


## Article

# Investigation on the Effects and Mechanisms of Alkaline Natural Mineral Water and Distilled Water on Ethanol-Induced Gastric Ulcers In Vivo and In Vitro

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**Abstract:** Studies have proven that alkaline water has a protective effect on gastric diseases. However, the underlying mechanism is not clear. Moreover, in some countries, especially in China, purified water (distilled water) is also an important form of drinking water, while its protective effect on gastric diseases is still unknown. This study aimed to compare the effects of distilled water ( $\text{pH} = 5.6 \pm 0.3$ ) and alkaline natural mineral water ( $\text{pH} = 9.3 \pm 0.6$ ) on ethanol-induced gastric ulcers in mice and to further clarify the underlying mechanisms. Pepsin activity, prostaglandin E-2 (PGE2) and heat shock protein 70 (HSP70), superoxide dismutase (SOD), reduced glutathione (GSH), and malondialdehyde (MDA), as well as the oxidative stress pathway related proteins such as nuclear factor erythroid-2 related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and NADH quinone oxidoreductase 1 (NQO1) were measured. After alkaline natural mineral water treatment, the levels of PGE2 and HSP70 were significantly increased ( $p < 0.05$ ). Antioxidant indexes (SOD, GSH, and MDA) and Western blot results (Nrf2, HO-1, and NQO1) showed that alkaline natural mineral water did not alleviate gastric ulcers by improving oxidative stress. Pepsin activity assay displayed that the pepsin activity was significantly declined after alkaline natural mineral water treatment compared with the distilled water treatment ( $p < 0.05$ ). This study indicated that alkaline natural mineral water may alleviate the ethanol-induced gastric ulcers in mice by inhibiting the pepsin activity and increasing the levels of PGE2 and HSP70.

**Keywords:** gastric ulcers; oxidative stress; alkaline natural mineral water; distilled water; pepsin activity



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## 1. Introduction

Gastrointestinal diseases have become a global health concern, among which peptic ulcer is the most common multi-factorial gastrointestinal disease; about 10% of people worldwide suffer from peptic ulcer disease each year [1]. Gastric ulcer, a type of pre-cancerous condition, is the most common peptic ulcer disease, which is mainly due to the imbalance of protective factors and aggressive factors in gastric mucosa [2]. There are many adverse factors (e.g., alcohol abuse, poor diet, drugs, and *Helicobacter pylori* infection), which can cause gastric ulcers [2]. Among these factors, ethanol is the most common cause of gastric ulcer disease, and alcoholics tend to be more susceptible to gastric ulcers than non-alcoholics [3]. Firstly, ethanol can directly destroy the structure of gastric mucosa to cause gastric ulcers. In addition, the formation of gastric ulcers is also the result of the digestion of gastric wall tissue by pepsin; this self-digestion process is the important cause of ulcers [4]. When gastric mucosa is damaged by ethanol, gastric acid secretion increases. Where a mass of pepsinogens is activated to pepsin, this further aggravates gastric ulcers [5]. Xie et al. reported that ethanol may also trigger gastric ulcers by increasing the levels of oxidative stress-related proteins [6]. The levels of the protective factors prostaglandin E-2 (PGE2) and heat shock protein 70 (HSP70) are imbalanced in gastric ulcers induced by ethanol. PGE2 is the most important repair factor regarding inflammation and injury in the

body, and it regulates bicarbonate secretion in the stomach to control gastric acid secretion in order to maintain the stability of the cellular mucosa in gastric tissue [7]. In addition, the synthesis of gastric mucus is also controlled by PGE2 [8]. The body will produce a stress response due to ethanol-induced gastric ulcers and protect gastric mucosa from injury by increasing the expression of HSP70 [9]. When ethanol is absorbed by the gastric mucosa, it decreases the expression of PGE2 and stimulates the cells to produce high expression of HSP70. At present, the treatment schemes for preventing or treating gastric ulcers mainly include acid inhibitors and proton pump inhibitors (PPI). However, long-term use of these drugs can lead to drug resistance and side effects [7]. Therefore, increasingly, alternative therapies, including lifestyle changes, healthy diet choices, and the use of natural products and functional water, have become hot topics of interest [10].

Water is an indispensable necessity in daily life, and people need to drink adequate enough water every day to maintain the normal physiological activities of the body. Moreover, some drinking water may have some auxiliary effects on improving human physiological function. It has been reported that alkaline water with pH 8.8 has good gastric acid buffer capacity, and can assist in the treatment of gastroesophageal reflux disease by irreversible inactivation of pepsin [11]. In addition, drinking alkaline natural mineral water rich in bicarbonate can effectively prevent gastric hemorrhagic injury induced by ethanol, and its high content of  $\text{HCO}_3^-$  is considered to be a key factor in exerting this function, which may involve the PEG2 increase and oxidative stress reduction [12]. However, the underlying protective mechanism of alkaline water against ethanol-induced gastric ulcers is still not clear. Purified water, including distilled water, is also an important form of drinking water in some countries, especially in China. Moreover, purified water, including distilled water, is widely considered by many consumers in China as one of the healthiest drinking waters because of its clean and free of impurities, although the purified water (distilled water) has a low pH and is commonly considered as acidic water. Therefore, the consumption of purified water, including distilled water, in the Chinese market is relatively large. However, the effect and the potential mechanism of purified water (distilled water) on ethanol-induced gastric ulcers are still unknown. Therefore, the aim of this study was to compare the effects and potential mechanisms of distilled water ( $\text{pH} = 5.6 \pm 0.3$ ) and alkaline natural mineral water ( $\text{pH} = 9.3 \pm 0.6$ ) on ethanol-induced gastric ulcers in mice, which may provide some new knowledge and scientific bases for exploiting drinking water as an auxiliary therapy in daily life to prevent and/or improve ethanol-induced gastric ulcers in combination with the study of Nassini et al. [12].

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

Biochemical kits, including superoxide dismutase (SOD), reduced glutathione (GSH), malondialdehyde (MDA), and pepsin enzyme activity, were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). PGE2 detection kit was purchased from Jiangsu Meimian industrial Co., Ltd. (Yancheng, China). Radio Immunoprecipitation Assay (RIPA) lysis buffer containing the inhibitors of protease and phosphatase and BCA protein assay kit were obtained from Shanghai Beyotime Co., Ltd. (Shanghai, China). Pepsin from porcine gastric mucosa (EC 3.4.23.1) was purchased from sigma (Shanghai, China). Alkaline natural mineral water ( $\text{pH} 9.3 \pm 0.6$ , Shilin Tianwaitian®) was purchased from local supermarkets (Kunming, China). Distilled water ( $\text{pH} 5.6 \pm 0.3$ ) was prepared by Heidolph Hei-VAP rotary evaporator.

### 2.2. Animal Experiment

Twenty-four Kunming male mice ( $20 \text{ g} \pm 10\%$ , SPF) were provided by Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China; Certificate No.: SCXK (Xiang) 2019-0004). Mice were fed in a standard animal feeding environment ( $23^\circ\text{C} \pm 2^\circ\text{C}$ , 40%–75% humidity, and 12 h light/dark cycle) with basic feedstuff (proteins, 23.07%. fats, 11.85%. carbohydrates, 65.08%.) purchased from Beijing Keao Xieli Feed Co., Ltd. After one week of adaptation,

mice were randomly divided into the following four groups: distilled water control (DWC), distilled water model (DWM), alkaline natural mineral water control (AWC), and alkaline natural mineral water model (AWM). The distilled water group and alkaline natural mineral water group were given the corresponding amount of water every day for 30 days at the beginning of the experiment. After 30 days of water feeding, all mice were fasted overnight (12 h), and then DWM and AWM mice were provided with ethanol at a dose of 0.2 mL/10g by gavage, while DWC and AWC mice were obtained with the corresponding amount of distilled water. After 1 h, mice were anesthetized with isoflurane (2%), and fresh blood was collected in anticoagulation tubes containing heparin sodium by eyeball harvesting and centrifuged for 5 min at  $1800\times g$  at  $4^{\circ}\text{C}$  to obtain plasma and stored at  $-80^{\circ}\text{C}$ . The gastric tissue of mice was cut open along the greater curvature of the stomach, and a piece of gastric tissue from the same area was cut and soaked in paraformaldehyde solution (4%) for further histopathological staining and analysis. The remaining gastric tissues were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Animal experiments were performed in strict compliance with the Guidelines for the Care and Use of Laboratory Animals and authorized by the Ethics Committee of Animal Experiments of Kunming University of Science and Technology (Ethical Approval No.: PZWH (Dian) K2020-0014-2).

### 2.3. Biochemical Index Analysis

PGE2 in gastric tissue was determined according to the instructions of enzyme-linked immunosorbent assay (ELISA) kit. Gastric tissue was prepared into 10% tissue homogenate according to our previous research method [13], and then GSH, SOD, and MDA levels in gastric tissue were determined according to the kit instructions.

### 2.4. Histopathological Assessment

Histopathological evaluation of gastric tissues was performed with hematoxylin-eosin staining (H&E staining). Firstly, gastric tissue was successively fixed (4% of paraformaldehyde solution), embedded, and then sliced ( $5\text{ }\mu\text{m}$ ). Thereafter, each section was dewaxed and stained. Finally, the specimens were observed and images were obtained using an Olympus IX83 microscope (Tokyo, Japan).

### 2.5. Immunohistochemical Staining

HSP70 expression in gastric tissues was detected by immunohistochemistry. Briefly, gastric tissues were sectioned and dewaxed, hydrated with gradient ethanol, then the sections were blocked with 5% bovine serum albumin (BSA) blocking solution for 15–30 min and incubated with HSP70 primary antibody overnight at  $4^{\circ}\text{C}$ . The sections were then washed 3 times with phosphate buffer saline (PBS) for 5 min each, and then incubated with secondary antibody. Finally, the sections were observed and photographed with an Olympus IX83 microscope (Tokyo, Japan).

### 2.6. Western Blot Analysis

The gastric tissue was mixed with cold Radio-Immunoprecipitation Assay (RIPA) cleavage buffer containing protease and phosphatase inhibitors at a ratio of 1:9 (*m/v*) to prepare 10% homogenate. After homogenization by tissue crusher, the supernatant was centrifuged at  $10,000\times g$  for 10 min. Protein content in tissue supernatant was determined by BioSharp BCA kit (Hefei, China). Western blot was performed according to our previous report [12]. The key proteins nuclear factor erythroid-2 related factor 2 (Nrf2), heme oxygenase-1 (HO-1) and NADH quinone oxidoreductase 1 (NQO1) detected in this study were purchased from ABclone (Wuhan, China). Protein expression analysis was performed using VILBER Fusion FX7 imaging system (Marne-la-Vallée, France).

### 2.7. Determination of Pepsin Activity

Firstly, 1.5014 g of glycine was weighed, dissolved in water, and fixed to 100 mL in a 100 mL volumetric flask to make a 0.2 mol/L glycine solution, which was adjusted to pH 3.0

with hydrochloric acid. Then, 80 mg of pepsin was dissolved in 25 mL of alkaline natural mineral water (Tianwaitian<sup>®</sup>, Shilin, pH 9.5), distilled water (pH 5.6), and 0.2 mol/L glycine solution (pH 3.0), and 25 mg of sodium chloride was added to each solution. After the three solutions were incubated for 1h, the pH was adjusted to 3.0 with 0.2 mol/L glycine (pH 2.0). Finally, the glycine solution (0.2 mol/L) at pH 3.0 was used to make the final volume of the three solutions equal, and the pepsin activity in the three solutions was determined with pepsin kit.

### 2.8. Statistical Analysis

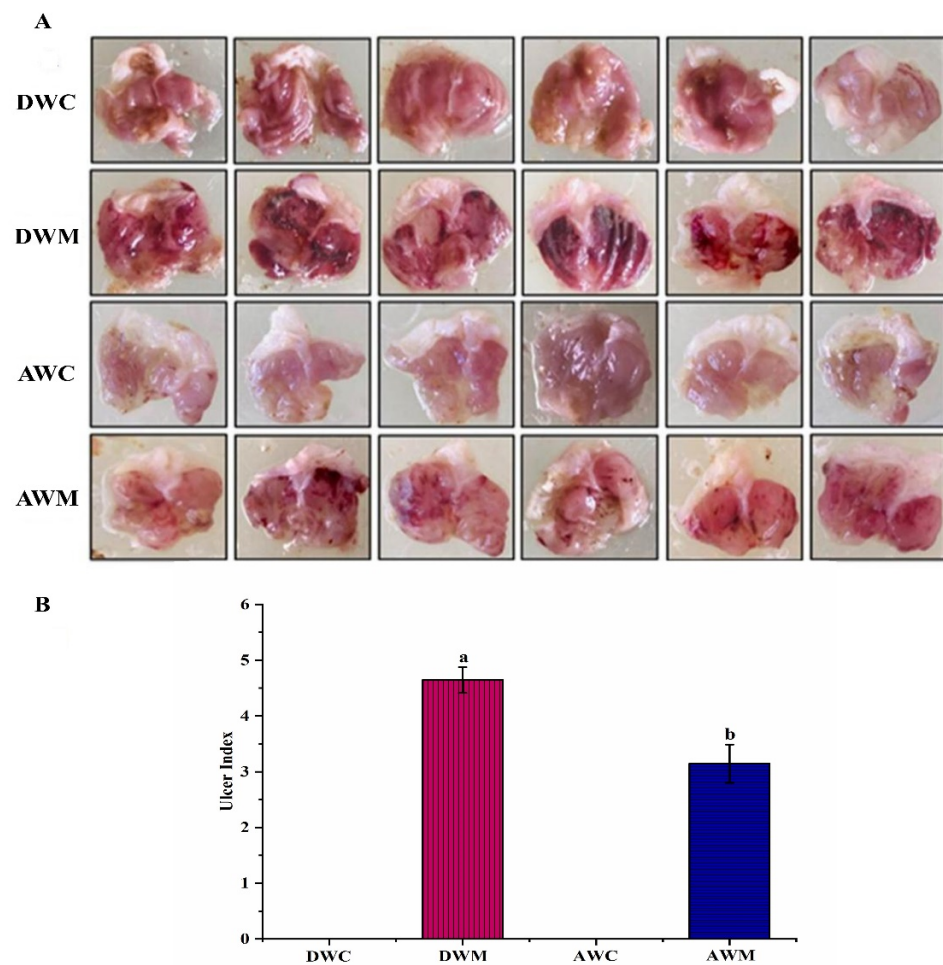
Data results are expressed as mean  $\pm$  standard error (S.E.). Tukey's test with one-way ANOVA was applied to detect significant difference ( $p < 0.05$ ) using Origin8.5 software (Northampton, MA, USA).

## 3. Results and Discussion

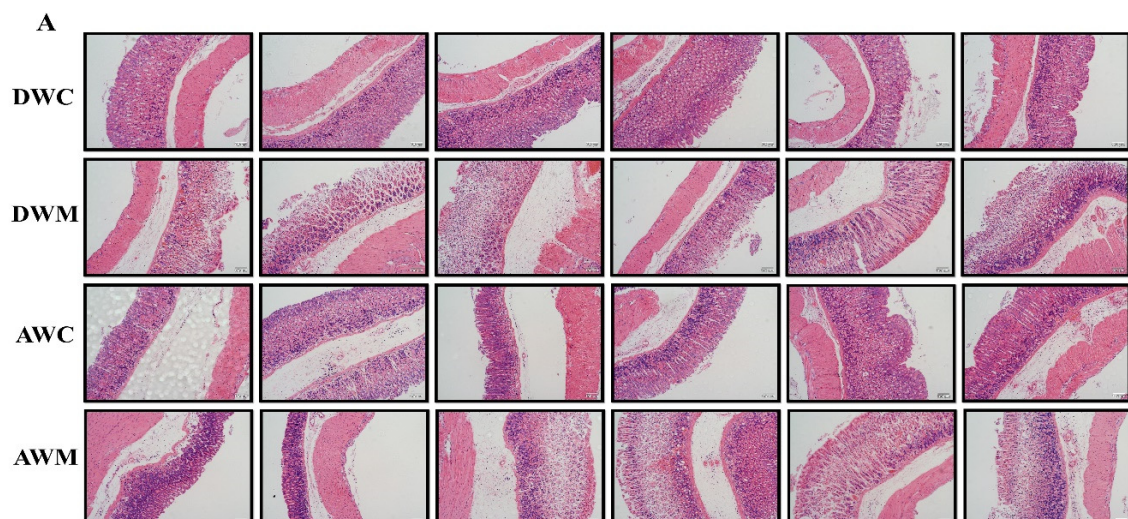
### 3.1. Morphological and Histopathological Examinations

The morphological results are shown in Figure 1. Compared with the groups DWC and AWC, gastric tissue of mice in the group DWM and AWM had significant changes in morphology and appearance. From the morphological point of view, whether DWC group or AWC group, gastric mucosal surface of mice was smooth and no obvious damage, whereas that in the two model groups had obvious strip bleeding, indicating that ethanol had damaged to the gastric tissue of mice in both model groups. Compared with DWM group, the bleeding points and ulcer area of gastric mucosal surface in group AWM were decreased. The results of gastric morphological tissue examination showed that compared with distilled water, alkaline natural mineral water could more effectively prevent gastric mucosal injury caused by ethanol. The ulcer index was scored according to the bleeding points on the surface of gastric mucosa and the degree of ulcer, so as to further determine the degree of gastric mucosal injury. The scoring criteria refer to previous literatures and are slightly modified [8], as follows: no damage is 0 points; congested 0.5–1 point; bleeding point was 1–5 points; 1–5 small ulcers 2–3 points; Multiple small ulcers were 3–4 points; 1–5 small ulcers and 1–3 large ulcers were 4–5 points; Multiple large ulcers were 5–6 points; and Gastric ulcer was 6 points. The ulcer index score results are shown in Figure 1B. Compared with the DWM group, the ulcer index of the AWM group was significantly decreased ( $p < 0.05$ ). The results of H&E in each group are shown in Figure 2. From the histopathological point of view, whether the groups of DWC or AWC, the gastric gland structure of mice was complete, the gastric mucosa cells were arranged in neat rows, and the morphology and structure of submucosa, muscle layer, and serosa were good and complete. However, the results of histopathological in the model group showed that the structure of gastric mucosa was destroyed, submucosal edema was evident, and there was a loss of gastric mucosal epithelial cells. However, compared to the DWM group, the pathological structure of gastric mucosa in mice fed with alkaline natural mineral water was significantly improved. The H&E scores are shown in Figure 2B. It indicates that compared with distilled water, drinking alkaline natural mineral water can significantly improve ethanol-induced gastric ulcers. According to the results of gastric morphological and histopathology of mice in the model group, ethanol was absorbed by the gastric mucosa, resulting in an ulcerated surface and cord-like bleeding. Meanwhile, the structure of the gastric mucosa was also destroyed, and the submucosa was swollen, epithelial cells were lost, and some structures were coagulated [14]. Therefore, the results of histopathological analysis proved that alkaline natural mineral water can alleviate ethanol-induced gastric ulcers when compared with distilled water.

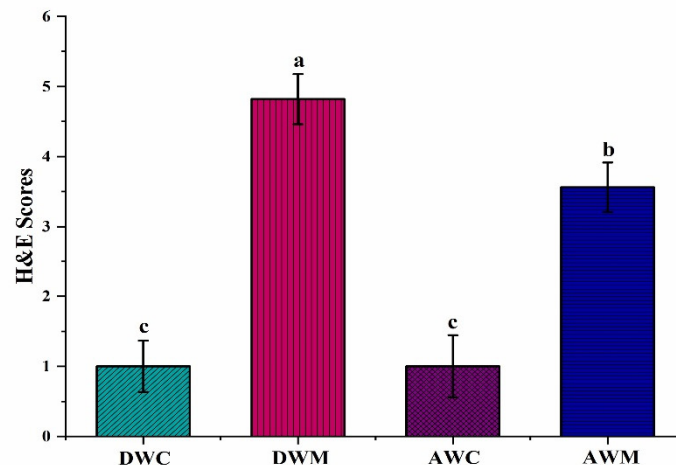




**Figure 1.** Histomorphology images (A) and ulcer index (B) of the gastric ulcer induced by ethanol in mice with two kinds of drinking water. DWC, distilled water control group; DWM, distilled water model group; AWC, alkaline natural mineral water control group; AWM, alkaline natural mineral water model group. In subfigure (B), different letters indicated significant difference ( $p < 0.05$ ). Ulcer index score was determined by bleeding point and degree of ulcer ( $n = 6$ ). Ulcer index scoring criteria: 0, no lesions; 0.5–1, hyperemia; 1–2, hemorrhagic spots; 2–3, 1–5 of small ulcers; 3–4, several small ulcers; 4–5, 1–5 of small ulcers and 1–3 of large ulcers; 5–6, several large ulcers; 6, full of ulcers in stomach or perforations.



**Figure 2.** Cont.

**B**

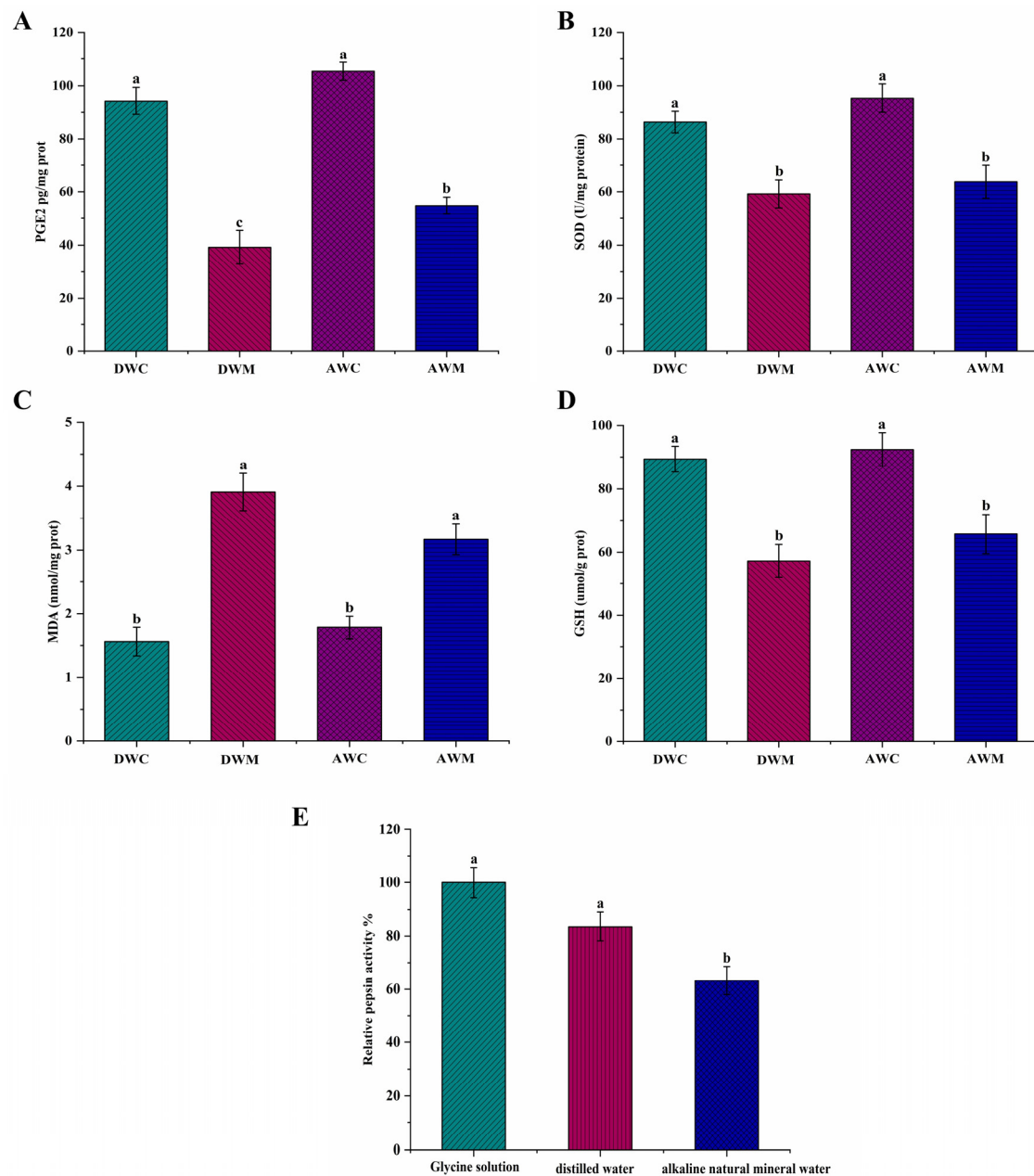
**Figure 2.** H&E staining ( $\times 200$ ) (A) and scores (B) of histological images of the gastric ulcer induced by ethanol in mice with two kinds of drinking water. DWC, distilled water control group; DWM, distilled water model group; AWC, alkaline natural mineral water control group; AWM, alkaline natural mineral water model group. In subfigure (B), different letters indicated significant difference ( $p < 0.05$ ). H&E score indicated the area of gastric mucosa injury ( $n = 6$ ): 0, no injury; 1, 0–10%; 2, 11–30%; 3, 31–50%; 4, 51–75%; 5, >75%.

### 3.2. Determination of Biochemical Parameters and Pepsin Activity in Tissues

The results of biochemical parameters in gastric tissue and pepsin activity are shown in Figure 3. The result of PGE2 in tissue was shown in Figure 3A, there was no significant difference between the two drinking water control groups ( $p > 0.05$ ). However, compared with those corresponding control groups, the PGE2 content in the two drinking water model groups were significantly decreased ( $p < 0.05$ ). Meanwhile, the PGE2 content in the group AWM was significantly higher than that in the DWM group ( $p < 0.05$ ). PGE2 is one of the most abundant prostaglandins (PGs) produced in the body, which has the most extensive characteristics in animal species; one of its important roles in the body is to participate in regulating the synthesis of gastric mucin, triggering the proliferation of mucosal cells, promoting angiogenesis and regulating gastric acid secretion [15–17]. Ethanol can damage gastric mucosa by damaging protective factors in gastric mucosa, such as PGE2. Compared with distilled water, alkaline natural mineral water can protect gastric mucosa by increasing the level of PGE2. The results of SOD, MDA, and GSH in gastric tissue are shown in Figure 3B–D. Compared with the two drinking water control groups, SOD and GSH in all model groups were significantly decreased ( $p < 0.05$ ), and MDA was significantly increased ( $p < 0.05$ ). Moreover, there was no significant difference between SOD, GSH, and MDA in gastric tissues of the groups AWM and DWM.

GSH, a compound containing mercaptans, is an important antioxidant in cells and has a protective effect against mucosal damage caused by peroxides. It also plays a crucial role as an electron donor for some antioxidant enzymes, such as glutathione peroxidase (GSH-PX) and glutathione S-transferase (GST) [18]. SOD can also protect gastric mucosa from oxidative damage by reducing the accumulation of free radicals in the body [19]. MDA is an important product of lipid peroxidation reaction, which can usually indirectly reflect the degree of lipid peroxidation reaction and cell damage in vivo. It also leads to cross-linked polymerization of proteins, nucleic acids, and other living macromolecules [20]. The results of this study showed that compared with DWM group, these biochemical parameters in group AWM had no significant improvement ( $p > 0.05$ ), suggesting that alkaline natural mineral water could not alleviate gastric ulcers by regulating antioxidant enzyme system in the body. However, Logozzi et al. found that alkaline water had a “sparing effect” on antioxidant enzymes such as SOD, which can directly inhibit the generation of free radicals in the body, thus having a good inhibitory effect on aging [21]. The difference between

findings about SOD of the present study and the study of Logozzi et al. may be due to that the different sources and/or consumption times of alkaline water.



**Figure 3.** Effects of two kinds of drinking water on the biochemical indexes of gastric tissue and plasma of mice induced by ethanol and the in vitro pepsin activity. Results of (A–D) are expressed as mean  $\pm$  S.E. ( $n = 6$ ), and results of (E) are expressed as mean  $\pm$  S.D. ( $n = 3$ ). In (E), the pH of distilled water was 5.6, the pH of alkaline natural mineral water was 9.5, and the pH of glycine solution was 3.0. Different letters indicated significant difference ( $p < 0.05$ ). PGE2, prostaglandin E2; GSH, glutathione; SOD, superoxide dismutase; MDA, malondialdehyde; DWC, distilled water control group; DWM, distilled water model group; AWC, alkaline natural mineral water control group; AWM, alkaline natural mineral water model group.

In addition, pepsinogen is converted to pepsin in acidic conditions, and excess pepsin can worsen gastric ulcers. A previous study has shown that the pH 8.8 alkaline water could protect larynx and esophagus from damage in laryngopharyngeal reflux and in gastroesophageal reflux diseases by irreversibly inactivating pepsin (in vitro) [11]. The



results of Figure 3E also showed that compared with the distilled water group, the activity of pepsin significantly decreased after alkaline natural mineral water treatment ( $p < 0.05$ ), and the decrease in pepsin activity is irreversible. Moreover, we also proved that boiled tap water ( $\text{pH} = 7.4 \pm 0.3$ ) and ordinary mineral water ( $\text{pH} = 7.2 \pm 0.4$ ) had no significant effect on the pepsin activity in vitro (data not shown), which may be used to explain from another angle the results obtained by Nassini et al. [12] that why tap water and reference water (ordinary water containing minerals) exhibited poor protection against ethanol-induced gastric ulcers. It is generally believed that an important mechanism for ethanol to destroy gastric mucosa is that ethanol can cause excessive gastric acid secretion. It has been reported that long-term administration of alkaline water can regulate the acid–base balance in the gastric tissue and balance gastric acid secretion to protect gastric mucosa from injury [12]. Moreover, gastric ulcers, as a type of pre-cancerous condition, could be alleviated by alkaline natural mineral water, indicating that long-term consumption of alkaline natural mineral water may be beneficial for preventing or slowing down gastric cancer caused by gastric ulcers, which, however, needs more studies to be further proved. Astigiano et al. have reported that the alkaline water treatment could be conducive to the alkalization of microenvironment to exert benefits on preventing or alleviating prostate cancer [22]. In addition, Azzarito et al. have also found that water alkalization can effectively control the growth of homologous melanoma in mice [23].

### 3.3. Expression of Oxidative Stress Pathway Related Proteins

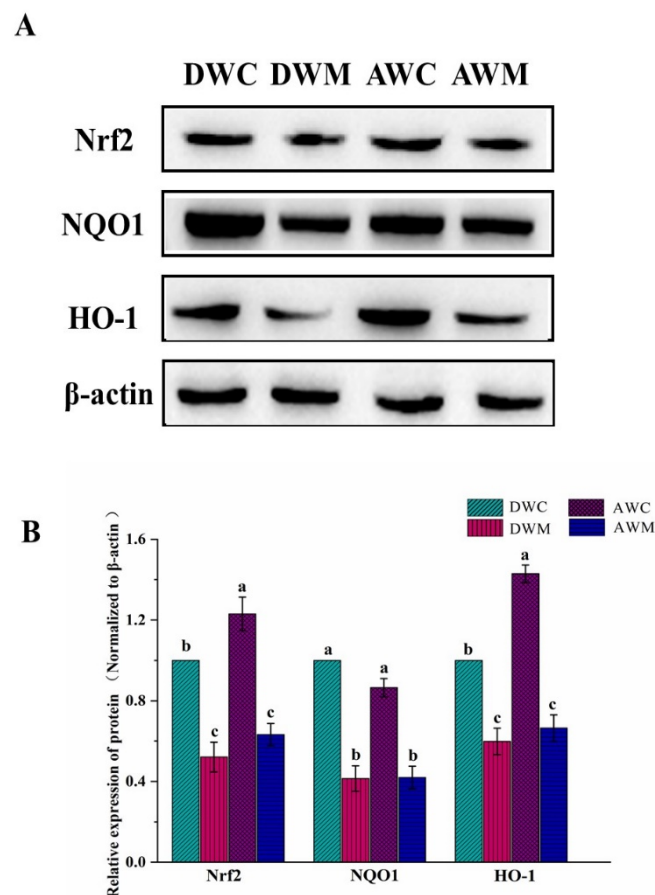
Oxidative stress is considered to be an important cause of ethanol-induced gastric ulcers. There are many proteins involved in the regulation of oxidative stress. Nrf2 is a redox-sensitive transcription factor that regulates the expression of genes encoding various antioxidant and detoxification enzymes; Nrf2 involved in oxidative stress has been confirmed to play a role in a variety of gastrointestinal diseases [24,25]. Nrf2/ Kelch-like ECH-associated protein 1 (Keap1) pathway is an important mechanism of collective defense against oxidative stress injury, which can regulate downstream phase II oxidase for antioxidant defense. When Nrf2 receives oxidative stress signal, it is separated from Keap1 and enters the nucleus. Then combined with antioxidant element antioxidant response element (ARE), regulate the expression of downstream genes HO-1, NQO1 to reduce oxidative stress [26]. HO-1 exerts its protective effect by degrading highly toxic heme, thereby maintaining the antioxidant capacity of cells [27]. NQO1 can catalyze the reduction in various active substances, which maintain redox state under oxidative damage stimulation [28]. In this study, the expression levels of oxidative stress-related proteins Nrf2, HO-1, and NQO1 were shown in Figure 4. Compared with the control group, the expression levels of Nrf2, HO-1, and NQO1 in the two drinking water models were significantly decreased ( $p < 0.05$ ). Compared with distilled water, alkaline natural mineral water cannot play a role by increasing the expression of oxidative stress pathway-related proteins. The results in Figure 3 also showed that alkaline natural mineral water had no significant effect on the regulation of antioxidant enzymes and lipid peroxidation in vivo as distilled water.

### 3.4. Expression of HSP70

When gastric mucosal cells are exposed to ethanol, gastric mucosal cells produce a stress response to ethanol and induce the expression of heat shock protein (HSP) to protect gastric mucosa. HSP70, a heat shock protein, which can promote wound healing and can be up-regulated by heat shock and other stresses; this protein shows markedly lower, or even undetectable, expression in unstressed normal cells and tissues [29,30]. When the body is stimulated by external stimuli, HSP70 is highly expressed to protect the body from injury [31]. Previous studies have reported that HSP70 family proteins have a very important protective effect on gastric mucosa. Some active compounds, such as 2'-hydroxy-4'-methoxychalcone and benzyl isothiocyanate, can improve peptic ulcer disease by increasing the expression of HSP70 in gastric tissue cells [9,32]. In ethanol-induced



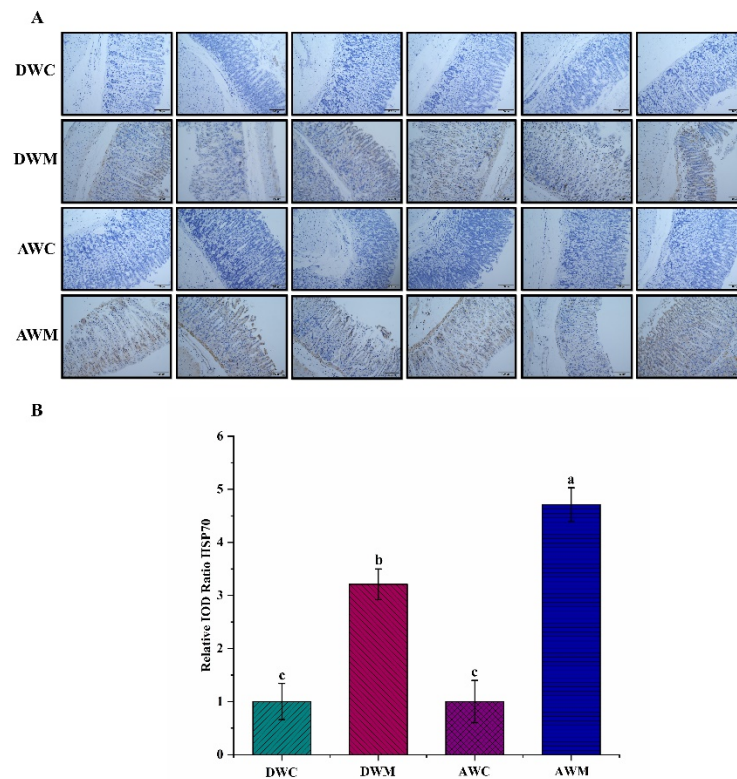
gastric ulcers, the expression of HSP70 is an important mechanism of body defense against ethanol injury, which can provide protection by retaining normal protein structure and removing damaged proteins [33]. In addition, it has been reported that the expression of HSP70 can accelerate the healing of gastric ulcers by increasing the level of PGE2 and the expression of growth factors, thereby stimulating cell proliferation at the edge of gastric ulcers and angiogenesis in granulation tissue [34]. Immunohistochemical results of HSP70 were shown in Figure 5. After ethanol stimulation, the expression levels of HSP70 in the stomach of mice in the DWM group and the AWM group were significantly higher than those in the control group without ethanol stimulation ( $p < 0.05$ ). In addition, the expression of HSP70 protein was significantly higher in the stomach of mice in the AWM group compared to the DWM group ( $p < 0.05$ ), which may be one of the reasons why gastric ulcers were significantly milder in the AWM group than in the distilled water group.



**Figure 4.** Western blot analysis (A) and quantification (B) of the expressions of Nrf2, NQO1, and HO-1 proteins in oxidative stress signaling pathway of ethanol-induced gastric ulcers in mice with two kinds of drinking water. Values are expressed as mean  $\pm$  standard error (S.E.) ( $n = 3$ ), and different letters of the same protein indicated significant difference ( $p < 0.05$ ). Nrf2, nuclear factor erythroid-2 related factor 2; NQO1, NADH, quinone oxidoreductase 1; HO-1, oxygenase-1; DWC, distilled water control group; DWM, distilled water model group; AWC, alkaline natural mineral water control group; AWM, alkaline natural mineral water model group.

This study compared the protective effects and potential mechanisms of distilled water (also known as acidic water) and alkaline natural mineral water on ethanol-induced gastric ulcers. However, both experiments were carried out in mice, which may be somewhat different to that in the human body. Therefore, in subsequent clinical trials, further studies about the effects of different drinking water, including acidic water (pure water or distilled water, pH = 5–6), boiled tap water and ordinary mineral water (pH = 6.5–8.5) and alkaline

mineral water (pH = 9.0–9.5), on patients with chronic gastric ulcers should be performed to prove the exactly protective effects of different drinking water on gastric ulcers.



**Figure 5.** HSP70 immunohistochemistry ( $\times 200$ ) (A) and quantification (B) of ethanol-induced gastric ulcers in mice with two kinds of drinking water. HSP70, heat shock protein 70; the values are expressed as mean  $\pm$  standard error (S.E.) ( $n = 6$ ), and different letters indicated significant differences ( $p < 0.05$ ); DWC, distilled water control group; DWM, distilled water model group; AWC, alkaline natural mineral water control group; AWM, alkaline natural mineral water model group.

#### 4. Conclusions

The results of this study show that compared with distilled water, alkaline natural mineral water has a certain mitigation and prevention effect on ethanol-induced gastric ulcers in mice, which may be due to its promotion of gastric mucosal protective factors (PGE2 and HSP70) expression and partial inhibition of pepsin activity. However, there was no significant difference between distilled water and alkaline natural mineral water in the regulation of SOD, MDA, and GSH levels and the expression of Nrf2/HO-1/ NQO1 related proteins in oxidative stress pathway. This study indicated that, compared with distilled water, alkaline natural mineral water has a certain mitigation and prevention effect on ethanol-induced gastric ulcers in mice, which may be by improving the levels of gastric mucous protective factor and inhibiting the pepsin activity. Thus, supplementing alkaline natural mineral water appropriately in daily life may be beneficial to improve ethanol-induced gastric ulcers.

**Author Contributions:** Investigation, Data curation, Y.S.; Writing—original draft, J.Z.; Methodology, J.Y.; Conceptualization, Supervision, Writing—review & editing, S.C. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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