment on cell viablity and apoptosis in EtOH induced injury

Cell viability and apoptosis were detected when Akt-Mdm2-p53 pathway was suppressed by LY294002 in EtOH-injured cells. CCK-8 assay, FCM, RT-qPCR and Western blot were prepared for the study. As a result, obvious reduction of
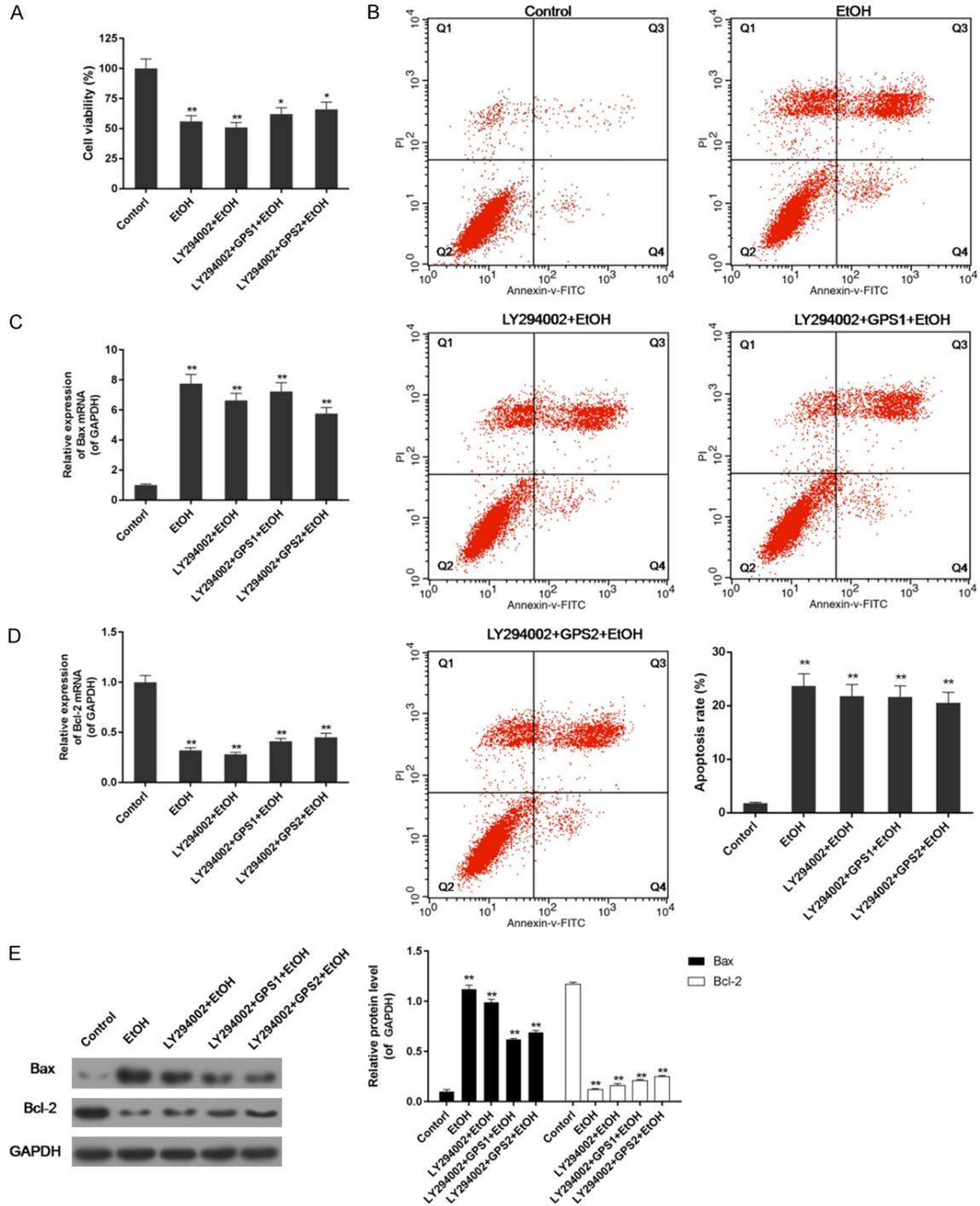
Figure 3. Influence of inhibition of Akt-Mdm2-p53 pathway on cell viability and apoptosis. A: Inhibition of Akt-Mdm2-p53 pathway suppressed the promoting effect of GPS pretreatment on viability of EtOH-injured GES-1 cells. B: Inhibiting Akt-Mdm2-p53 by LY294002 suppressed the protective effect of GPS pretreatment in EtOH induced damages to GES-1 cells. C, D: Inhibition of Akt-Mdm2-p53 pathway narrowed the differences in Bax and Bcl-2 mRNA expression between EtOH group and GSP treated + EtOH groups. E: Inhibition of Akt-Mdm2-p53 pathway affected the effect of GSP pretreatment on Bax and Bcl-2 protein levels. Data were presented as mean ± SD, n=3, $^{*}P<0.05$ and $^{**}P<0.01$ vs. control group, $^{\#}P<0.05$ vs. EtOH model group (0.8 M).

viability and increase of apoptosis rate were observed in 0.8 M EtOH treated cells ($P<0.01$).

With inhibition of Akt phosphorylation by in LY294002 + GPS1 + EtOH group and LY294002

+ GPS2 + EtOH group, cell viability and apopto-sis rate were both slightly reduced and were both not significantly different in comparison with EtOH model group (P>0.05) (**Figure 3A**, **3B**). Furthermore, inhibition of Akt phosphoryla-tion affected the regulatory effects of GSP pre-treatment on Bax and Bcl-2 expression, which proved the important role of the Akt-Mdm2-p53 pathway in the study.

Discussion

Damages of EtOH to gastric mucosa involve in multi-aspects such as direct injury to gastric mucosa cells, damage to protective mucous layer, disruption of gastric acid secretion, etc. [1-3]. At present, most of researches construct-ed animal injury model of gastric mucosa burn by direct gavage with absolute EtOH to investi-gate damage effects and mechanisms of EtOH to gastric mucosa. However, as there may be difference between human and animal cells in reaction to drugs, pharmacological functions which proved in animal cells may not be con-firmed. GES-1 cells, which were derived from normal fetal gastric mucosa epithelial cell then transformed by SV40 to human gastric epithe-lial immortal cell lines, remain the basic charac-ters and functions of human normal gastric mucosal epithelial cells. GES-1 cells have stable genetic state, positive reaction with mucin, and normal cytoskeletal structure including microfilament, microtubule and kera-tin [20-22].

The study applied GES-1 cell line as object of study to establish *in vitro* injury model of human gastric epithelial cell under the condition of EtOH. The surface epithelium of human gastric mucosa, constantly refreshed, are renewed nearly once a day. Old cells are lifted from base-ment membrane by extrusion of regenerative cells [23]. The gastric mucosal epithelium has certain restoration function to repair mechani-cal and chemical damages caused in the pro-cess of digesting food [24]. Basement mem-brane inhered by gastric mucosal epithelium is extremely sensitive to acid induced injury. When gastric mucosal epithelium is damaged, epithelial cells are able to move injured base-ment membrane and make up compound pro-tective barrier formed by mucus, cell debris, etc. so as to prevent the injury from the proba-ble second damage induced by gastric acid [25]. Thus, it is because of self-healing and self-renewing function of gastric mucosal epitheli-um that gastric mucosa maintains integrity [24, 26-29]. GlycyrrhizauralensisFisch has been known as "the king of herbs" due to its widely medicinal and officinal properties for thousands of years. GPS, one of the main active ingredients of glycyrrhiza is attributed to many healing properties of the herb. Recently, it has been reported that GPS has many functions such as immunity regulation, phagocytosis, anti-complement, anti-virus, anti-tumor, and it has low cellular toxicity [15-19].

Our study proved inhibiting effect of EtOH on cell viability of GES-1 cells. When the concen-tration of EtOH reached to 0.6 M, cell growth obviously slowed down. With the increase of concentration, apoptosis rate of GES-1 cells was apparently decreased. Because of volatile physical property of EtOH, action duration of 0.6 M EtOH was comparatively short, and the damage was limited. Therefore, treatment with 0.8 M EtOH for 4 h was selected as the injury model in the study. We observed that GPS pre-treatment (100 µg/mL and 400 µg/mL) for 6 h apparently reduced EtOH induced injury to GES-1 cells, and decreased apoptosis rate.

Bcl-2 gene family is one of the earliest studied and important apoptosis related gene family to regulate cell apoptosis. In 1997, Koshida et al found that the expression levels of Bcl-2 and Bax was closely associated with the apoptosis rate: when Bcl-2 was highly expressed or Bax was weakly expressed, the apoptosis rate was low; in turn, the rate was high [30]. Anti-apoptotic gene Bcl-2 and pro-apoptotic geneB-ax are a couple of key apoptosis related genes. When cells are stimulated by death signals, pro-apoptotic protein will undergo conforma-tional change, transposition from cytoplasm to membranes of organelles and reaction with anti-apoptotic proteins so as to deprive them of inhibiting effect on apoptosis and release of a series of pro-apoptotic factors, eventually resulting in apoptosis [31]. The sensitivity of cells to apoptosis signals is dependent on competitive dimer process of Bcl-2/Bax. High expression of Bcl-2 makes the formation of homodimer and/or heterodimer of Bcl-2/Bax to inhibit apoptosis while high level of Bax pro-motes apoptosis through composing Bax/Bax homodimer. Thereby, the ratio of Bcl-2 and Baxis key to determine cell apoptosis [32, 33]. In our research, we observed high expression

level of pro-apoptotic gene Bax and weak level of anti-apoptotic gene Bcl-2 in EtOH model group with increasing apoptosis rate compared with normal cells. GPS pretreatment could markedly down-regulate the expression level of Bax and up-regulate the level of Bcl-2 in EtOH injury model, and the effect was in a concentration-dependent manner, which implied that the protective effect of GPS on EtOH induced apoptosis was related to regulation of apoptotic pathway.

Akt is a junction of numerous cell signal pathways. pAkt, the active form of Akt, plays a key role in improving cell proliferation, promoting cell cycle development and inhibiting apoptosis, and is also strongly linked to tumorigenesis and tumor metastasis [34-36]. Oncogene Mdm2 is located in chromosome 12q13-14 with a coding region of 1.476 kb. Mdm2 protein combined with p53 to p53-Mdm2 compound to degrade p53 and inhibit its transcriptional activity as well. Mdm2 can also inhibit the activity of Rb, act on target DNA to promote cell division and proliferation [12]. p53, located in chromosome 17q13.1, is a cancer suppressor gene with the highest correlation to tumor [37]. p53 is a transcription factor in which converge many cellular stress pathways such as oncogene activation, hypoxia, DNA damage, and endoplasmic reticulum stress, to induce different biological cell responses such as cell cycle arrest in G1 or G2, DNA repair, senescence, or even apoptosis [38]. As a result of these features, p53 is named "the guardian of the genome". In a normal healthy cell, p53 remains at a very low concentration, but after cellular stress, its level increases [39]. Mdm2 mediation can continuously degrade a majority of p53 proteins to maintain low intracellular p53 protein level [12-14]. In the study, increasing ratio of p-Akt and Akt, which meant higher phosphorylation level of Akt, was detected in EtOH model cells in comparison to control group. Moreover, the protein level of Mdm2 was significantly decreased while that of p53 was dramatically increased. GPS pretreatment to GES-1 cells in EtOH induced injury was observed to apparently mitigate the changes in their expression. With GPS pretreatment, p-Akt/Akt level was decreased, the expression of Mdm2 was up-regulated and that of p53 was down-regulated in GPS1 + EtOH group and GPS2 + EtOH group. To verify our conjecture that the protective effect of GPS pretreatment in EtOH induced injury may be rel-

evant to Akt-Mdm2-p53 signal pathway, we inhibited phosphorylation of Akt by LY294002 and detected apoptosis rate under different conditions. The results showed no significant difference between EtOH model group and GPS pretreated groups when Akt-Mdm2-p53 pathway was inhibited.

Conclusion

EtOH is one of the great causes of diseases of digestive tract [1]. GPS pretreatment was found to be able to improve cell viability and proliferation, and decrease apoptosis to protect GES-1 cells from EtOH induced damage. Akt-Mdm2-p53 pathway was activated to contribute to the repair processes, and inhibition of Akt phosphorylation was proved to substantially reduce the protective effect of GPS. GPS protected GES-1 cells from EtOH induced damages through regulating the expression Akt-Mdm2-p53 pathway.

Disclosure of conflict of interest

None.

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