

# Protective Effect and Mechanism of DHQ On Ethanol-induced Gastric Mucosal Injury in GES-1 Cells

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**Abstract:** It is reported that the incidence of gastric mucosal injury diseases is very high in the world, among which ethanol is one of the most important predisposing factors. In this study, DHQ effectively inhibited ethanol-induced apoptosis of GES-1 cells in a concentration-dependent manner. DHQ can down-regulate the expression of various pro-apoptotic proteins and up-regulate the expression of anti-apoptotic proteins, which may be related to the activation of mitochondrial apoptosis pathway. Therefore, DHQ can protect gastric mucosal epithelial cells to a certain extent, and finally achieve the protective effect of protecting gastric mucosa. Our study provides new insights for the prevention and treatment of gastric mucosal injury diseases, and DHQ can be used as a new therapeutic drug.

**Keywords:** GES-1, DHQ, Gastric mucosal injury, Apoptosis.

## 1. Introduction

Epidemiological survey results show that in China, the incidence of stomach disease is as high as 85%, of which gastric cancer ranks in the top 5 of urban men and women with a high incidence of cancer, a serious threat to human health[1]. One of the important manifestations of the occurrence and development of gastric disease is gastric mucosal injury[2], which mainly induces acid-base imbalance, *Helicobacter pylori* invasion and chemical damage of gastrointestinal mucosa, which can be attributed to the imbalance between protective factors and aggressive factors of gastric mucosa[3]. As a result, the protective factors in gastric mucosa are weaker than attack factors, resulting in gastric mucosal damage[4]. However, there are still many problems to be solved, such as long treatment time, slow ulcer healing, high recurrence rate, many side effects and so on[5]. Among them, the occurrence and development of gastric disease are related to gastric mucosal injury to a certain extent[6]. Patients with gastric mucosal injury, first cure the mucosal surface damage, and then prevent its further infection, usually the effect of drugs at this stage is very obvious, many patients feel that their symptoms have been alleviated, no longer continue treatment, then it may lead to the recurrence of gastritis[7]. In recent years, the application of proton pump inhibitors in the treatment of drug-induced digestive tract injury has reached a consensus at home and abroad[8]. However, the clinical application of PPIs in digestive tract injury has not been confirmed[9]. Stress gastric mucosal injury should be characterized by upper gastrointestinal bleeding, even perforation and acute abdomen, with high mortality and dangerous prognosis[10]. Therefore, drugs to reduce apoptosis and oxidative stress can be used as an effective therapeutic strategy for ethanol-induced gastric mucosal injury[11].

Apoptosis is programmed cell death, which plays a very important role in the development of the body, the maintenance of homeostasis in the internal environment and the occurrence of a variety of diseases. Gastric mucosal lesions caused by stress injury are not only accompanied by the decline of gastric mucosal cell proliferation, but also

accompanied by a large number of cell apoptosis[12]. At the same time, the development of apoptosis also depends on the dynamic balance of the expression of genes that enhance the process of apoptosis (such as Bax) and other genes that inhibit the process of death (such as Bcl-2)[13]. Therefore, agents that can stabilize the structure and function of mitochondria are considered to have the ability to protect gastric mucosa from injury[14].

Dihydroquercetin (Dihydroquercetin, DHQ) is a kind of dihydroflavonoids. DHQ is widely distributed in nature, such as larch, Douglas pine, *Pinus sylvestris* var. *mongolica* and olive oil all contain more DHQ, especially in the neck of larch root[15]. With the increase of storage time, the content of DHQ in the used plants decreased. DHQ, a kind of glucoside originally extracted by Japanese scholars, was then deeply studied, and its pharmacological effect was gradually recognized[16].

Reactive oxygen species (ROS) are important substances to keep healthy[17]. Excessive production of ROS or insufficient antioxidant function of cells will cause endogenous oxidation, resulting in more synthesis of some or some proteases in the body, resulting in the formation of oxidation products[18]. It has been reported that DHQ can reduce the activity of xanthine oxidase to achieve the purpose of antioxidation[19]. DHQ can inhibit inflammation by reducing the expression of pro-inflammatory mediators and reducing the activity of inflammation-related protein genes and inflammation-related enzymes[20]. The anti-tumor mechanism of DHQ is not clear, but some studies have reported that DHQ can inhibit the continuous production of tumor necrosis factor by preventing the continuous proliferation and differentiation of tumor cells, affect the intracellular signal transmission, and then inhibit the stage of tumor proliferation and development[21].

Apoptosis is the end of life activities, is a gene-coded suicide process, which occurs in the process of cell death in the body[22]. It has been written that DHQ may play an anti-apoptotic effect by inhibiting the activation of Caspase-8-dependent pathway and attenuating protease inhibitor-induced PC12 cells. Some studies have also reported that DHQ can also achieve the purpose of anti-apoptosis by

regulating the Bcl-2 family[23].

## 2. Materials and Methods

### 2.1. Materials

DHQ (purity  $\geq 98\%$  by HPLC) was obtained from Prof. Kun Hu (Changzhou University). Analytical grade ethanol, petroleum ether, methylene chloride, ethyl acetate and butyl alcohol were purchased from Energy Chemistry (Shanghai, China). Dulbecco's modified Eagle's medium with high glucose (DMEM), fetal bovine serum (FBS), and antibiotics were obtained from Gibco (USA); Annexin V-FITC apoptosis detection kit was obtained from PROTEINBIO (Nanjing, China). MTT and Hoechst 33258 were obtained from sigma Aldrich (USA). MDC were obtained from Beyotime Biotechnology (China). All antibodies were obtained from Beyotime Biotechnology (China).

### 2.2. Cell culture

Human Gastric Mucosal Cells (GES-1) were purchased from Keygen Biotech (China). All cells were cultured in the DMEM containing 10% fetal bovine serum (FBS, NEWZERUM, New Zealand) and 1% penicillin/streptomycin at 5% CO<sub>2</sub> and 37 °C.

### 2.3. MTT assay

GES-1 cells were inoculated into 96-well plates at a density of  $1.62 \times 10^5$  cells per well, incubated overnight in a CO<sub>2</sub> incubator. When the cells are in good condition and grow to more than 90% of the bottom of the petri dish, the following experiments are carried out.

In order to detect the toxicity of DHQ, 10  $\mu$ L of a final concentration of 6.25, 12.5, 25, 50, 100 and 200  $\mu$ M DHQ solution was added to each well according to the gradient dilution method for 6 hours. Five replicate wells were set up.

In order to establish the stimulation model of ethanol, 10  $\mu$ L of ethanol at final concentrations of 4%, 5% and 6% was added uniformly to each well, and incubation is continued for the appropriate time (2, 3, or 4 h).

In order to detect the protective effect of DHQ on ethanol-induced GES-1 cell injury, 10  $\mu$ L of the final concentration of 1, 10, 50 and 100  $\mu$ M was added into each well for 3 hours, then 10  $\mu$ L of ethanol with the final concentration of 6% was added to each well for 1.5 h.

At the incubation end point, 10  $\mu$ L MTT solution was added to each well for 4 h, and 100  $\mu$ L 10% SDS solution was added. The next morning, absorbance values (570 nm to 630 nm) were measured using Microplate Reader.

### 2.4. Hoechst 33258 staining assay

Cover slips were preplaced in 24-well culture plates at an inoculation density of  $2.0 \times 10^5$  cells/ mL in a volume of 1 mL and incubated overnight in a cell incubator. On the second day, the experimental groups were incubated for 3 h with 100  $\mu$ L of final concentrations of 20, 40 and 80  $\mu$ M DHQ solution, respectively; except for the blank control group, 100  $\mu$ L of the corresponding ethanol stimulation concentration was added and the incubation was continued for 1.5 h. Hoechst 33258 assay were carried out according to the product instruction. After staining, a small amount of glycerol was taken to make sections, and the staining in the nuclei was observed using a fluorescent inverted microscope (IX73 OLYMPUS) and photographed.

### 2.5. Annexin V-FITC/PI stained fluorescence-activated cell sorter (FACS)

After the cells were treated by various concentration of DHQ for 3 h, 100  $\mu$ L of ethanol at a final concentration of 3% was added uniformly to each well and incubated for 1.5 h. Annexin V-FITC/PI assay were carried out according to the product instruction. Apoptosis was detected using Flow cytometer (Accuri C6 Plus, BD) after 30 min at room temperature and protected from light.

### 2.6. Determination of mitochondrial membrane potential ( $\Delta\Psi$ m)

After the cells were treated by various concentration of DHQ for 3 h, 300  $\mu$ L of ethanol at a final concentration of 3% was added to each well and the culture was continued for 1.5 h. Rhodamine 123 assay were carried out according to the product instruction. Apoptosis was detected using Flow cytometry (Accuri C6 Plus, BD) after 30 min at room temperature and protected from light.

### 2.7. Western Blotting

The density of GES-1 cells was adjusted to  $2 \times 10^5$  cells/mL, and 3 mL per well was inoculated in a 6-well culture plate. Give the medicine as mentioned earlier. After 3% ethanol stimulation, the cells were collected and WIP protein lysate was added. The nuclear protein was extracted by protein extraction kit, and the protein was collected and quantified by centrifugation. The corresponding proteins were taken for SDS-PAGE gel electrophoresis, transferred to membrane, and incubated with 5% skimmed milk powder for 1 hour. The first antibody reagent corresponding to the protein to be tested was added and incubated overnight at 4°C. The next day, the second antibody corresponding to the primary antibody was added, incubated at room temperature for 1 hour, and the ECL chemiluminescence developer was uniformly covered. The exposure was placed in 5200 Tanon. The Image J software was used to analyze the optical density value of the strip.

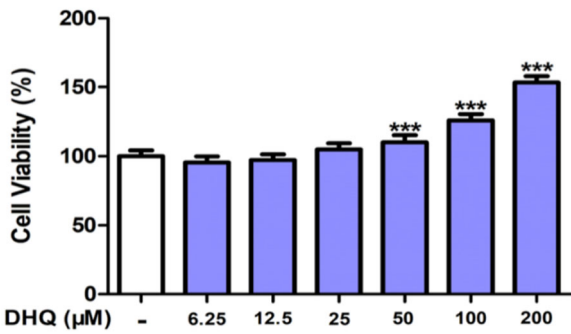
### 2.8. Statistical analysis

GraphPad Prism 5 Software (Version 8.0, GraphPad Software, Inc. San Diego, USA) was used for statistical processing of all data, and all data were expressed as mean  $\pm$ SD of independent experiments. In addition, P-value  $\leq 0.05$  or less is considered statistically significant in a single comparative statistical analysis using the T-test method. All measurements were performed in triplicates.

## 3. Results

### 3.1. Effect of DHQ on GES-1 cells

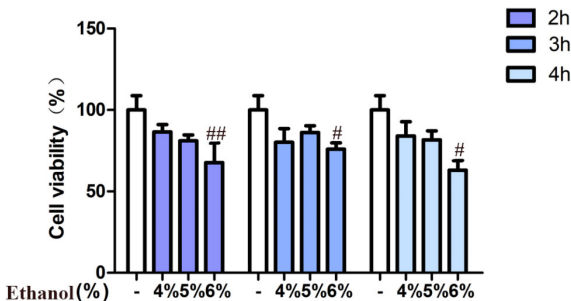
GES-1 cells were treated with 6.25, 12.5, 25, 50, 100, 200  $\mu$ M DHQ respectively to detect the toxic effect of DHQ on GES-1 cells. As can be seen from Figure 1, compared with the normal control group, the treatment with DHQ in the concentration range of 6.25-50  $\mu$ M had no effect on the survival rate of GES-1 cells, and even the GES-1 cells treated with DHQ in the concentration range of 100-200  $\mu$ M increased the survival rate compared with the normal control group. Therefore, it shows that DHQ has no toxic effect on cells and meets the requirements of biosafety.



**Figure 1.** Effect of DHQ on the survival rate of GES-1 cells (B) Effect of ethanol on the survival rate of GES-1 cells; Values = mean  $\pm$  SD, n = 3 (\*\*\*) p < 0.001 Compared to the control group, compared with ethanol alone group)

### 3.2. Effect of ethanol on GES-1 cells

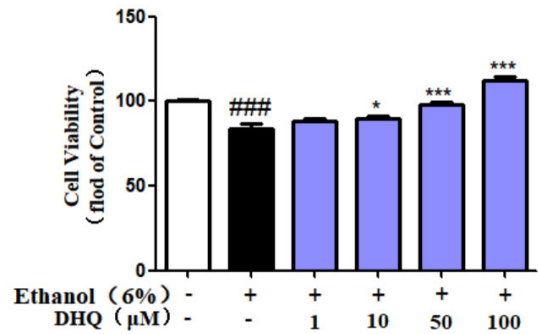
The effects of ethanol on GES-1 cells were detected by stimulation with 4%, 5% and 6% ethanol for 2 h, 3 h and 4 h, respectively. As can be seen from Figure 2, compared with the normal control group, with the increase of ethanol concentration, the survival rate of GES-1 cells is lower and smaller, indicating that ethanol stimulation is successful. When the cells were stimulated with 6% ethanol for 3 h, the cell survival rate was 75.96%, and the degree of injury was moderate, so in the later stage, when detecting the activity of DHQ on ethanol-induced GES-1 cells, 6% ethanol was selected to stimulate for 3 h to establish a stimulation model.



**Figure 2.** Effect of DHQ on survivability of ethanol-induced GES-1 cells. Values = mean  $\pm$  SD, n = 3 (#p < 0.05, ##p < 0.01, ###p < 0.001, Compared to the control group)

### 3.3. Effect of DHQ on ethanol-induced GES-1 cells

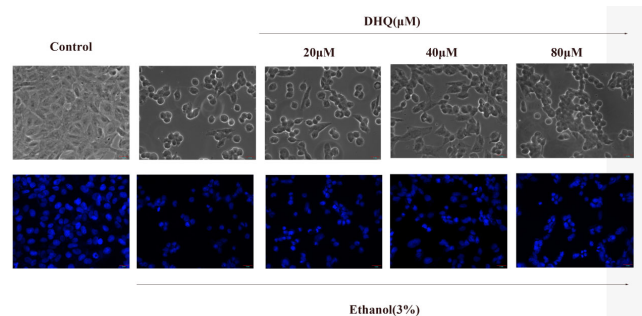
GES-1 cells were pretreated with 1, 10, 50 and 100  $\mu$ M DHQ, respectively, and 6% ethanol was added to stimulate GES-1 cells for 3 h to detect the effect of DHQ on ethanol-stimulated GES-1 cells. As can be seen from Figure 3, compared with the normal control group, the survival rate decreased significantly after being stimulated with 6% ethanol for 3 hours, but after pretreatment with different concentrations of DHQ, the survival rate of each group increased, especially when the concentration of DHQ was 100  $\mu$ M. It is suggested that DHQ has a certain protective effect on ethanol-induced GES-1 cells. In the future, 100  $\mu$ M DHQ will be selected for the experiment.



**Figure 3.** Effect of DHQ on the survival rate of GES-1 cells after ethanol stimulation; Values = mean  $\pm$  SD, n = 3 (\*p < 0.05, \*\*\*p < 0.001; Compared to the control group, ###p < 0.0001; compared with ethanol alone group)

### 3.4. Effect of DHQ on the morphology of GES-1 cells induced by ethanol

According to the above experimental results of the effect of DHQ on ethanol-induced GES-1 cells, GES-1 cells were pretreated with 100  $\mu$ M DHQ for 3 h, and then stimulated with 6% ethanol for 3 h, a series of apoptosis-related experiments were carried out, but the experimental results were far from the expected results. It has been reported that ethanol in the process of stimulating GES-1 cell injury will be accompanied by cell apoptosis and necrosis, but a high concentration of ethanol will directly contact the gastric mucosa, resulting in cell degeneration and necrosis, which is disadvantageous to the recovery of normal body. Therefore, this study takes this as a starting point to further screen the stimulation model and reduce the concentration of ethanol stimulation to study apoptosis. It was found that 3% ethanol stimulation for 1.5 h was a good choice to establish the stimulation model. The results of Hoechst 33258 fluorescence staining (Fig 4) showed that compared with the normal control group, the nuclear morphology of 3% ethanol treated group changed from ellipse to crescent shape, and there were more dense blue concentration points (apoptotic bodies) in the center, while DHQ pretreatment groups with different concentrations (20  $\mu$ M, 40  $\mu$ M, 80  $\mu$ M) were compared with ethanol group. The morphology of nucleus recovered to some extent, and the degree of recovery was related to the concentration.

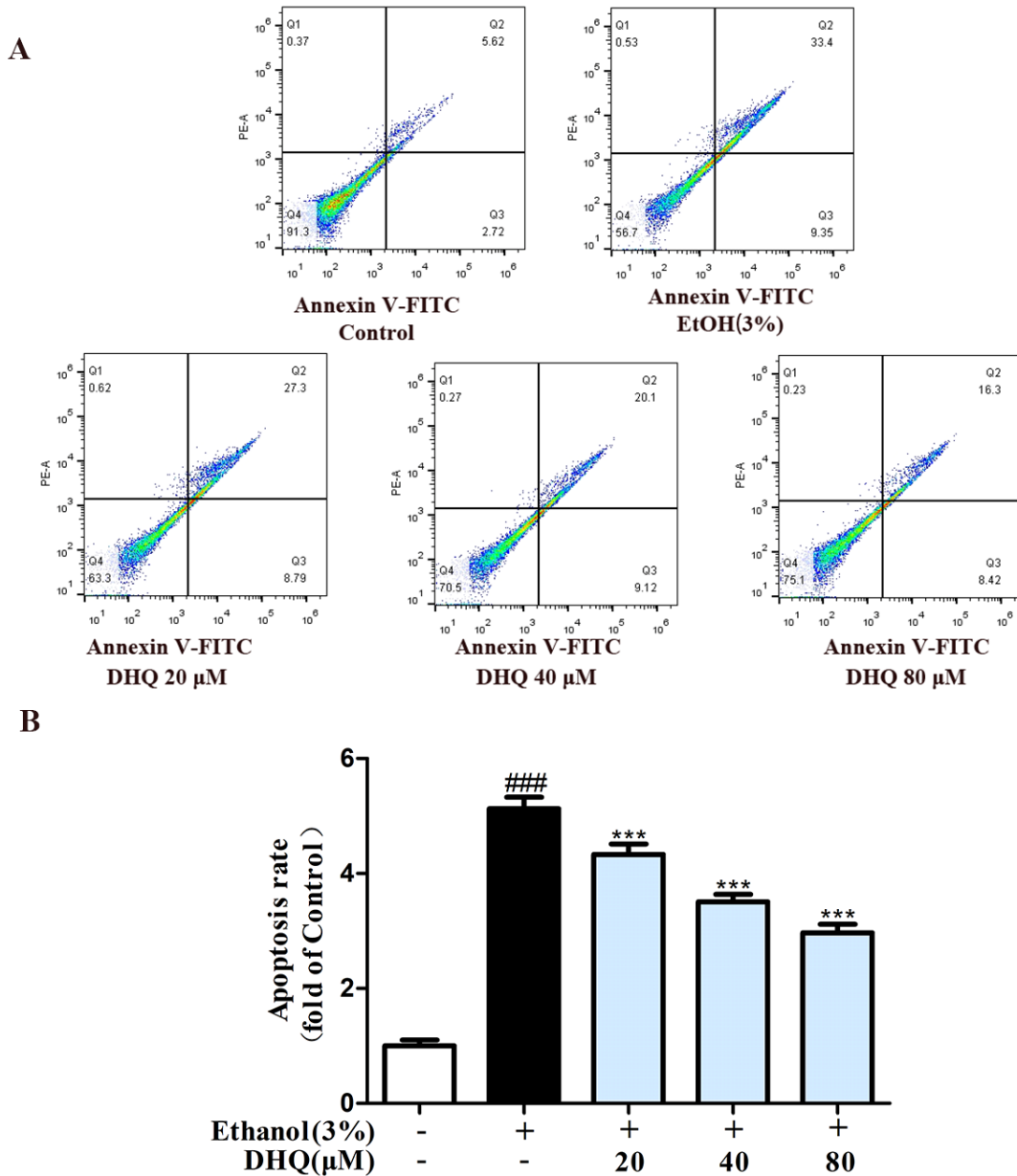


**Figure 4.** Effects of DHQ pretreatment on GES-1 cells

### 3.5. Effect of DHQ on ethanol-induced apoptosis of GES-1 cells detected by Annexin V-FITC/PI double staining

Annexin V-FITC/PI double staining flow cytometry is a technique for high-speed quantitative analysis and sorting of single cells or other biological particles in suspension by

detecting the fluorescence signals labeled by Annexin V-FITC/PI. Flow cytometry showed that (Fig 5) compared with the normal control group, 3% ethanol alone could significantly increase the apoptosis rate, and the DHQ pretreatment groups of 20  $\mu\text{M}$ , 40  $\mu\text{M}$  and 80  $\mu\text{M}$  could significantly reduce the apoptosis rate by induced by 20 ethanol stimulation.

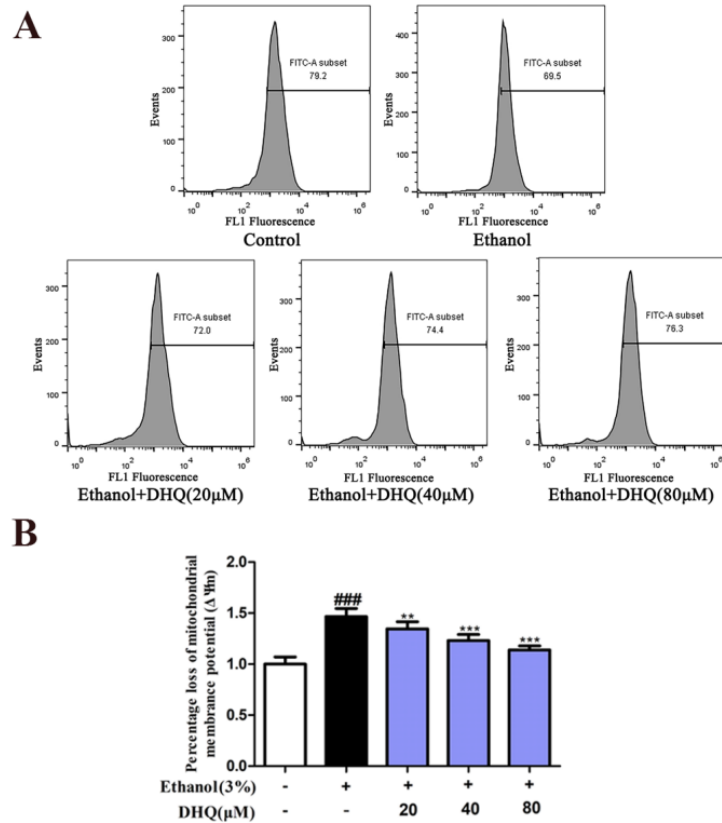


**Figure 5.** Effect of DHQ on apoptosis of GES-1 cells after ethanol stimulation detected by flow cytometry; (A) Apoptotic distribution of GES-1 cells pretreated with DHQ for 3 h and treated with 3% ethanol for 1.5 h; (B) Apoptosis rate of GES-1 cells pretreated with DHQ for 3 h and treated with 3% ethanol for 1.5 h. Values = mean  $\pm$  SD, n = 3 (#p<0.001 compared to normal control; \*p<0.05, \*\*\*p<0.001 compared to ethanol alone group)

### 3.6. Rhodamine 123 staining to detect the effect of DHQ on ethanol-induced mitochondrial membrane potential in GES-1 cells

The decrease of mitochondrial membrane potential occurs in the early stage of apoptosis. Through the determination of

mitochondrial membrane potential in each group, it was also found that the pretreatment of DHQ could slow down the decrease of mitochondrial membrane potential induced by ethanol stimulation (Fig 6), indicating that DHQ has a protective effect in the early stage of ethanol-induced apoptosis of GES-1 cells. It can be seen that DHQ can inhibit the apoptosis of GES-1 cells after ethanol stimulation.



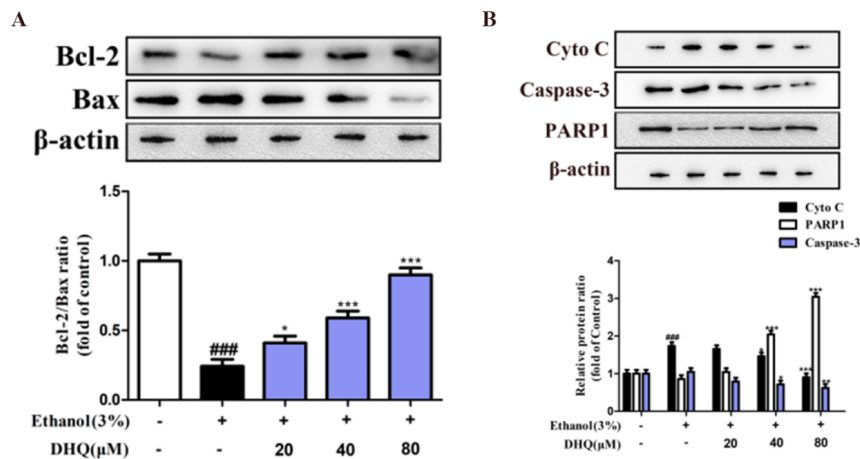
**Figure 6.** Effect of on DHQ mitochondrial membrane potential of GES-1 cells by flow cytometry; (A, B) Effect of on DHQ mitochondrial membrane potential of GES-1 cells by flow cytometry; Values = mean  $\pm$  SD, n = 3 (#p<0.001 compared to normal control; \*p<0.05, \*\*\*p<0.001 compared to ethanol alone group)

### 3.7. Effect of DHQ on apoptosis-related proteins in GES-1 cells

In order to explore whether the inhibitory effect of DHQ on ethanol-induced apoptosis of GES-1 cells is through the related pathway regulated by Bax protein family, we used Western blot method to detect the expression of mitochondrial apoptosis pathway-related proteins in order to further explore the anti-apoptosis mechanism of DHQ. As can be seen from Figure 7A, after being stimulated with 3% ethanol for 1h, compared with the normal control group, the expression of Bax increased, and the expression of Bax decreased after

DHQ treatment, indicating that DHQ inhibits ethanol-induced apoptosis of GES-1 cells by affecting the Bax protein family.

Further study found that, as shown in Figure 7B, the expression of apoptosis family related proteins Cyto C and Caspase-3 increased significantly after ethanol stimulation, while the increase of Cyto C and Caspase-3 could be alleviated in a concentration dependent manner after pretreatment with DHQ. It can be seen that DHQ protects apoptosis by reducing the release of Cyto C from mitochondria and regulating the expression of Caspase-3 and its product PARP1.



**Figure 7.** Effect of DHQ on mitochondrial apoptosis pathway-related proteins in GES-1 cells; (A) Effect of DHQ on Bax and Bcl-2 proteins of mitochondria apoptosis pathway in GES-1 cells; (B) Effects of DHQ on proteins of the caspase family of mitochondrial apoptotic pathway in GES-1 cells; Values = mean  $\pm$  SD, n = 3 (###p<0.001 compared to normal control; \*p<0.05, \*\*\*p<0.001 compared to ethanol alone group)

## 4. Conclusion

Dihydroquercetin (Dihydroquercetin, DHQ) is a polyphenolic flavonoid extracted from the root of larch[24]. It has a variety of pharmacological activities, such as antioxidant, anti-tumor, anti-radiation, anti-virus and so on[25]. In recent years, the abuse of ethanol has become more and more common in daily life[26]. It is well known that ethanol is a kind of ulcer agent, which can directly absorb and weaken gastrointestinal mucosa, and drinking a lot of alcohol will lead to excessive secretion of gastric acid in the body[27], stimulate the release of pepsin, resulting in an imbalance between protective factors such as bicarbonate and prostaglandins, resulting in serous, ulcerative lesions and petechial bleeding in the gastric mucosa. The mechanism of ethanol-induced gastric mucosal injury is relatively complex, and so far, the specific protective mechanism is not completely clear[28].

In this paper, through experimental study, the model of ethanol-induced gastric mucosal injury was established, and different research methods were used to study the protective effect of DHQ on ethanol-induced GES-1 injury cells from different aspects, and some of the protective mechanisms were explored. The results show that DHQ has a certain potential in inhibiting apoptosis and protecting gastric mucosal injury induced by ethanol[29].

In order to study the protective effect of DHQ on ethanol-induced gastric mucosal injury and its mechanism, the ethanol injury model of GES-1 cells of human gastric mucosal epithelial cells was established. MTT method is often used to detect living cells as a reliable method. In this study, MTT method was used to verify the safety of DHQ with a concentration of less than 200  $\mu\text{M}$ . a stimulation model was established with 6% ethanol stimulation for 3 h, and it was preliminarily confirmed that DHQ had a certain protective effect on ethanol-induced GES-1 damage cells. The morphological changes of blue fluorescent GES-1 nucleus stained by Hoechst33258 were observed under fluorescence inverted microscope after GES-1 cells were stimulated by different concentrations of ethanol for different times, and then the specific conditions of apoptosis and proliferation of GES-1 cells were judged. It was found that the inhibition of cell proliferation was moderate after 1.5 h stimulation with 3% ethanol, and the cell morphology could be recovered to a certain extent after pretreatment with DHQ, so it was established that 1.5 h stimulation with 3% ethanol concentration was the best model for follow-up mechanism study.

Then a series of studies were carried out from the point of view of apoptosis to explore the protective effect of DHQ on ethanol-induced GES-1 damage. The mechanism of apoptosis is the key to understand the protective effect of DHQ. Flow cytometry can efficiently detect the specific situation of apoptosis. In this experiment, two dyes of Annexin V-FITC and PI were used to label the cells, and it was found that compared with the normal control group, the apoptosis rate of the stimulation group increased, and the apoptosis rate of GES-1 cells treated with DHQ was lower than that of the stimulation group. The effect of DHQ on ethanol-induced mitochondrial membrane potential of GES-1 cells was also detected, and the results were consistent with the expectation. The above results further confirmed that DHQ can inhibit the

apoptosis of GES-1 cells and protect GES-1 cells from injury.

Mitochondria are the main places of cell respiration and energy metabolism, and mitochondrial pathways are particularly important in apoptosis[30]. There are many apoptosis-related factors in this pathway. When cells are stimulated, the mitochondrial pathway is activated, resulting in a decrease in mitochondrial membrane potential ( $\Delta\psi$ ) and further release of Cytochrome C (Cyto C), activates procaspase9/3, splits poly (ADP- ribose) polymerase PARP, and causes cells to enter apoptosis. Western blot results showed that DHQ pretreatment reversed the expression of these apoptotic factors, indicating that DHQ can inhibit ethanol-induced apoptosis of GES-1 cells through caspase-dependent mitochondrial apoptosis pathway.

## 5. Author's Contributions

The experiments for this work were designed by Prof. Jie Ren. The experiments were done by Qianwen Wang. Qianwen Wang analyzed the data, edited the graphs and tables, and wrote the manuscript. Prof. Kun Hu reviewed the manuscript. Other authors provided reagents.

## 6. Conflict of Interest

The authors declared that no conflict of interest.

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