Chronic Administration with Electrolyzed Alkaline Water Inhibits Aspirin-induced Gastric Mucosal Injury in Rats through the Inhibition of Tumor Necrosis Factor-α Expression

Yuji NAITO,* Tomohisa TAKAGI, Kazuhiko UCHIYAMA, Naoya TOMATSURI, Kiichi MATSUYAMA, Takaaki FUJII, Nobuaki YAGI, Norimasa YOSHIDA, and Toshikazu YOSHIKAWA

First Department of Medicine, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan

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Summary Neutrophils activation and tumor necrosis factor-α (TNF-α) induction play critical roles in aspirin-induced gastric mucosal injury. The aim of the present study was to determine whether electrolyzed alkaline water (EAW) can ameliorate aspirin-induced gastric mucosal injury in rats, and whether EAW can inhibit the increased gastric TNF-α expression associated with neutrophil accumulation and gastric epithelial cell apoptosis. EAW (pH 10.5, oxidation-reduction potential -450 mW) was produced by electricity resolution of tap water with a device that used a platinum electrode. EAW was administered to rats via free drinking for 14 days. Aspirin-induced injury was produced by the intragastric administration of aspirin (200 mg/kg) and HCl (0.15 N, 8.0 ml/kg). After 3 h the animals were killed, and the gastric mucosal tissue was used for the assessment of macroscopic damage and tissue-associated myeloperoxidase (MPO) activity, the quantitation of TNF-α protein, and the assay of epithelial cell apoptosis. The expression of TNF-α mRNA was determined by reverse transcription polymerase chain reaction (RT-PCR) 1 h after aspirin administration. In the group drinking tap water, intragastric administration of acidified aspirin induced hyperemia and hemorrhagic erosions in rat stomachs. The increase in the total gastric erosive area after aspirin administration was significantly inhibited by pretreatment with EAW. The increases in MPO activity and epithelial cell apoptosis after aspirin administration were significantly inhibited by treatment with EAW. The gastric content of TNF-α increased and the expression of TNF-α mRNA was up-regulated after aspirin treatment. However, the peak TNF-α mRNA expression 1 h after aspirin administration was inhibited by EAW. Based on these data, we conclude that the

*To whom correspondence should be addressed.: First Department of Medicine, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan. E-mail: ynaito@koto.kpu-m.ac.jp
beneficial effects of EAW on aspirin-induced gastric mucosal injury may be attributed to its anti-inflammatory properties via inhibition of TNF-α expression.

**Key Words:** electrolyzed alkaline water, cytoprotection, aspirin, inflammation, tumor necrosis factor-α

Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine, mainly produced by activated macrophages, which signals cytotoxic and inflammatory activities and up-regulates the expression of adhesion molecules on leukocytes and endothelial cells [1–3]. Recent reports have hypothesized that TNF-α is a crucial mediator of aspirin-induced gastric mucosal injury associated with inflammation. Five lines of evidence support this hypothesis: 1) aspirin administration in rats results in an early increase in plasma TNF-α concentrations [4], 2) aspirin causes a time- and concentration-dependent increase in macrophage TNF-α mRNA accumulation and cytokine release [5], 3) gastric mucosal injury induced by oral aspirin administration is caused by TNF-α-dependent activation of an apoptosis signaling cascade [6], 4) TNF-α exerts direct cytotoxic effects on gastric epithelial cells [6, 7], as well as endothelial cells via the induction of apoptosis, and 5) the addition of a TNF-α-processing enzyme inhibitor prevents aspirin-induced TNF-α release and protects against gastric muscle injury in rats [5]. These data show that blockade of the TNF-α activation pathway may represent a valuable therapeutic strategy for treating aspirin-induced gastropathy.

Electrolyzed alkaline water (EAW), which is produced by electricity resolution, has been shown to be clinically effective in the treatment of patients with irritable bowel syndrome or non-ulcer dyspepsia [8]. In Japan, EAW is well known as “alkaline-ion-water,” and it is used as a health promotion aid, although the mechanism causing its effectiveness has never been elucidated. In this study, we show that continuous administration with EAW attenuates the gastric mucosal injury and gastric inflammation induced by aspirin. Moreover, EAW interferes with the expression of TNF-α in vivo and therefore may represent a preventive and therapeutic agent against aspirin-induced gastric mucosal injury.

**MATERIALS AND METHODS**

**Reagents.** All chemicals were prepared immediately before use. Electrolyzed alkaline water (EAW; pH 10.5, oxidation-reduction potential −450 mV) was obtained from a cathode chamber of Alkaline-Ion-Water Electrolyzer (TK780, National Ltd., Osaka, Japan), which is a commercial electrolyzer in Japan. 3,3',5,5'-Tetramethylbenzidine was obtained from Wako Pure Chemicals (Osaka, Japan). Isogen was purchased from Nippon Gene (Toyama, Japan), and Taq DNA polymerase was from Takara Shuzo Co. (Shiga, Japan). A TNF-α enzyme-linked immunosorbent assay (ELISA) kit was obtained from BioSource Int. (Camarillo, CA), and a Cell Death Detection ELISA kit was from Roche Diagnostics Co. (Indianapolis, IN). All other chemicals used were of reagent grade.

**GASTROPROTECTION BY ELECTROLYZED ALKALINE WATER**

Measurements of gastric acid secretion and serum level of gastrin. Gastric acid secretion was evaluated by the pylorus-ligation method. Under brief ethyl ether anesthesia, a ventral abdominal incision was made along the midline and the pylorus was ligated. Four hours after the treatment, rats were reanesthetized with ethyl ether and killed. The stomachs were removed, and the gastric contents were collected and centrifuged. The volume of gastric juice was measured and the acidity was determined by titration with 0.01 N NaOH to a pH of 7.0. Gastric acid output was calculated by the following formula; gastric acid output (mEq/h)=volume (ml/4 h)×acidity (mEq/ml)/4 h. Serum gastrin levels were determined using a Gastrin RIA-kit II (Dainabot, Tokyo, Japan).

Gastric injury model. Male Sprague-Dawley rats weighing 180–200 g were obtained from Keari Co., Ltd. (Osaka, Japan). The animals were housed at 22°C in a controlled environment with 12 h of artificial light per day. They were fed a standard diet (Oriental Yeast, Tokyo, Japan) and EAW, alkaline solution adjusted to pH 10.5 with NaOH, calcium lactate solution (40 ppm Ca²⁺), or tap water ad libitum. The rats were not fed for the 18 h prior to the experiments, but they were allowed free access to water. Aspirin-induced injury was produced by the intragastric administration of aspirin (200 mg/kg) and HCl (0.15 N, 8.0 ml/kg).

Evaluation of gastric mucosal lesions. Rats were anesthetized with urethane (1 mg/kg, i.p.) before they were killed by exsanguination from the abdominal aorta and the stomach was removed. To estimate the severity of the gastric erosions induced by aspirin-HCl, the total area of the red gastric lesions was measured using a dissecting microscope (×10 magnification) by a person blinded to the experimental treatment. For histological evaluation, formalin-fixed tissues were stained with hematoxylin and eosin and evaluated light-microscopically by a pathologist unaware of the experimental conditions.

Measurement of tissue-associated myeloperoxidase (MPO) activity. Tissue-associated MPO activity was determined as an index of neutrophil accumulation by a modification of the method of Grisham et al. [9]. The rats were killed by rapid exsanguination after the experiments, and the stomachs were removed. The gastric mucosa was scraped off using two glass slides and homogenized with 1.5 ml of 10 mM potassium phosphate buffer (pH 7.8) containing 30 mM KCl in a Teflon Potter-Elvehjem homogenizer. Two milliliters of mucosal homogenate was centrifuged at 20,000×g for 15 min at 4°C to precipitate the insoluble cellular debris. The supernatant was used for the measurement of gastric cytokine concentration. The pellet was rehomogenized in an equal volume of 0.05 M potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide. The samples were centrifuged at 20,000×g for 15 min at 4°C, and the supernatants were saved. MPO activity was assessed by measuring the H₂O₂-dependent oxidation of 3,3',5,5'-tetramethylbenzidine, and it was expressed as units per mg protein. One unit of enzyme activity was defined as a change in absorbance of 1.0/min at 655 nm and 25°C. The total protein concentration in the tissue homogenates was measured by the method of Lowry et al. [10].

Determination of gastric content and mRNA expression of TNF-α. The concentrations of TNF-α in the serum and the supernatant of the mucosal homogenates were determined by an ELISA kit specific for rat TNF-α with a sandwich method. The assay was
performed according to the manufacturer’s instructions. After color development, optimal densities were measured at 450 nm with a micro plate reader (MPR A4i, Tosoh, Tokyo). The minimum detection level of TNF-α was 4 pg/ml, and the assay has no cross-reactivity with other cytokines. The gastric concentration of TNF-α was expressed as pg per mg protein.

For the quantification of TNF-α mRNA, rats were killed at 1 h after aspirin administration. The gastric wall was rinsed in phosphate-buffered saline (PBS), snap-frozen in liquid nitrogen, and then stored at −80°C until the time of RNA extraction. Total RNA was extracted with the single-step guanidium thiocyanate-phenol-chloroform method using Isogen. The concentration of RNA was determined by the absorbance at 260 nm relative to that at 280 nm. The RNA was used for reverse-transcription polymerase chain reaction (RT-PCR) amplification. The amplification was carried out in a 50-ml mixture containing 2 ml of the RT product, 0.6 mM of both the sense and antisense primers, 0.4 mM dNTP mixture, and 0.5 ml Taq DNA polymerase. The reaction was performed as follows: 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 60 s) followed by a final extension step of 7 min at 72°C. The primers had the following sequences: for TNF-α, sense 5'-ATGAGCACAGAAAGCATGATC-3' and antisense 5'-TACAGGCTTGTCACTCAATT-3'; for β-actin, sense 5'-ATCGTGGGCCGCCCTAGGCA-3' and antisense 5'-TGGCCTTAGGGTTTCAGAGGGG-3' [11]. The PCR reaction products were separated electrophoretically in a 2.5% agarose gel and stained by ethidium bromide.

DNA fragmentation assay. Quantitative measurement of epithelial cell apoptosis was done with a sandwich enzyme immunoassay directed against cytoplasmic histone-associated DNA fragments using a Cell Death Detection ELISA kit. The mucosal scrapings were dispersed by homogenization and settled by centrifugation. The cell aliquots were then subjected to incubation in the lysis buffer in accordance with the manufacturer’s instruction, and the lysates were centrifuged at 20,000×g for 10 min, after which the diluted supernatant containing the cytoplasmic histone-associated DNA fragments reacted in the microtitrator wells with immobilized anti-histone antibody. After being washed, the retained complex was reacted with anti-DNA peroxidase, and the immunocomplex-bound peroxidase was probed with 2,2-azino-bis-ethylbenzthiazoline sulfonate reagent for spectrophotometric detection.

Statistics. The results are presented as the mean±SEM. The data were compared by two-way analysis of variance (ANOVA), and differences were considered significant if the p value was less than 0.05 based on two-tailed Scheffe’s multiple comparison test. All analyses were performed using Stat View 5.0-J program (Abacus Concepts, Inc., Berkeley, CA) on a Macintosh computer.

Ethical considerations. Maintenance of animals and experimental procedures were carried out in accordance with the U. S. National Institutes of Health Guidelines for the Use of Experimental Animals. All experiments were approved by the Kyoto Prefectural University of Medicine Animal Care Committee (Kyoto, Japan).
RESULTS

Effect of EAW on general appearances and gastric acid secretion

We firstly checked the general appearances of the mice administered with vehicle alone or EAW alone for 2 weeks by measuring body weight, liver and renal function tests, and peripheral blood cell counts. As shown in Table 1, there were no significant differences between the tap water group and the EAW group. Next we determined the effect of EAW on gastric acid secretion and serum level of gastrin. As shown in Tables 1 and 2, there were no differences in these data between two groups.

Effect of EAW on aspirin-induced gastric mucosal injury

Neither the vehicle alone nor EAW alone produced any macroscopic lesions in the rat stomachs. In the rats treated with the vehicle plus aspirin-HCl, multiple erosions and bleeding developed in the glandular stomach after aspirin administration in a time-dependent manner (Fig. 1a). EAW treatment by gastric intubation 1 h before aspirin administration did not affect the total area of gastric erosions induced by aspirin; however, continuous treatment with EAW for 14 days significantly reduced the size of the gastric erosions (Fig. 1b). The total area of gastric erosions in animals receiving tap water was 45.8±6.3 mm² 3 h after aspirin administration. EAW significantly reduced the lesion area by 57% (Fig. 1b). The protective effect of EAW was confirmed histologically. Aspirin

Table 1. Effects of electrolyzed alkaline water (EAW) on body weight, biochemical parameters, and peripheral blood cell counts.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>EAW</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>376±15</td>
<td>383±14</td>
</tr>
<tr>
<td>Biochemical parameters</td>
<td></td>
<td></td>
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<tr>
<td>Gastrin (pg/ml)</td>
<td>116.8±18.4</td>
<td>110.8±14.0</td>
</tr>
<tr>
<td>ALT (KU)</td>
<td>140±16</td>
<td>149±21</td>
</tr>
<tr>
<td>AST (KU)</td>
<td>33±3</td>
<td>31±4</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>9.8±1.4</td>
<td>17.0±1.2</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.56±0.03</td>
<td>0.51±0.04</td>
</tr>
<tr>
<td>Peripheral blood cell counts</td>
<td></td>
<td></td>
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<tr>
<td>WBC (/mm³)</td>
<td>10,225±1,172</td>
<td>12,133±1,329</td>
</tr>
<tr>
<td>Neutrophil (/mm³)</td>
<td>6,521±862</td>
<td>7,634±1,032</td>
</tr>
<tr>
<td>RBC (×10⁶/mm³)</td>
<td>930±40</td>
<td>922±21</td>
</tr>
<tr>
<td>PLT (×10⁴/mm³)</td>
<td>131±13</td>
<td>131±20</td>
</tr>
</tbody>
</table>

Table 2. Gastric acid secretion and serum levels of gastrin in vehicle- and electrolyzed alkaline water (EAW)-treated rats.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Secretion volume (ml/4 h)</th>
<th>Acid output (mEq/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>4</td>
<td>4.43±0.46</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>EAW</td>
<td>4</td>
<td>5.14±1.25</td>
<td>0.14±0.04</td>
</tr>
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administration resulted in large areas of epithelial crypt loss, predominantly neutrophilic infiltrate throughout the mucosa and submucosa, erosion, and mucosal bleeding. In contrast, chronic administration with EAW resulted in smaller erosions with few neutrophils (Fig. 2).

To confirm the gastroprotective action of EAW, we compared the effects of EAW (pH 10.5, 40 ppm Ca²⁺), alkaline solution adjusted to pH 10.5, and calcium lactate solution (40 ppm Ca²⁺) on aspirin-induced gastric mucosal injury. Alkaline solution, calcium lactate solution, or alkaline plus calcium solution did not attenuate the total area of gastric erosions after aspirin administration, but these injuries were significantly inhibited by the treatment with EAW (Fig. 3).

**Effect of EAW on myeloperoxidase activity in the gastric mucosa**

Tissue-associated myeloperoxidase (MPO) activity in the gastric mucosa significant-

![Fig. 1. Time-course changes in the area of gastric erosions after the administration of aspirin-HCl (a) and the effects of chronic or acute administration of electrolyzed alkaline water (EAW) (b). Treatment with EAW was performed by gastric intubation 1 h before aspirin administration or by providing a supply for free drinking for 14 days. Values are the mean±SE for 6 to 8 rats. *p<0.01 when compared with control rats receiving tap water alone, and #p<0.01 when compared with rats receiving tap water plus aspirin-HCl.](image)
Fig. 2. Effect of electrolyzed alkaline water (EAW) on acute gastric mucosal injury induced by aspirin-HCl in rats. Multiple hemorrhagic erosions with acute edema developed in the glandular stomach of rats 3 h after administration of aspirin-HCl (A), and chronic administration with EAW markedly reduced these erosions and edematous lesion (B).

Fig. 3. The effect of electrolyzed alkaline water (EAW), tap water, alkaline solution (NaOH), calcium lactate solution (Ca), and NaOH plus Ca on the total area of gastric erosions induced by aspirin-HCl. Values are the mean±SE for 6 to 8 rats. *p<0.01 when compared with rats receiving tap water plus aspirin-HCl.
ly increased 1 h after aspirin administration, and this increase in MPO activity tended to precede the development of complete lesion (Fig. 4). The increase in MPO activity in the gastric mucosa after aspirin administration was significantly inhibited by treatment with EAW (Fig. 4).

**Effects of EAW on DNA fragmentation of gastric mucosa**

By measuring cytosolic histone-associated DNA fragments using a specific ELISA system, the extent of epithelial cell apoptosis caused by aspirin administration was determined. Aspirin caused a significant increase in gastric epithelial cell apoptosis. Pretreatment with EAW significantly inhibited the increase in epithelial cell apoptosis-associated DNA fragmentation (Fig. 5).

![Graph](image1)

**Fig. 4.** The effect of electrolyzed alkaline water (EAW) on neutrophil accumulation in the gastric mucosa induced by aspirin-HCl. Myeloperoxidase (MPO) activity was determined as an index of neutrophil accumulation. Values are the mean±SE for 6 to 8 rats. *p<0.01 when compared with control rats receiving tap water alone, and 5p<0.05 when compared with rats receiving tap water plus aspirin-HCl.

![Graph](image2)

**Fig. 5.** The effect of electrolyzed alkaline water (EAW) on DNA fragmentation of gastric mucosa after aspirin-HCl administration. Values are the mean±SE for 6 to 8 rats. *p<0.01 when compared with control rats receiving tap water alone, and 5p<0.01 when compared with rats receiving tap water plus aspirin-HCl.
Effects of EAW on serum levels of TNF-α and the gastric TNF-α expression

Serum concentration of TNF-α significantly increased 1 h after aspirin administra-
tion and the increase in TNF-α tended to paralleled in the development of erosive lesions (Fig. 6a). The gastric concentration of TNF-α increased significantly from a basal concentration of 0.425±0.100 pg/mg protein to 0.758±0.075 pg/mg protein 3 h after aspirin treatment. EAW treatment significantly decreased the concentration of TNF-α compared with the effect of drinking only tap water (Fig. 6b). To further analyze the effects of EAW on experimental gastric inflammation, we assessed gastric mRNA expression for TNF-α using semiquantitative RT-PCR yielding a 276-bp product to identify TNF-α gene expression. As shown in Fig. 7, we found TNF-α gene expression in control animals to be negligible or faint. In contrast, the expression of TNF-α mRNA was up-regulated in the stomach 1 h after aspirin treatment. The increased expression of TNF-α mRNA was inhibited by EAW treatment for 14 days (Fig. 7).

DISCUSSION

The present study demonstrate for the first time that continuous administration of EAW for 14 days attenuates aspirin-induced gastric muscle injury in rats. Neither acute treatment of EAW 1 h before aspirin administration nor chronic administration of alkaline water, which was adjusted to pH 10.5 by NaOH, showed gastroprotective properties against aspirin-induced gastric muscle injury. Because EAW contains 40-ppm of calcium ions, we compared the effects of EAW with those of calcium lactate solution. Neither calcium lactate solution nor alkaline plus calcium lactate solution caused gastroprotective actions. We found by in vitro study that EAW did not cause cytoprotection against cultured gastric mucosal cell injury induced by aspirin or other cytotoxic agents (data not shown). These results indicate that gastroprotective action is indirectly induced by the continuous treatment with EAW but not by the direct action of EAW.

Because the gastric acidity seems to be one of the important factors on absorption of aspirin, gastric injury was produced by the intragastric administration of aspirin and HCl in the present study. In addition, there was no significant difference in gastric acid secretion between the tap water alone group and the EAW alone group. These results indicate that the gastroprotective action of EAW was not induced by reducing the gastric acidity which affects absorption of aspirin.

Researchers recently have hypothesized that neutrophil-mediated inflammation is involved in the development of aspirin-induced gastric mucosal injury. Five lines of evidence support this hypothesis: 1) neutrophil depletion by intraperitoneal injection of antineutrophil serum significantly attenuates the gastric muscle injury induced by aspirin [12], 2) intravital microscopic evaluation of the mesenteric circulation has shown that aspirin promotes leukocyte adherence and emigration in postcapillary venules [13, 14], 3) immunoneutralization of the CD11/CD18 adherence complex on neutrophils attenuates aspirin-induced injury [15], 4) aspirin directly promotes neutrophil adherence to endothelium via CD11b/CD18-dependent interactions with ICAM-1 [16, 17], and 5) the expression of the adhesion molecules CD11a and ICAM-1 on the normal gastric mucosa and the number of stained blood vessels containing ICAM-1 increase rapidly after aspirin treatment [18]. At first we compared the counts of peripheral blood cells including neu-
trophil, however, there were no significant differences in these counts between the tap water group and the EAW group. The present study shows that MPO activity, an index of tissue-associated neutrophil accumulation, increases in the gastric mucosa 1 h after aspirin administration in rats treated with vehicle solution before the lesion is completed, and that the increase in MPO activity is significantly inhibited by treatment with EAW. These results indicate that the inhibition of neutrophil accumulation by EAW may be one of the protective factors decreasing aspirin-induced gastric muscle injury.

Previous investigations have shown that apoptosis pathways are involved in the pathogenesis of gastric mucosal injury induced by non-steroidal anti-inflammatory drugs [6, 19].

Slomiany et al. [19] have shown that indomethacin causes extensive multiple hemorrhagic lesions accompanied by a dramatic enhancement in gastric epithelial cell apoptosis. Fiorucci et al. [6] have demonstrated that short- and long-term (7 days) aspirin administration results in a time- and dose-dependent gastric injury that is associated with apoptosis and caspase up-regulation. The present results showed that oral aspirin administration resulted in an increase in gastric mucosal cell apoptosis as detected by DNA fragmentation, and that this enhancement of apoptosis was significantly reduced by procurement with EAW. This effect of EAW may be also reflected in the ability to prevent the gastric mucosal damage caused by aspirin.

The mechanism responsible for neutrophil accumulation and gastric epithelial cell apoptosis after aspirin administration remains to be determined. TNF-α is one of the candidate factors involved in these phenomena observed after aspirin administration. TNF-α is a proinflammatory cytokine that stimulates the production of several cytokines such as interleukin 1β and interleukin 8 [20], and augments neutrophil-endothelial cell interaction through up-regulation of the expression of adhesion molecules on these cells [3]. In addition, gastric mucosal apoptosis after oral aspirin administration is caused by TNF-α-dependent activation of interleukin 1β-converting enzyme-like cysteine protease. TNF-α directly induces apoptosis of gastric epithelial cells [6, 7] as well as endothelial cells [4]. More directly, portal infusion of TNF-α causes gastric intestinal damage in rats, with nearly total loss of the mucosa [21].

The present study has demonstrated that continuous treatment with EAW inhibits the increase in the TNF-α concentration as well as the expression of TNF-α mRNA in the gastric mucosa after aspirin administration. These findings support the hypothesis that EAW attenuates aspirin-induced neutrophil accumulation and gastric epithelial cell apoptosis by inhibiting the expression of TNF-α. However, the mechanism by which EAW causes the inhibition of TNF-α expression remains unclear. Previous studies have implicated redox signaling and the nuclear transcription factor nuclear factor-κB in macrophage activation [22, 23]. Recent studies revealed the high concentrations of hydrogen particles as a colloidal solution in the EAW [24], which may affect the intracellular redox state of gastric inflammatory cells, such as monocytes and macrophages. Further studies will be required to clarify the precise mechanism responsible for the EAW-induced decrease in TNF-α expression.

In summary, EAW significantly inhibits the acute gastric mucosal injury induced by
aspirin in rats. This effect may be due, in part, to a reduction in the neutrophil infiltration into the gastric mucosa and to a decrease in gastric epithelial cell apoptosis, possibly via the inhibition of TNF-α production. These results indicate that modulation of TNF-α expression will promote the development of novel strategies to treat aspirin-induced gastropathy.

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