Research Article

The Total Flavonoid Extract from Glycyrrhiza inflat Bat. Suppresses Atrophic Gastritis in Rats through the Akt/MAPK Pathway

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1. Introduction

Atrophic gastritis (AG) is a histopathological entity which may be caused by Helicobacter pylori infection, bile reflux, diet, alcohol, and tobacco. The basic pathological characteristics of AG include reduction of glands, thickening of the muscularis mucosa, and inflammatory response in the gastric mucosa. According to the World Health Organization report, AG is considered as a precancerous lesion with 2.55% to 7.46% incidence of gastric cancer [1–3]. Therefore, developing effective therapeutic approaches to block AG is of great importance to prevent the occurrence and development of gastric cancer.

Until now, no effective drugs are available for the treatment of AG. Although iron and vitamin B12 supplements are proposed to treat AG, they only partially reverse the atrophy of gastric mucosa and gastric function injury [4]. Glycyrrhiza inflat Bat. has been used to treat gastric ulcer and gastritis in clinic in traditional Chinese medicine. However, the underlying mechanisms are still unclear. Our previous study revealed that the total flavonoid extract (TFE) from G. inflat Bat. protected against gastric mucosal injury in chronic superficial gastritis rats through the upregulation of serum prostaglandin E2 (PGE2) [5]. Other recent research has reported that flavonoids from Glycyrrhiza have antitumor, anti-Helicobacter pylori, hypnotic, and anticonvulsant activities [6–9]. In fact, TFE can be available in the commercial market in China, which is called Anweiyang Capsule. This drug is used to treat gastric ulcer and gastritis with a good efficacy [10, 11].

For better clarifying the rationale of Glycyrrhiza in clinic use, we investigated protective effects and possible
mechanisms of TFE from *G. inflat* Bat. on AG rats. Our results suggested that TFE could protect gastric mucosa in AG rats through the Akt/MAPK signaling pathway. These findings provided a rationale for the clinical application using *G. inflat* Bat. for the treatment of AG.

2. Materials and Methods

2.1. Reagents and Chemicals. TFE from *G. inflat* Bat. was supplied by the Jiuhiui Pharmaceutical (Batch no. 101110, Huizhou, China). Vitacoenzyme tablets were purchased from Sunshine Pharmaceutical (Beihai, China). Sodium taurodeoxycholate hydrate was purchased from Amresco (Solon, OH). TUNEL apoptosis kit was purchased from KeyGEN (Nanjing, China). All antibodies were purchased from Cell Signaling Technology (Beverly, MA), except Bcl-2 and Bax antibodies (Bioworld Technology, St. Louis Park, MN). GTVISIONTM III Detection System/Mo & Rb was purchased from Gene (Shanghai, China). All other agents were purchased from Sigma (St Louis, MO).

2.2. Animals. SPF Sprague-Dawley rats (6 weeks old, half males and half females) were obtained from the Laboratory Animal Centre of Southern Medical University (Certificate no. SCXK2011-0015, Guangzhou, China) and were housed in a controlled environment with a room temperature of 24 ± 0.5°C, humidity of 50%-60 % and alternate 12 hours of light-dark cycles. The animal study procedures were conducted in accordance with the Guidelines for the Care and Use of laboratory animals published by the National Institutes of Health and were approved by the Laboratory Animals Care and Use Committee of Southern Medical University.

2.3. Establishment of AG Animal Models and Animal Treatments. Sixty SD rats were randomly assigned into five groups with 12 rats in each group. The first group was administered with water once a day to serve as the normal group. The rest groups were provided for free access to water containing 20 mmol/L deoxycholate sodium (pH = 7–7.8) for 30 days. At an interval of 10 days, 60% ethanol (v/v, 2 ml/rat) was administered to each rat. Then, the rats were provided with 10 mmol/L deoxycholate sodium or 30% ethanol (v/v) at an interval of 7 days [12]. At day 100, AG rat model was well established according to the degree of gastric mucosal damage by histopathological analysis. The second group was served as the model group to give water by intragastric administration once a day. The third group (vitacoenzyme group) was fed with vitacoenzyme (0.35 g/kg/day i.g.). The fourth group (TFE high-dose group, H-TFE group) and the fifth group (TFE low-dose group, L-TFE group) were treated with TFE (0.6 g/kg/day i.g. or 0.15 g/kg/day i.g.). All the treatments lasted for 30 days. Behavior and food intake of the rats were carefully observed, and the body weights were measured every fifteen days throughout the treatment period.

2.4. Hematoxylin-Eosin (H-E) and Alcian Blue-Periodic Acid Schiff (AB-PAS) Staining Analysis. Parts of gastric tissues were immediately fixed by 10% buffered formalin (v/v) and then embedded with paraffin. Paraffin sections (5 μm) were deparaffinized and routinely stained with H-E or AB-PAS, respectively. Pathological changes and mucosubstances of gastric mucosa in the AG rat model were observed by microscopy (BH-2, Olympus, Japan). The thickness of lamina propria and lamina muscularis mucosae of gastric body and pylorus was analyzed using image analysis software package Image-Pro Plus 6.0 (IPP 6.0) in each of the five microscopic fields.

2.5. TUNEL Apoptosis Assay and Immunohistochemical Analysis. The gastric specimens were fixed by formalin, embedded with paraffin, and cut into 3 μm sections. Then, the sections were deparaffinized with xylene, dehydrated, quenched with 3% H₂O₂ in methanol, and treated with sodium citrate buffer (pH = 6.0) in a microwave oven at 500 W for 15 min to retrieve antigen. Then, the apoptosis appearance of gastric mucosa was detected by the TUNEL assay according to the manufacturer’s manual. Immunohistochemical analysis was conducted to detect the protein expression levels of Bcl-2 and Bax. The primary monoclonal antibodies against Bcl-2 or Bax (1:100) were applied overnight at 4°C after the nonspecific antigen was blocked with 5% bovine serum albumin (BSA) in PBS. Then, the sections were washed and incubated with secondary antibodies. The reaction products were visible by immersing the sections in diaminobenzidine and washing them twice with water. The slides were counterstained with hematoxylin, dehydrated using graded alcohols and xylene, and observed under a light microscope (Olympus). Brown granules represented positive staining. The integrated optical density (IOD) of the positive cells in five randomly chosen fields was determined by IPP 6.0. The mean IOD (MIOD) was calculated by the following formula: MIOD = IOD/area.

2.6. Western Blot Analysis. Freshly isolated gastric tissues were homogenized in a RIPA buffer containing 50 mmol/L DTT, 20 × phosphatase inhibitor, and 1 mM PMSF for 30 min on ice. After centrifugation at 12,000 × g at 4°C for 30 min, the supernatant of the tissue homogenate was collected and the protein concentration was determined using the BCA protein assay kit. Electrophoresis and immunoblotting analysis were performed as described before [13]. The relative expression levels of proteins were quantified by IPP 6.0.

2.7. Statistical Analysis. The data were expressed as mean ± SEM and analyzed by using SPSS 13.0 software package (SPSS, Chicago, IL, USA). Data were statistically significant when *P* < 0.05.

3. Results

3.1. TFE Attenuated Pathological Changes of Gastric Mucosa in the AG Rat Model. Throughout the treatment, rats did not
show abnormality in food intake and behavior except the model group. Histological changes in gastric mucosa were investigated under a light microscope. In the normal group, gastric glands were well organized with the normal shape. Few inflammatory cells in the gastric mucosa were observed, while in the AG model group, the gastric glands were irregularly arranged and were atrophic. Massive neutrophils and lymphatic cells were distributed in the gastric glands. After TFE treatment, the arrangement of histological structures in gastric mucosa was improved and the number of inflammatory cells and the degree of mucosa atrophy were reduced. Similar effects were also observed in the vitacoenzyme treatment group (Figure 1(a)). Moreover, in the AG model group, lamina propria of rat gastric mucosa at body and pylorus was thinned, while muscularis mucosae were thickened. The degrees of these changes were attenuated after TFE treatment (Figures 1(b)–1(c)). These results indicate that TFE attenuates AG in rats.

3.2. TFE Protects the Secretion Capability of Gastric Mucosa in the AG Rat Model. As shown in Figures 2(a)–2(b), the acetic mucosubstances (outer layer) of the gastric mucosa was blue, and the mucosubstances near epithelial cells (inner layer) were fuchsia. The epithelial cell area was pink. In the normal group, the outer layer is arranged regularly. In the AG model group, the outer layer was decreased significantly. However, the TFE treatment attenuated the damage of the acetic mucosubstances, which were consistent with the vitacoenzyme group. Taken together, these results demonstrate that TFE protects the capability of gastric mucosa to secrete mucus in the AG rat model.

3.3. TFE Induces Apoptosis in Epithelial Cells in the AG Rat Model through Disrupting the Balance of Bcl-2/Bax Ratio. In AG, the imbalance between cell proliferation and apoptosis of epithelial cells is a critical event and epithelial cells are usually found in lack of apoptosis leading to the accumulation of mutant cells which may induce the appearance of gastric cancer. Therefore, we first detected whether TFE could regulate apoptosis in epithelial cells in AG rats. As shown in Figure 3, some epithelial cells in the normal group were apoptotic. The appearance of apoptosis in epithelial cells in the model group was decreased significantly, whereas apoptosis induction was increased after TFE treatment, suggesting TFE could inhibit the overproliferation of epithelial cells.

Both Bcl-2 and Bax belong to the Bcl-2 family, which are important in the regulation of apoptosis. Our data showed Bcl-2 and Bax (brown) were mainly located in the cytoplasm. Bcl-2 level was increased, whereas Bax level was decreased in the model group. The phenomena were attenuated after TFE treatment (Figures 4(a)–4(c)). The results were consistent with those detected by western blotting (Figures 4(d) and 4(e)). In summary, these data demonstrate that TFE induces apoptosis in epithelial cells in AG rats through regulating the Bcl-2/Bax ratio.

3.4. TFE Protects Rats against AG through the Akt/MAPK Pathway. Recent research has suggested that both Akt and MAPK are linked to AG. We detected the key proteins in the Akt/MAPK pathway by western blot analysis. In AG rats, the levels of p-p38Thr180/tyr182, JNK, and p-JNKThr183/tyr185 were increased, whereas the degrees of p-Erk1/2Thr202/tyr204 and p-AktThr308 were decreased after TFE treatment (Figure 5). These data together imply that TFE protects rats against AG through Akt/MAPK signaling.

4. Discussion

AG is one kind of gastritis with the characteristics of atrophy of the gland, dysplasia, and intestinal metaplasia [2, 3]. It has been recognized that AG is closely related to gastric cancer. Therefore, AG is thought as precancerous lesions [1]. However, the pathogenesis of AG is not yet completely identified and there is no effective drug to treat AG, until now. Traditional Chinese medicine G. inflata Bat. is used to treat gastric ulcer and gastritis in China with the significant clinical efficacy. TFE from G. inflata Bat. is also show great antiagastic ulcer and antigastritis effects in clinic [10, 11]. RP-HPLC analysis found that there are four main compounds in TEF, including licochalcone A, liquiritigenin, isoliquiritigenin, and isoliquiritiside [14]. In this study, we demonstrated that TFE of G. inflata Bat. significantly inhibited AG without weight loss or any obvious adverse reactions in the treatment.

Our results showed that TFE treatment could suppress AG by reducing the infiltration of inflammatory cells. Recent studies found that flavonoids from Glycyrrhiza such as licochalcone A, glabridin, and isoliquiritigenin showed obvious anti-inflammatory effects [15, 16]. However, whether these flavonoids play anti-AE effects still needs to be explored.

Present studies show that AG in rats is related to the damage of the gastric mucosa barrier [1]. Our results showed that TFE treatment could suppress AG by decreasing the atrophy of mucosa and the thickness of the muscularis mucosae and increasing of the thickness of the lamina propria of gastric mucosa, suggesting the protective effects of TFE on gastric mucosa of AG rats. Moreover, we found that TFE could repair the total and acetic mucosubstances of rats with AG. Considered that mucus on the surface of gastric mucosa is a barrier to defense against gastric acid attack, we speculated that the protective activity in mucus might partially account for the inhibitory effects of TFE on AG.

Although epithelial cells could protect gastric mucosa through the secretion of mucus, the overproliferation of epithelial cells will interrupt the balance between cell survival and death. It has been identified that there are apoptotic epithelial cells in the stomach of healthy rats and the apoptotic rate is decreased in AG rats [17]. We also observed similar results and TFE treatment increases apoptosis to reduce epithelium dysplasia.

Bcl-2 and Bax are the two most critical species in the Bcl-2 family, which exert antiapoptotic and proapoptotic
activities, respectively [18]. Therefore, the balance between Bcl-2 and Bax is critical in apoptosis regulation. It has been suggested that Bcl-2 only expresses in a few new epithelial cells of normal gastric mucosa, whereas it overexpresses in epithelium in patients with AG and intestinal metaplasia [19]. Recent study has also demonstrated that Bax is crucial in prognosis to the evolvement and clinical course of AG [20]. These results reveal that Bcl-2 and Bax may play a critical role in the progression of AG. Our result showed that the TFE treatment could decrease the ratio of Bcl-2/Bax, suggesting this agent facilitates epithelial cell apoptosis by regulating the ratio of Bcl-2/Bax.

Both Akt and MAPK pathways govern cell proliferation, differentiation, and apoptosis [21]. Inhibition of the Akt pathway can attenuate inflammation through apoptosis induction which is involved in the activation of Bax, while the activation of the MAPK pathway induces apoptosis by modulating the activity of apoptotic regulatory proteins, such as Bcl-2 [22]. Therefore, we speculated that TFE regulated the expressions of Bax and Bcl-2 through the Akt/MAPK pathway.
Figure 3: TFE induces epithelial cell apoptosis in gastric tissues of AG rats. (a) The gastric mucous was detected by TUNEL assay. Arrows indicate apoptotic cells (original magnification: 200×). (b) The statistical analysis of apoptotic cells in gastric mucous. $## P \leq 0.01$ vs. normal; $** P \leq 0.01$ vs. model. One-way ANOVA, post hoc comparisons, Tukey’s test. Columns, means; bars, SEs.

Figure 4: Continued.
Akt has two phosphorylated forms. Among them, Akt Ser473 is linked to apoptosis by controlling subcellular localization of proapoptotic proteins, whereas AktSer308 phosphorylation regulates cell shape, proliferation, and protein synthesis [23]. We found that the phosphorylation of Akt at Thr308 in AG rats was significantly reduced, while the phosphorylation of Akt at Ser473 was not changed obviously (data not shown). These results suggested that Akt Ser308 may play a protective role in TFE-induced apoptosis in epithelial cells.

There are three major MAPKs: p38, JNK, and Erk. p38 and JNK are sensitive to stress which can induce apoptosis, while Erk is generally response to growth factors which is linked to cell survival and proliferation [24]. Upon the activation of the MAPK pathway, activated transcription factors regulate the expressions of target genes resulting in a biological response, such as anti-inflammatory effects and apoptosis. Previous research found that licochalcone A inhibits lipopolysaccharide-induced inflammatory response through suppression of
Our results found that all three proteins were changed, suggesting that MAPK signaling is involved in the inhibitory effects of TFE on AG.

Increasing evidence has demonstrated that the cross-talk between Akt and MAPK pathways is complex. Moreover, the cross-talk is context-dependent on the levels of growth factors. The activation of MAPK (ERK) inhibits PI3K/Akt pathway at the high concentration of growth factors, whereas PI3K/Akt can positively facilitate the MAPK cascade in response to physiological stimuli at the low level of growth factors [15].

Figure 5: The Akt/MAPK pathway involves in the anti-AG effects of TFE. (a) The expression levels of key proteins in the Akt/MAPK pathway in the gastric tissues were detected by western blotting. β-actin was used as a loading control. (b) The statistical analysis of key proteins in the Akt/MAPK pathway in gastric tissues by IPP 6.0. *P ≤ 0.05, **P ≤ 0.01 vs. normal; *P ≤ 0.05, **P ≤ 0.01 vs. model. One-way ANOVA, post hoc comparisons, Tukey’s test. Columns, means; bars, SEs.
5. Conclusion

We demonstrated the anti-AG effects of TFE from G. inflat Bat. in vivo. We elucidated the underlying mechanism that involves the protective effect on gastric mucosa via the Akt and MAPK pathway (Figure 6). Our study provides a rationale for the application of Glycyrrhiza for the treatment of AG.

Abbreviations

AB-PAS: Alcian blue-periodic acid Schiff
BSA: Bovine serum albumin
AG: Atrophic gastritis
H&E: Hematoxylin-eosin
PBS: Phosphate-buffered saline
TFE: Total flavonoid extract.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have declared that no competing interests exist.

Authors’ Contributions

Junshan Liu, Jun Zheng and Xiaochun Lin contributed equally to this work. Junshan Liu analyzed the data, wrote the manuscript, and provided the fund for the study. Jun Zheng was involved in study conception and designed the manuscript and participated in animal experiment. Xiaochun Lin participated in animal experiment. Shuntao Bai and Hongling Zhou interpreted the data. Qiudi Deng revised the manuscript. Wanjiao Gao participated in data acquisition. Li Tong was involved in study supervision, revised the manuscript, and provided the fund for the study. Besides, all authors read and agreed to the final manuscript.

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References


Figure 6: A proposed model of web interaction to delineate the anti-AG mechanisms of TFE.


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