The Study of Anti-inflammatory Effect of Curcumae Longae Rhizoma 30% EtOH Extract on Chronic Acid Reflux Esophagitis Rats

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Research

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Abstract

Background: Gastroesophageal reflux disease (GERD) is associated with hypotensive lower esophageal sphincter, frequent transient lower esophageal sphincter relaxation and esophageal hypersensitivity, and often precedes the development of Barrett's esophagus and esophageal adenocarcinoma. GERD is a very common diseased, and is on the rise worldwide. The purpose of this study is to investigate the pharmacological efficacy of Curcumae Longae Rhizoma 30% EtOH extract (CLR) on the chronic acid reflux esophagitis (CARE) rats.

Methods: CLR was measured antioxidant activity, such as total polyphenol and total flavonoid contents, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), and 2, 2′-azinobis-3-ethyl-enzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity. And, to confirm the effect of CLR on esophageal in CARE-model, gross esophageal tissue was observed, and esophageal mucosal infiltration and inflammatory cell changes were observed under a microscope. We measured the level of reactive oxygen species (ROS) and peroxynitrite ($ONOO^-$) in serum. Also, we measured the level of 2-thiobarbituric acid-reactive substance (TBARS) in serum and esophageal tissue. In addition, the antioxidant and inflammatory protein levels were investigated western blot analysis.

Results: Administration of CLR to rats of induction of CARE was found to reduce esophagus tissue injury. ROS, $ONOO^-$, and TBARS levels were significantly decreased in CLR compared with Veh rats. In addition, CLR was effectively reduced inflammatory protein, and increase antioxidant-related factors.

Conclusions: Overall, CLR treatment suggested that markedly ameliorated the inactivation of NF-κB by blocking the phosphorylation of IκBα led to the inhibition of the expressions of proinflammatory proteins.

Background

The gastroesophageal reflux disease (GERD) is a disease involving epithelial metaplasia and mucosal damage, which is caused by duodenal and gastric contents with or without vomiting or regurgitation and the typical symptoms of GERD consist regurgitation, heartburn, belching, nausea, dysphagia, and angina-like retrosternal chest pain [1–4]. GERD is common gastrointestinal disorders with a recurrent or chronic apparatus, the prevalence corresponds about 2.5 ~ 25%, especially, Western countries are much higher than that of Asia [4–6]. The cause of GERD are a reduction in lower esophageal sphincter (LES) function, alteration of esophageal peristalsis, impairment of esophageal clearance, and increase from the transient LES relaxation (TLESR) [7, 8]. Furthermore, family history and lifestyles such as westernized diet, smoking, alcoholism, and social stresses increase the risk factor of GERD [6].

Various agents to treat GERD included proton pump inhibitors (PPIs) and non-steroidal anti-inflammatory drug (NSAID) [9, 10]. Among these agents, PPIs has an excellent effect on esophageal mucosal healing by blocking HCl secretion and inhibiting H+/K + ATPase [11, 12]. However, long-term use of PPI has a high relapse rate of 11–35%, with complications and side effects. For the reason, there are careful about the

use of PPIs by patients and prescribing physicians [13, 14]. Therefore, many studies focused on finding a stable natural material with fewer side effects and effective efficacies [15].

Curcuma longa L. (Curcumae Longae Rhizoma) is belonging a genus of Curcuma in the ginger family (Zingberaceae), has been used in Korean medicine as a treatment for peptic ulcer. *Curcuma longa* L. is cultivated throughout Asia and has been used to reduce the acid release, treat gastric mucosal damage, and prevent diseases including liver injury [16–18]. In addition, a recent study confirmed that *Curcuma longa* L. has a protective effect on the esophageal mucosa in an animal model of acute reflux esophagitis [19]. *Curcuma longa* L. contains curcuminoids such as curcumin, demethoxycurcumin, and isdemethoxycurcumin, among which curcumin the lipophilic polyphenol compound which is quite stable in the acidic pH of stomach [20]. Also, Curcumin is exhibited anti-oxidant, and anti-inflammatory properties. Free radical-mediated peroxidation and damage caused by oxidative stress leads to a variety of chronic pathological complications, and curcumin is known to exhibit anti-inflammatory effects by inhibiting inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [21].

Inflammation, which is the compensatory response about tissue injury is induced in the increase of leukocytes and inflammatory factors such as cytokines and chemokines [22]. Furthermore, excessive inflammation can lead to systemic or chronic inflammatory diseases [23]. Especially, inflammatory response by nuclear factor-κB (NF-κB) activation promotes the expressions of iNOS, COX-2, and cytokines such as TNF-α, IL-6, and IL-1β to induce an inflammatory response. So, many studies have been conducted as a new strategy to develop various anti-inflammatory drugs targeting NF-κB [24, 25].

The purpose of this study is to demonstrate whether *Curcuma longa* L. exerts the protective effect via the inhibition of NF-κB activation in reflux esophagitis rats.

Methods

Materials

L-(+)-ascorbic acid and diethylene glycol were purchased from Alfa Aesar (Lancashire, UK). Folin-ciocalteu's phenol reagent, 2,2-Diphenyl-1-picrylhydrazyl, potassium persulfate, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 2-Thiobarbituric acid, gallic acid, naringin, phenyl methane sulfonyl fluoride (PMSF), and 1,1,3,3-Tetramethoxypropane were purchased from Sigma-Aldrich (St, Louis, MO, USA). Sodium carbonate was purchased from daejung chemicals & metals Co., Ltd. (Siheung, Korea). Sodium hydroxide was purchased from OCI Company Ltd. (Seoul, Korea). Phosphoric acid was purchased from Duksan company (Ansan, Korea). 2',7'-Dichloro fluorescein diacetate (DCF-DA) was purchased from Molecular Probes (Eugene, OR, USA). The protease inhibitor mixture solution and ethylene diamine tetra acetic acid (EDTA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The pierce BCA protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). ECL Western Blotting Detection Reagents and pure nitrocellulose membranes were purchased from GE Healthcare (Chicago, IL, USA). Rabbit polyclonal antibodies against nuclear factor

erythroid-derived 2-related factor 2 (Nrf2), NADPH oxidase 4 (NOX4), $p22^{phox}$, heme oxygenase-1 (HO-1), superoxide dismutase (SOD), catalase, glutathione peroxidase-1/2 (GPx-1/2), and inhibitor of nuclear factor κ B α (IκBα); Goat polyclonal antibodies against tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β); Mouse polyclonal antibodies against nuclear factor-κ B p65 (NF-κBp65), phospho-inhibitor of nuclear factor kappa B α (p-IκBα), inducible nitric oxide synthase (iNOS), cycloxygenase-2 (COX-2), histone, and beta-actin were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). And, goat anti-rabbit, rabbit anti-goat, and goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated secondary anti-bodies were purchased from GeneTex, Inc. (Irvine, LA, USA).

Preparation of the plant material

Curcumae longae Rhizoma was purchased from Daemyung Pharm. Co. Ltd (Seoul, Korea), and the origin is India. A voucher herbarium specimen was verified at the college of Korean medicine in Daegu haany university. The herb dried CLR (100 g) was extracted by 10 times of EtOH : distilled water (3 : 7) during 24 h at room temperature. The mixture was concentrated in evaporated *in vacuo*, and dried completely using a freeze dryer to obtain a powder (the yield rate of CLR ; 7.1%). Powder stored at -80℃.

CLR analysis by HPLC chromatogram

The extract of CLR (1 mg) was dissolved in 1 mL of 70% methanol. 10 μL of sample was injected into high performance liquid chromatography (HPLC) using YMC-pack pro C18 RS (4.6 × 150 mm, 5 μm). The mobile phase was composed of Acetonitrile (A) and Acetic acid (B), and its flow rate was 1.0 mL/min. The UV absorbance from 420 nm was monitored using a Waters e2695 (Water Corporation, MA, USA), and the column temperature was kept at a constant 25℃ throughout the analysis. The peak areas were used to calculate the sample contents of the compounds. Representative HPLC results are illustrated in Fig. 1. The amount was as follows: Curcumin : 4.05 μg/mL.

DPPH free radical scavenging activity

The anti-oxidative effect of CLR was determined by the DPPH radical scavenging assay [26]. 100 μL of CLR (blank; 100 μL of distilled water) was added to equal volumes of an ethanolic solution of DPPH (60 μM) in a 96-well microplate. L-ascorbic acid was used as a standard sample. The reaction mixtures were incubated at 20℃ for 30 min in the dark and the optical density was determined using a UV-VIS spectrophotometer, model infinite M200 Pro (Tecan, Switzerland) at 540 nm. The antioxidant activity of each sample was expressed by IC_{50}. The radical scavenging activity was calculated as %.

ABTS free radical scavenging activity

The antioxidative effect of CLR was determined by the ABTS radical scavenging assay [27]. L-ascorbic acid was used as a standard sample. The ABTS solution was dissolved in water at 7.4 mM concentration. ABTS free radical cation ($ABTS^{+}$) was produced by reacting ABTS stock solution with 2.45 mM and potassium sulfate and leaving the mixture in the dark for 16 ~ 18 h at 20℃. Calibrate the ABTS

solution with ethanol to have an absorbance of 0.70 ± 0.02 at 415 nm. After adding 95 μL of ABTS solution to 5 μL of each of the sample, the mixture was left for 15 min at 20℃ in the dark. And, the optical density was determined using a UV-VIS spectrophotometer, model infinite M200 Pro at 415 nm. The antioxidant activity of each sample was expressed by IC_{50}. The radical scavenging activity was calculated as %.

Total polyphenol and total flavonoid contents

Total polyphenol content was measured by method of Folin-Denis [28]. 10 μL of each sample was mixed with 790 μL of distilled water and 50 μL of Folin-Ciocalteu's phenol reagent, followed by reaction at 20℃ for 1 min, and then 150 μL of 20% sodium carbonate was added. After reacting with 2 h at 20℃, absorbance was measured at 765 nm using a UV-VIS spectrophotometer, model infinite M200 Pro. Gallic acid was used to plot a standard calibration curve and calculate the total polyphenol content of the sample.

Total flavonoid content was measured by the method of Lister *et al.* [29]. 1 mL of diethylene glycol, 100 μL of each sample, and 10 μL of 1 N NaOH were mixed well and reacted at 37℃ for 1 h, and then the absorbance was measured at 420 nm using a UV-VIS spectrophotometer, model infinite M200 Pro. Naringin was used to plot a standard calibration curve and calculate the total flavonoid content of the sample.

Chronic acid reflux esophagitis model (CARE)

The animal experiments were performed according to the ‘Guidelines for Animal Experiment’ approved by Ethics Committee of the Daegu Haany University (Approval No. DHU2019-118). 5-week-old male Sprague-Dawley rats (B.W. 130 ~ 150 g) were purchased from DaehanBioLink (Eumseong, Korea) and used for experiments after being adapted to environment for 1 week. Environmental conditions were set to 12 h light/dark cycle, controlled humidity (50 ± 5%) and temperature (22 ± 2℃). After 1 week adaptation, rats were fasted 18 h prior to surgery and maintained with a raised mesh-bottom cage to prevent co-propagation, and water was supplied until surgery. Before surgery, rats were anesthetized by intraperitoneal injection of 30 mg/kg doses of zoletil (Virbac S.A., France) + 10 mg/kg doses of rompun (Bayer, Germany). After that, a midline laparotomy was performed to expose the stomach and the transitional region (i.e., limiting ridge) between the fundus and the glandular portion of the stomach was ligated with 2–0 silk thread in order to restrict the compliance of the stomach. Additionally, a latex ring (2 mm in thickness; ID, 4 mm, made from 18-Fr Nelaton catheter) was placed at siting the pyloric sphincter and restricted the emptying of gastric contents [30]. After surgery, dexamethasone and gentamicin sulfate were injected for 4 days to prevent infection, and rats were given water after 24 h and ingested feed after 48 h. All animals had a surgical recovery period for 7 days after surgery. After that, a total of 32 rats were randomly divided into 4 groups as follows; Nor (normal group), Veh (water administered and chronic acid reflux esophagitis rats), CL (CLR 100 mg/kg body weight administered and chronic acid reflux esophagitis rats), and CH (CLR 200 mg/kg body weight administered and chronic acid reflux esophagitis rats). After group separation, body weight and food intake were measured for 14 days, and drugs were

orally administered. On the 15th day, mice were sacrificed by inhalation anesthesia (isoflurane, Telangana, India), blood and esophageal tissue were collected, and the esophageal tissue was immediately stored at -80 ℃.

Esophageal ulcer ratio

After sacrifice, the rat esophagus was cut from the gastroesophageal junction to the pharynx after sacrifice. The dissected esophagus was taken using an optical digital camera and then analyzed using the I-Solution Lite software program (Innerview Co., Korea).

The gross mucosal ulcer ratio (%) = [width of area with esophageal mucosal ulcer (mm^2)/width of total area of esophagus (mm^2)]×100.

Measurement of ROS and $ONOO^-$ levels in serum

Reactive oxygen species (ROS), an oxidative stress biomarker, was measured according to the method of Ali *et al.* [31]. After mixing serum and 1 mM EDTA-50 mM sodium phosphate buffer (pH 7.4), 25 mM DCFH-DA was added, and after incubation for 30 min, the changes in fluorescence values were determined at emission of 535 nm and excitation of 485 nm using a UV-VIS spectrophotometer.

Peroxynitrite ($ONOO^-$), an oxidative stress biomarker, was measured according to the method of Kooy *et al.* [32]. After mixing serum and DHR123 buffer (rodamin buffer, 5 mM DTPA, 10 mM DHR123), and after incubation for 5 min at 37℃. After that, the changes in fluorescence values were determined at emission of 535 nm and excitation of 485 nm using a UV-VIS spectrophotometer.

Measurement of TBRAS levels in serum and tissue

The 2-thiobarbituric acid-reactive substance (TBARS) levels were measured according to the method of Mihara and Uchiyama [33]. 1,1,3,3,-tetramethoxypropane was used as a standard sample. After mixing sapmle and 1% phosphoric acid, 0.67% thiobarbituric acid was added, and boiling for 45 min at 95℃. After that, mixed butanol, and centrifuge (3000 rpm, 10 min) to use supernatant. Dispense the supernatant and absorbance was measured at 540 nm using a UV-VIS spectrophotometer.

Preparation of nuclear and cytosol factions

The extraction of protein was performed according to the method of Komatsu with modifications [34]. For cytosol fractions, esophageal tissues were homogenized with 250 mL ice cold lysis buffer A containing 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and 1,250 μL protease inhibitor mixture solution. The tissue homogenates incubated (4℃ for 30 min), and then 10% NP-40 was mixed well. After centrifugation (12,000 rpm at 4℃ for 2 min) using Eppendorf 5415R (Hamburg, Germany), the supernatant (cytosol fractions) was separated new Eppendorf tubes. The pellets were washed twice by the lysis buffer and discard the supernatant. After that, the pellets were suspended with 20 mL ice cold lysis buffer C containing 300 mM NaCl, 50 mM HEPES (pH 7.8), 50 mM KCl, 1 mM DTT, 0.1 mM PMSF, 0.1 mM EDTA, 1% (v/v) glycerol and 100 μL protease inhibitor mixture

solution suspended and incubated (4℃ for 30 min). And, centrifugation (12,000 rpm at 4℃ for 10 min), the supernatant (nuclear fractions) was collected new tubes. Both cytosol and nuclear fractions were stored at -80℃ before the analysis.

Immunoblotting analyses

For the estimation of Nrf2, NF-κBp65, and histone 12 μg of proteins from each nuclear fraction were electrophoresed through 8–12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane, and incubated with primary anti-bodies (Nrf2, NF-κBp65, and histone) overnight at 4℃. After the membranes were washed, the membranes were incubated with anti-rabbit or anti-mouse IgG HRP-conjugated secondary antibody for 2 h at room temperature (20℃). In addition, for the estimation of NOX4, $p22^{phox}$, HO-1, SOD, Catalase, GPx-1/2, p-IκBα, IκBα, iNOS, COX-2, TNF-α, IL-6, IL-1β, and β-actin 12 μg of proteins from each cytosol fraction were electrophoresed through 12% SDS-PAGE. Antigen-antibody complex was visualized using an ECL Western Blotting Detection Reagents and detected by chemiluminescence with Sensi-Q 2000 Chemidoc (Lugen Sci Co., Ltd., Gyeonggi-do, Korea). Band densities were measured using ATTO Densitograph Software (ATTO Corporation, Tokyo, Japan), and quantified as the ratio to histone or β-actin. The protein levels of the groups are expressed relative to those of the normal rat (represented as 1).

Histological examination

Histological examination micro-scopic was performed to evaluate the separated esophagus tissues. The separated esophagus was fixed through a 10% neutral-buffered formalin and embedding in paraffin, and cut into 2 μm sections and stained using hematoxylin&eosin (H&E) for micro-scopic evaluation. The stained slices were observed under an optical microscope and then analyzed using the I-Solution Lite software program (Innerview Co., Korea).

Statistical analysis

In vitro values were expressed as means ± SEM and *in vivo* values as means ± SD. Statistical comparisons were analyzed by One-way ANOVA tests followed by the least significant difference (LSD) test using SPSS (version 25.0, IBM, Armonk, NY, USA). Values of $p < 0.05$ were considered significant.

Results

DPPH radical scavenging activity

The antioxidant effect of CLR was measured by DPPH radical scavenging assay, are expressed in IC_{50} (μg/mL). The IC_{50} value of DPPH radical scavenging activity of CLR was 36.04 ± 0.59 μg/mL, and the IC_{50} value of L-ascorbic acid (standard sample) was 1.19 ± 0.04 μg/mL (Fig. 2).

ABTS radical scavenging activity

The antioxidant effect of CLR was measure by ABTS radical scavenging assay, are expressed in IC_{50} (μg/mL). The IC_{50} value of ABTS radical scavenging activity of CLR was 44.08 ± 1.09 μg/mL, and the IC_{50} value of L-ascorbic acid (standard sample) was 3.47 ± 0.01 μg/mL (Fig. 3).

Total polyphenol and total flavonoid contents

Total polyphenol content of CLR was 35.46 ± 0.04 mg/g, and total flavonoid content of CLR was 31.49 ± 0.19 mg/g (Table 1).

Table 1
Total Polyphenol and Total Flavonoid Contents of CLR

Sample	Total polyphenol (mg/g)	Total flavonoid (mg/g)
Curcumae longae Rhizoma 30% EtOH extract	35.46 ± 0.04	31.49 ± 0.19
All values are expressed mean ± SEM of three replications.		

Change in body weight and food intake

Body weight gain and change into food intake of the experimental periods were examined. Before the experiment, body weight was not significant between groups. After 14 days, the weight change was significantly increased to Veh rats compared to Nor rats, and higher weight gain in CLR-treated rats than in Veh rats. Also, daily food intakes of CLR-treated rats were higher than daily food intakes of Veh rats (Table 2). This is not as normal as Nor rats, but suggests that reflux esophagitis is being recovered by treatment with CLR.

Table 2
Changes in Body Weight and Food Intake in CARE

Group	Body weight			Food intake (g/day)
	Initial (g)	Final (g)	Change (g/14 day)	
Nor	144 ± 22.52	203.25 ± 19.94	48.50 ± 6.02	13.56 ± 0.49
Chronic acid reflux esophagitis model				
Veh	136.44 ± 18.83	153.81 ± 29.67$^{###}$	19.00 ± 15.80$^{##}$	5.30 ± 1.27$^{###}$
CL	140.17 ± 23.13	163.33 ± 31.22	22.20 ± 19.95	6.10 ± 1.17
CH	136.89 ± 8.74	173.17 ± 37.08	35.00 ± 14.61*	6.43 ± 1.39
Data are presented as mean ± SD (n = 8). $^{###}p < 0.001$ vs. Nor group, $^{*}p < 0.05$ vs. Veh group.				

Esophageal lesion ratio

As a result of esophagus tissues damage in rats with chronic acid reflux esophagitis, morphological changes such as various erosion and hyperemia were observed in Veh rats compared with Nor rats. And mucosal damage was found in CLR treated rats, whereas CLR treatment significantly decreased compared with Veh rats (Fig. 4).

Measurement of ROS and $ONOO^-$ levels in serum

As a result of ROS and $ONOO^-$ levels measurement in serum, significantly increased to Veh rats compared to Nor rats. On the other hand, ROS and $ONOO^-$ levels were significantly decreased in all CLR treated rats compared with Veh rats (Fig. 5).

Measurement of TBARS levels in serum and tissue

As a result of TBARS levels measurement in serum and esophagus tissue, TBARS levels of serum and esophagus tissue were significantly increased to Veh rats compared with Nor rats. In contrast, TBARS level in serum was significantly decreased to CH rats compared with Veh rats, and was decreased to CL rats compared with Veh rats. Also, TBARS level in esophagus tissue was significantly decreased to all CLR treated rats compared with Veh rats (Fig. 6).

Expressions of NADPH oxidase proteins

The change of NADPH oxidase proteins such as NOX4 and $p22^{phox}$ were examined. Veh rats showed significantly increased expressions of NOX4 in esophagus compared with Nor rats. However, CH rats CLR administrated were significantly decreased to compared with Veh rats. CL rats were also down-regulated compared with Veh rats. And, the $p22^{phox}$ protein expression was significantly increased to Veh rats compared with Nor rats whereas CLR treatment significantly down-regulated $p22^{phox}$ expression (Fig. 7).

Expressions of anti-oxidation related proteins

The change of anti-oxidation related proteins such as Nrf2, HO-1 were examined. The expressions of Nrf2 and HO-1 in Veh rats were own-regulated compared with Nor rats, whereas CLR treatment significantly increased expression of Nrf2 and HO-1 (Fig. 8).

Expressions of anti-oxidant enzymes

The change of anti-oxidant enzymes such as SOD, Catalase, and GPx-1/2 were examined. Veh rats showed decreased expressions of SOD, Catalase, and GPx-1/2 in esophagus compared with Nor rats, whereas CH rats, administrated CLR, significantly increased antioxidant enzymes including SOD,

Catalase, and GPx-1/2 compared with Veh rats. Also, Catalase and GPx-1/2 were significantly up-regulated in CL rats (Fig. 9).

Expressions of inflammation related proteins

The change of inflammation related proteins such as NF-κBp65 and p-IκBα were examined. The expressions of NF-κBp65 and p-IκBα in Veh rats were significantly up-regulated compared with Nor rats. However, the administration of CLR was significantly decreased compared with Veh rats (Fig. 10).

Expressions of proinflammatory enzymes

The change of proinflammatory enzymes such as iNOS and COX-2 were examined. The expressions of iNOS and COX-2 in Veh rats were significantly up-regulated compared with Nor rats. On the other hand, the administration of CLR suppressed significantly the expressions of proinflammatory enzymes compared with Veh rats (Fig. 11).

Expressions of inflammatory cytokines

The change of inflammatory cytokines such as TNF-α and IL-6, and IL-1β were examined. In Veh rats, the expressions of TNF-α, IL-6, and IL-1β were significantly up-regulated compared with Nor rats. On the other hand, CH rats, the expressions of TNF-α and IL-6, and IL-1β were significantly down-regulated compared with Veh rats, also IL-6 was significantly down-regulated in CL rats (Fig. 12).

Esophagus histological examination

As a result of H&E staining confirmed the condition of esophageal tissue, the esophageal mucosa of Nor rats were normal and showed few inflammatory cells, whereas Veh rats showed mucosal thickening and basal cell proliferation. However, CLR treated rats had less damage to the submucosa and inflammatory cells than the Veh rats (Fig. 13).

Discussion

In the past decades, PPIs has been prescribed a lot for the treatment of GRED, however, in recent years, there is a growing awareness and problems associated with side effects of PPIs [35]. Several herbals from Traditional Korea Medicine (TKM) have been shown the therapeutic effect through the inhibition of inflammation, particularly in the treatment of gastroesophageal reflux disease (GERD) [36]. *Curcuma longa L.* has been used to reduce the acid release and treat gastric mucosal damage in TKM. So, we conducted an experiment to determine the effect on experimentally induced chronic acid reflux esophagitis using Curcumae longae Rhizoma possess stronger inhibitory effect against inflammation as a natural herb [37, 38].

First, DPPH and ABTS free radical scavenging activities, total polyphenol, and total flavonoid contents of CLR were measured. The results showed that CLR could improve the oxidative stress caused by CARE (Fig. 2–3, Table 1). In this study, CARE rats had increased esophageal ulcer ratio compared to normal rats, while CL and CH treated rats significantly reduced esophageal ulcer ratio. This indicated the potential possibility of CLR for the treatment of CARE (Fig. 4).

It was known that the cause of CARE was mucosal damage caused by acid reflux, but the major cause of CARE discovered recently has mediated by free radicals derived from oxygen [39]. Oxidative stress (OS) is defined as cellular damage by the overproduction of reactive oxygen species (ROS) such as H_2O_2, O_2^-, and HO^- and reactive nitrogen species such as (NO and $ONOO^-$). The excessive increase of OS can damage DNA, protein, and membranes of normal cells and tissues [40]. Also, not only ROS but also malonic dialdehyde (MDA), one factor of lipid peroxidation may contribute to OS [41]. MDA is a reactive marker of membrane injury and forms a color complex with thiobarbituric acid (TBA) and its level can be measured spectrophotometrically [42]. Moreover, NO by the induction of iNOS reacts with superoxide ($^{\cdot}OO^-$) form peroxynitrate ($ONOO^-$). $ONOO^-$ is a potent oxidant and on consequent OS [43]. Herein, we measured ROS and $ONOO^-$ in serum and MDA content through 2-thiobarbituric acid-reactive substance (TBARS) assay in serum and esophagus tissue. In the present study, ROS and $ONOO^-$ level in serum of Veh rats were markedly higher than those of normal rats, whereas, the elevated ROS and $ONOO^-$ levels were significantly lower than that of normal rats by CLR treated (Fig. 5). Likewise, TBARS in serum and esophagus tissue showed a significant reduction by CLR treated (Fig. 6).

The other production source about OS is the NADPH oxidase (NOX) family. NOX is critical regulators of cell differentiation, growth, and proliferation. Previous studies have reported that the suppression of NOX prevents the apoptosis of tissue [44]. These findings indicate that NOX could contribute to apoptosis of esophagus tissue with CARE [45]. Therefore, up-regulation of NOX as NOX4 and $p22^{phox}$ may cause damage of esophageal mucosa [46, 47]. Also, previous study was known that down-regulation of NOX protects tissue from ROS mediated inflammation and cell death [45]. As a result of measuring NOX such as NOX4 and $p22^{phox}$, CARE rats showed significantly increased to expressions of NOX proteins in esophagus compared with normal rats. However, CLR treated rats were significantly down-regulated compared with CARE rats (Fig. 7).

Nrf2 is a transcription factor that prevents OS caused by ROS or RNS. Nrf2 prevents OS by activating expressions of anti-oxidant proteins such as HO-1, SOD, Catalase, and GPx-1/2 catalyzes the conversion to O_2^- to H_2O_2, and H_2O_2 is subsequently neutralized to H_2O by Catalase and GPx. In addition, HO-1, one of the anti-oxidant enzymes, works functions as a key component of cytoprotection against oxidative stress in various cell types [46–48]. Also, the increase in OS levels to activate NF-κB. NF-κB, which is the transcription of gene induces proinflammatory, cytokines, chemokines, and enzymes. The OS activates NF-κB which is bound to IκB in the cytoplasm. And then NF-κB translocate into the nucleus [46, 49]. NF-κB in nucleus promotes the expressions of iNOS, COX-2, and cytokines such as TNF-α, IL-6, and IL-1β to induce an inflammatory response [50]. In the present study, the expression of Nrf2 was significantly

down-regulated in CARE rats compared with normal rats, whereas CLR treated rats were significantly increased Nrf2 expression compared with CARE rats. Moreover, the expressions of anti-oxidant enzymes such as HO-1, SOD, Catalase, and GPx-1/2 were also significantly increased in CLR treated rats (Fig. 8–9). Also, NF-κB was significantly up-regulated in CARE rats, whereas CLR treated rats were significantly down-regulated. In addition, proinflammatory enzymes and inflammatory cytokines were down-regulated in CLR treated rats compared with CARE rats (Fig. 10–12).

Our results are shown that the administration of CLR significantly decreased the factors associated with oxidative stress and increased the antioxidant enzymes such as SOD, Catalase, GPx-1/2 and HO-1 in CARE model. Furthermore, the anti-inflammatory effects of CLR suggested that the inactivation of NF-κB by blocking the phosphorylation of IκBα led to the inhibition of the expressions of proinflammatory proteins.

Conclusion

Taken together, it suggests that CLR could suppress excessive production of oxidative stress and activate the expression of anti-oxidant factors by Nrf2 pathway. Also, it could inactivation of NF-κB by blocking the phosphorylation of IκBα led to the inhibition of the expressions of proinflammatory proteins. Thus, the results of this study suggest that CLR may be a new material for the treatment of GERD including chronic acid reflux esophagitis.

Abbreviations

GERD: gastroesophageal reflux disease; CARE: chronic acid reflux esophagitis; DPPH: 1, 1-diphenyl-2-picrylhydrazyl; ABTS: 2, 2’-azinobis-3-ethyl-enzothiazoline-6-sulfonic acid; ROS: reactive oxygen species; $ONOO^-$: peroxynitrite; TBARS: 2-thiobarbituric acid-reactive substance; Nrf2: nuclear factor erythroid-derived 2-related factor 2; NOX4: NADPH oxidase 4; HO-1: heme oxygenase-1; SOD: superoxide dismutase; GPx-1/2: glutathione peroxidase-1/2; IκBα: inhibitor of nuclear factor κ B α; p-IκBα: phosphor-inhibitor of nuclear factor κ B; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; IL-1: interleukin-1; NF-κBp65: nuclear factor-κ B p65; iNOS: inducible nitric oxide synthase; COX-2: cycloxygenase-2

Declarations

Availability of data and materials

All data are contained within the article.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

SSR conceived and designed the experiments. JAL wrote and revised the manuscript. JAL and MJK carried out the laboratory experiments. MRS and BIS carried out the statistical analysis and data analysis. All authors read and approved the final manuscript.

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Not applicable.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Five-week-old male Sprague-Dawley rats were used according to the ‘Guidelines for Animal Experiment’ approved by Ethics Committee of the Daegu Haany University (Approval No. DHU2019-118)

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References

1. Barnhart DC. Gastroesophageal reflux disease in children. Semin Pediatr Surg. 2016;25(4):212–8.
2. Zhuang ZH, Xie JJ, Wei JJ, Tang DP, Yang LY. The effect of n-3/n-6 polyunsaturated fatty acids on acute reflux esophagitis in rats. Lipids Health Dis. 2016;15(1):172.
3. Gong Y, Zeng Q, Yan Y, Han C, Zheng Y. Association between Lifestyle and Gastroesophageal Reflux Disease Questionnaire Scores: A Cross-Sectional Study of 37 442 Chinese Adults. Gastroenterol Res Pract. 2019:5753813.
4. Savarino E, Zentilin P, Marabotto E, Bodini G, Della Coletta M, Frazzoni M, Savarino V. A review of pharmacotherapy for treating gastroesophageal reflux disease (GERD). Expert Opin Pharmacother. 2019;18(13):1333–43.
5. Darvishmoghadam S, Zahedi MJ, Hayatbakhsh Abbasi MM, Haghdoost AA, Khalilyzade M, Karimi Goughari E. Review of Clinical Spectrum of Gastroesophageal Reflux Disease in a General Population; A Study from South-East Iran. Middle East J Dig Dis. 2016;8(4):310–7.
6. Pohle T, Domschke W. Results of short- and long-term medical treatment of gastroesophageal reflux disease (GERD). Langenbecks Arch Surg. 2000;385:317–23.

7. Nicolau AE, Lobonţiu A, Constantinoiu S. New Minimally Invasive Endoscopic and Surgical Therapies for Gastroesophageal Reflux Disease (GERD). Chirurgia (Bucur). 2018;113(1):70–82.
8. Torresan F, Mandolesi D, Ioannou A, Nicoletti S, Eusebi LH, Bazzoli F. A new mechanism of gastroesophageal reflux in hiatal hernia documented by high-resolution impedance manometry: a case report. Ann Gastroenterol. 2016;29(4):548–50.
9. Nakahara K, Fujiwara Y, Tsukahara T, Yamagami H, Tanigawa T, Shiba M, Tominaga K, Watanabe T, Urade Y, Arakawa T. Acid reflux directly causes sleep disturbances in rat with chronic esophagitis. PLoS One. 2014;9(9):e106969.
10. Scarpignato C, Gatta L, Zullo A, Blandizzi C. Effective and safe proton pump inhibitor therapy in acid-related diseases - A position paper addressing benefits and potential harms of acid suppression. BMC Med. 2016;14(1):179.
11. Ament PW, Dicola DB, James ME. Reducing adverse effects of proton pump inhibitors. Am Fam Physician. 2012;86(1):66–70.
12. Ang D, How CH, Ang TL. Persistent gastro-oesophageal reflux symptoms despite proton pump inhibitor therapy. Singapore Med J. 2016;57(10):546–51.
13. Malfertheiner P, Kandulski A, Venerito M. Proton-pump inhibitors: understanding the complications and risks. Nat Rev Gastroenterol Hepatol. 2017;14(12):697–710.
14. Harris RA, Kuppermann M, Richter JE. Prevention of recurrences of erosive reflux esophagitis: a cost-effectiveness analysis of maintenance proton pump inhibition. Am J Med. 1997;102(1):78–88.
15. Lee JA, Park HJ, Kim SH, Kim MJ, Kim KJ, Shin MR, Roh SS. Evaluation of Evodiae Fructus Extract on the Chronic Acid Reflux Esophagitis in Rats. Kor J Herbol. 2019;34(2):15–23.
16. Orona-Ortiz A, Medina-Torres L, Velazquez-Moyado JA, Pineda-Pena EA, Balderas-Lopez JL, Bernad-Bernad MJ, Tavares Carvalho JC, Navarrete A. Mucoadhesive effect of Curcuma longa extract and curcumin decreases the ranitidine effect, but not bismuth subsalicylate on ethanol-induced ulcer model. Sci Rep. 2019;9(1):16622.
17. Mun J, Kim S, Yoon HG, You Y, Kim OK, Choi KC, Lee YH, Lee J, Park J, Jun W. Water Extract of Curcuma longa L. Ameliorates Non-Alcoholic Fatty Liver Disease. Nutrients. 2019;11(10):pii: E2536.
18. Choi Y, Ban I, Lee H, Baik MY, Kim W. Puffing as a Novel Process to Enhance the Antioxidant and Anti-Inflammatory Properties of Curcuma longa L. (Turmeric). Antioxidants (Basel). 2019;8(11):pii: E506.
19. Lee JA, Shin MR, Kim MJ, Lee JH, Park HJ, Roh SS. Protective Effects of Inflammation of Curcumae Longae Rhizoma 30% EtOH Extract on Acute Reflux Esophagitis Rats. Biomed Res Int. 2021;2021:8854945.
20. Kocaadam B, Şanlier N. Curcumin, an active component of turmeric (Curcuma longa), and its effects on health. Crit Rev Food Sci Nutr. 2017;57(13):2889–95.
21. Menon VP, Sudheer AR. Antioxidant and anti-inflammatory properties of curcumin. Adv Exp Med Biol. 2007;595:105–25.

22. Somade OT, Ajayi BO, Safiriyu OA, Oyabunmi OS, Akamo AJ. Renal and testicular up-regulation of pro-inflammatory chemokines (RANTES and CCL2) and cytokines (TNF-α, IL-1β, IL-6) following acute edible camphor administration is through activation of NF-κB in rats. Toxicol Rep. 2019;6:759–67.
23. Guo H, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat Med. 2015;21(7):677–87.
24. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol. 2009;1(6):a001651.
25. Kim YS, Young MR, Bobe G, Colburn NH, Milner JA. Bioactive food components, inflammatory targets, and cancer prevention. Cancer Prev Res (Phila). 2009;2(3):200–8.
26. Blois MS. Antioxidant Determinations by the Use of a Stable Free Radical. Nature. 1958;181:1199–200.
27. Re R. Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999;26(9–10):1231–7.
28. Folin O, Denis W. On phosphotungastic-phosphomolybdic compounds as color reagent. J Biol Chem. 1912;12(2):239–43.
29. Lister CE, Lancaster JE, Sutton KH, Walker JR. Developmental changes in the concentration and composition of flavonoids in skin of a red and a green apple cultivar. J Sci Food Agric. 1994;64(2):155–61.
30. Omura N, Kashiwagi H, Chen G, Suzuki Y, Yano F, Aoki T. Establishment of surgically induced chronic acid reflux esophagitis in rats. Scand J Gastroenterol. 1999;34(10):948–53.
31. Ali SF, LeBel CP, Bondy SC. Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. Neurotoxicology. 1992;13:637–48.
32. Kooy NW, Royall JA, Ischiropoulos H, Beckman JS. Peroxynitrite-mediated oxidation of dihydrohodamine 123. Free Radic Biol Med. 1994;16:149–56.
33. Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem. 1978;86(1):271–8.
34. Komatsu S. Extraction of nuclear proteins. Methods Mol Biol. 2007;355:73–7.
35. Sandhu DS, Fass R. Current Trends in the Management of Gastroesophageal Reflux Disease. Gut Liver. 2018;12(1):7–16.
36. Zikri NN, Riedl KM, Wang LS, Lechner J, Schwartz SJ, Stoner GD. Black raspberry components inhibit proliferation, induce apoptosis, and modulate gene expression in rat esophageal epithelial cells. Nutr Cancer. 2009;61(6):816–26.
37. Okuda-Hanafusa C, Uchio R, Fuwa A, Kawasaki K, Muroyama K, Yamamoto Y, Murosaki S. Turmeronol A and turmeronol B from Curcuma longa prevent inflammatory mediator production by lipopolysaccharide-stimulated RAW264.7 macrophages, partially via reduced NF-κB signaling. Food Funct. 2019;10(9):5779–88.

38. Senthilkumar A, Joshua AJ, Bharathi B, Amit A, Jung EB. Anti-inflammatory Effects of Turmeric (Curcuma longa L.) Extract on Acute and Chronic Inflammation Models. J Korean Soc Food Sci Nutr. 2014;43(1):612–7.
39. Oh TY, Lee JS, Ahn BO, Cho H, Kim WB, Kim YB, Surh YJ, Cho SW, Hahm KB. Oxidative damages are critical in pathogenesis of reflux esophagitis: implication of antioxidants in its treatment. Free Radic Biol Med. 2001;30(8):905–15.
40. Haynes RL, Brune B, Townsend AJ. Apoptosis in RAW 264.7 cells exposed to 4-hydroxy-2-nonenal: dependence on cytochrome C release but not p53 accumulation. Free Radic Biol Med. 2001;30(8):884–94.
41. Xu Y, Zhu J, Hu X, Wang C, Lu D, Gong C, Yang J, Zong L. CLIC1 Inhibition Attenuates Vascular Inflammation, Oxidative Stress, and Endothelial Injury. PLoS One. 2016;11(11):e0166790.
42. Shin MR, Seo BI, Son CG, Roh SS, An HJ. Banhasasim-Tang Treatment Reduces the Severity of Esophageal Mucosal Ulcer on Chronic Acid Reflux Esophagitis in Rats. Biomed Res Int. 2017:7157212.
43. Pall ML. The NO/ONOO-cycle as the central cause of heart failure. Int J Mol Sci. 2013;14(11):22274–330.
44. Cheng L, Harnett KM, Cao W, Liu F, Behar J, Fiocchi C, Biancani P. Hydrogen peroxide reduces lower esophageal sphincter tone in human esophagitis. Gastroenterology. 2005;129(5):1675–85.
45. Meng XM, Ren GL, Gao L, Yang Q, Li HD, Wu WF, Huang C, Zhang L, Lv XW, Li J. NADPH oxidase 4 promotes cisplatin-induced acute kidney injury via ROS-mediated programmed cell death and inflammation. Lab Invest. 2018;98(1):63–78.
46. Chen H, Fang Y, Li W, Orlando RC, Shaheen N, Chen XL. NFκB and Nrf2 in esophageal epithelial barrier function. Tissue Barriers. 2013;1(5):e27463.
47. Lee HG, Li MH, Joung EJ, Na HK, Cha YN, Surh YJ. Nrf2-Mediated heme oxygenase-1 upregulation as adaptive survival response to glucose deprivation-induced apoptosis in HepG2 cells. Antioxid Redox Signal. 2010;13(11):1639–48.
48. Cavanagh EM, Inserra F, Ferder L, Romano L, Ercole L, Fraga CG. Superoxide dismutase and glutathione peroxidase activities are increased by enalapril and captopril in mouse liver. FEBS Lett. 1995;361(1):22–4.
49. Ebrahimi R, Sepand MR, Seyednejad SA, Omidi A, Akbariani M, Gholami M, Sabzevari O. Ellagic acid reduces methotrexate-induced apoptosis and mitochondrial dysfunction via up-regulating Nrf2 expression and inhibiting the IκBα/NFκB in rats. Daru. 2019;27(2):721–33.
50. Gius D, Botero A, Shah S, Curry HA. Intracellular oxidation/reduction status in the regulation of transcription factors NF-kappaB and AP-1. Toxicol Lett. 1999;106(2–3):93–106.

Figures

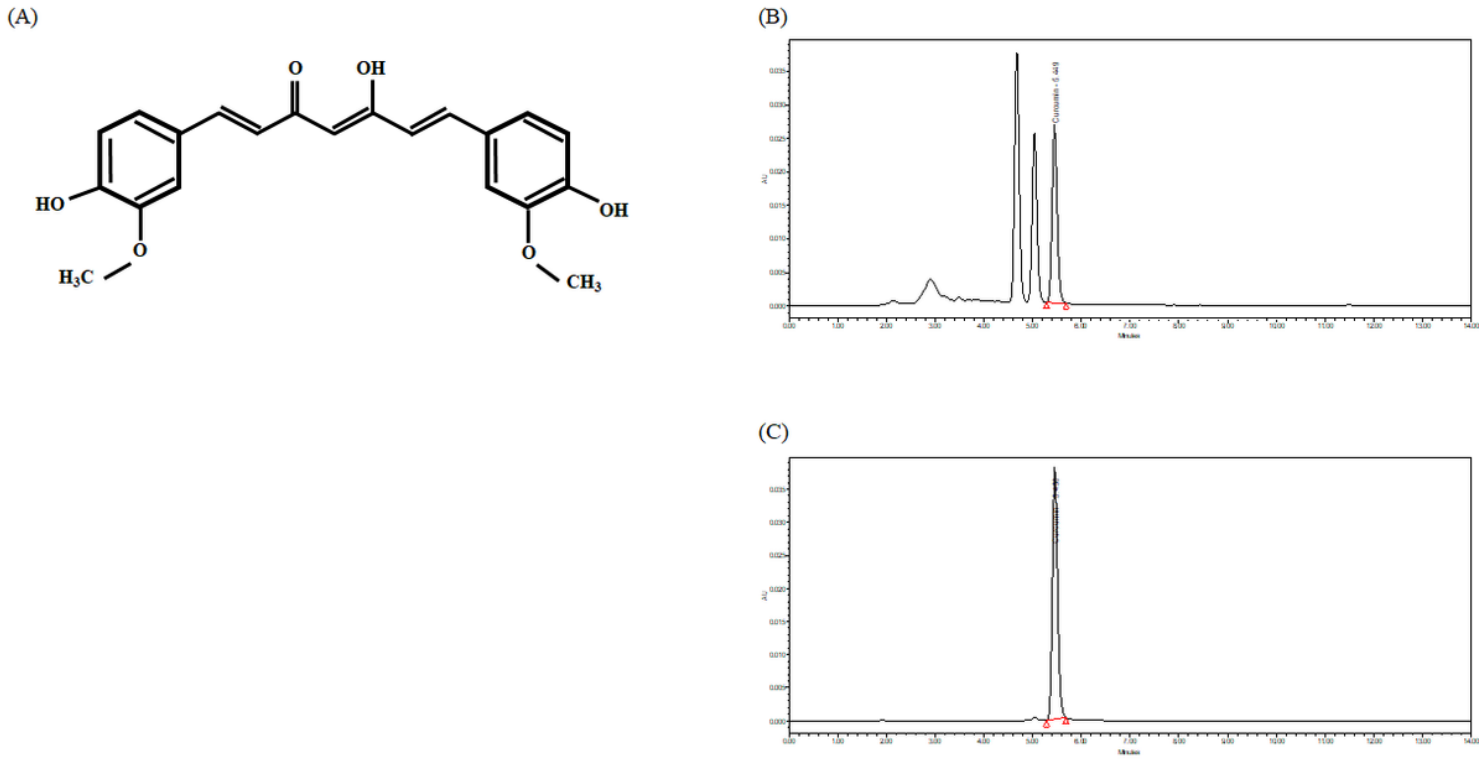

Figure 1

HPLC profile of Curcumae Longae Rhizoma (CLR). Curcumin (C21H20O6 : 368.38 g/mol); (A), HPLC profile of Curcumin; (B), HPLC profile of Curcumae Longae Rhizoma extract; (C).

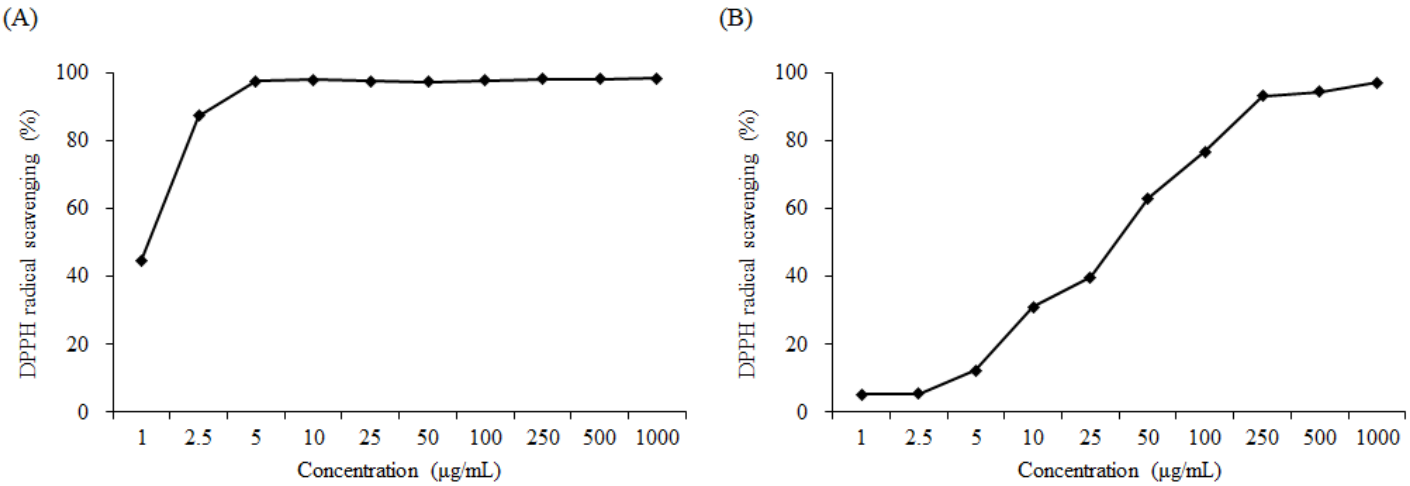

Figure 2

DPPH radical scavenging activity of CLR. DPPH radical scavenging activity of L-ascorbic acid; (A), DPPH radical scavenging activity of Curcumae longae Rhizoma 30% EtOH extract; (B). All values are expressed mean±SEM of three replications.

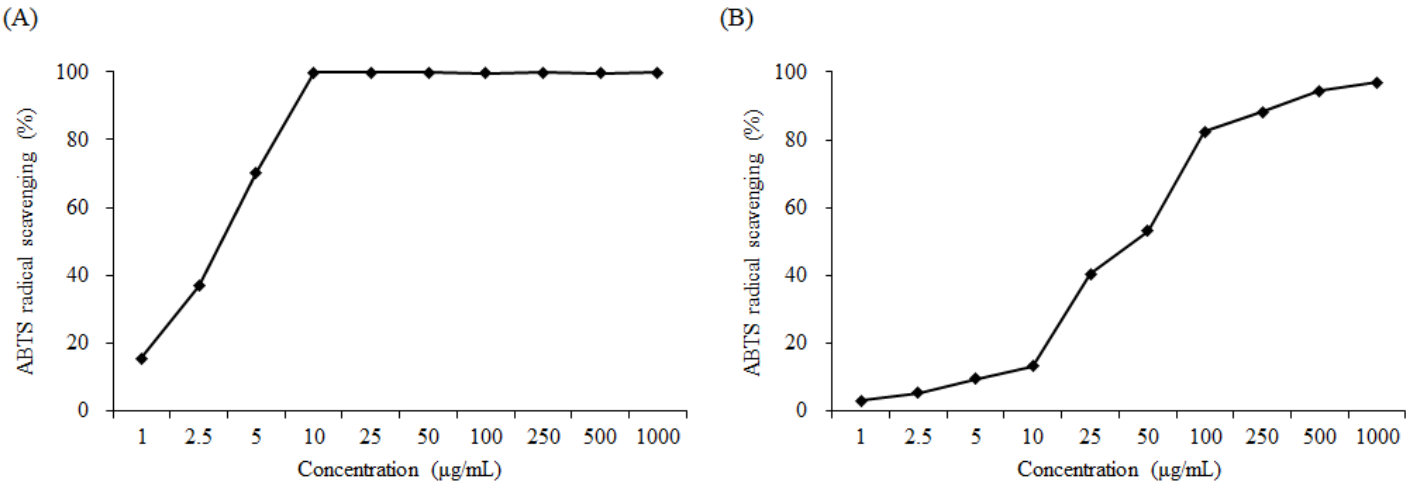

Figure 3

ABTS radical scavenging activity of CLR. ABTS radical scavenging activity of L-ascorbic acid; (A), ABTS radical scavenging activity of Curcumae longae Rhizoma 30% EtOH extract; (B). All values are expressed mean±SEM of three replications.

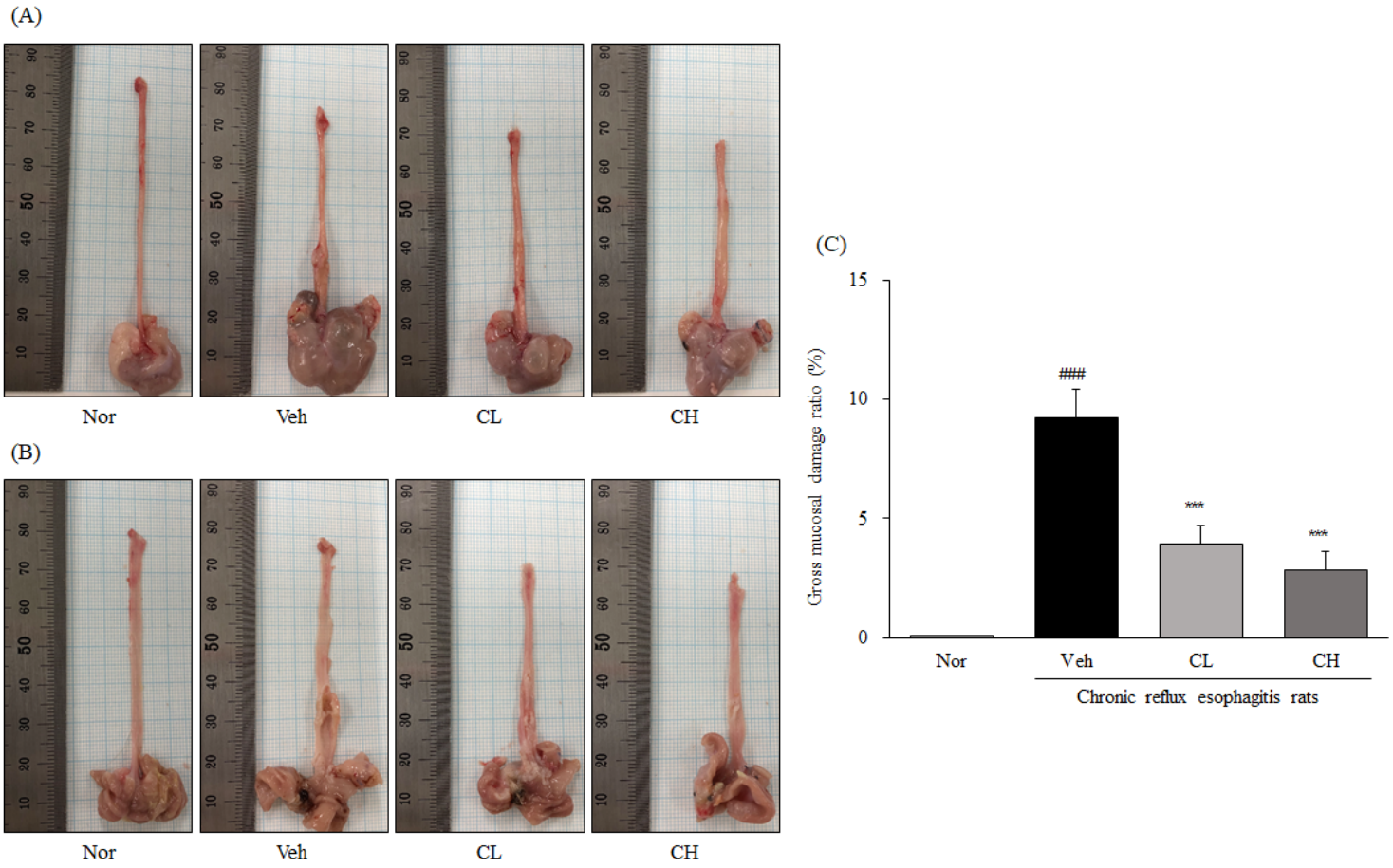

Figure 4

Esophagus tissue damage. A representative gross image of surgical induction of CARE; (A), the opened gross esophageal ulcer; (B), esophageal ulcer ratio; (C). Nor; normal group, Veh; water administered and chronic acid reflux esophagitis rats, CL; CLR 100 mg/kg body weight administered and chronic acid reflux esophagitis rats, CH; CLR 200 mg/kg body weight administered and chronic acid reflux esophagitis rats. Data are mean±SD (n=8). Significance: ###p < 0.001 vs. Nor group, ***p < 0.001 vs. Veh group.

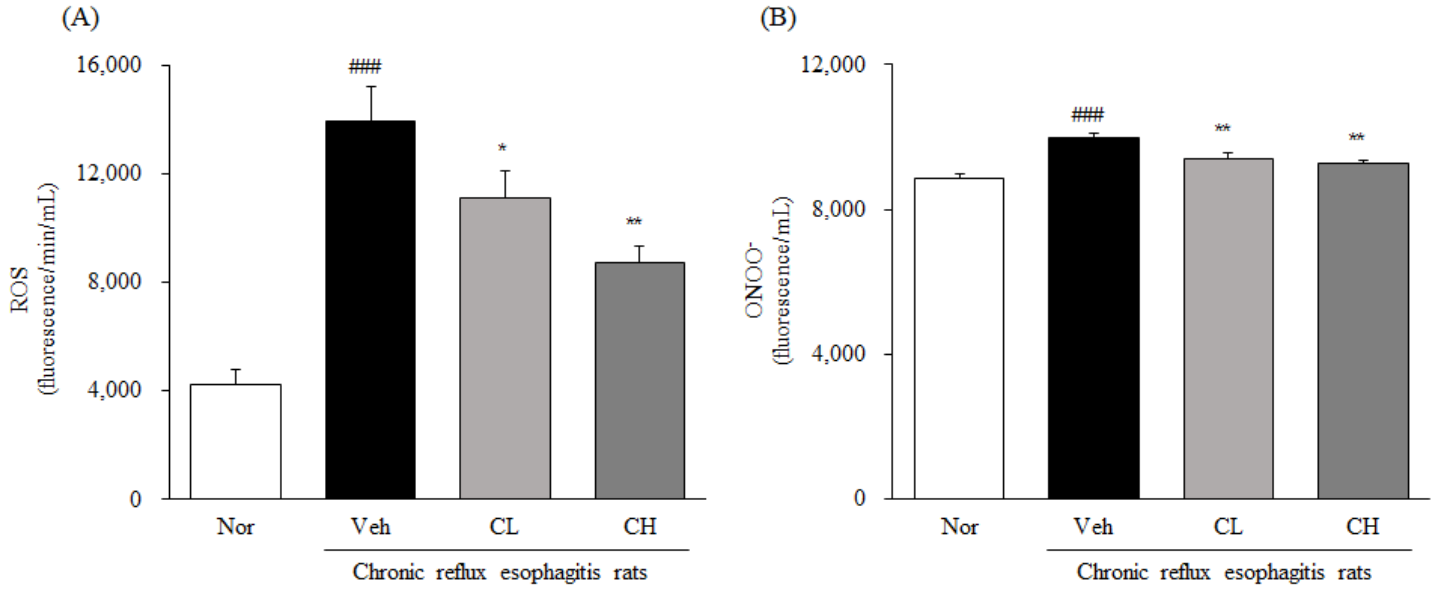

Figure 5

Serum ROS and ONOO- levels. Reactive oxygen species (ROS); (A), peroxynitrite (ONOO-); (B). Nor; normal group, Veh; water administered and chronic acid reflux esophagitis rats, CL; CLR 100 mg/kg body weight administered and chronic acid reflux esophagitis rats, CH; CLR 200 mg/kg body weight administered and chronic acid reflux esophagitis rats. Data are mean±SD (n=8). Significance: ###p < 0.001 vs. Nor group, *p < 0.05, **p < 0.01 vs. Veh group.

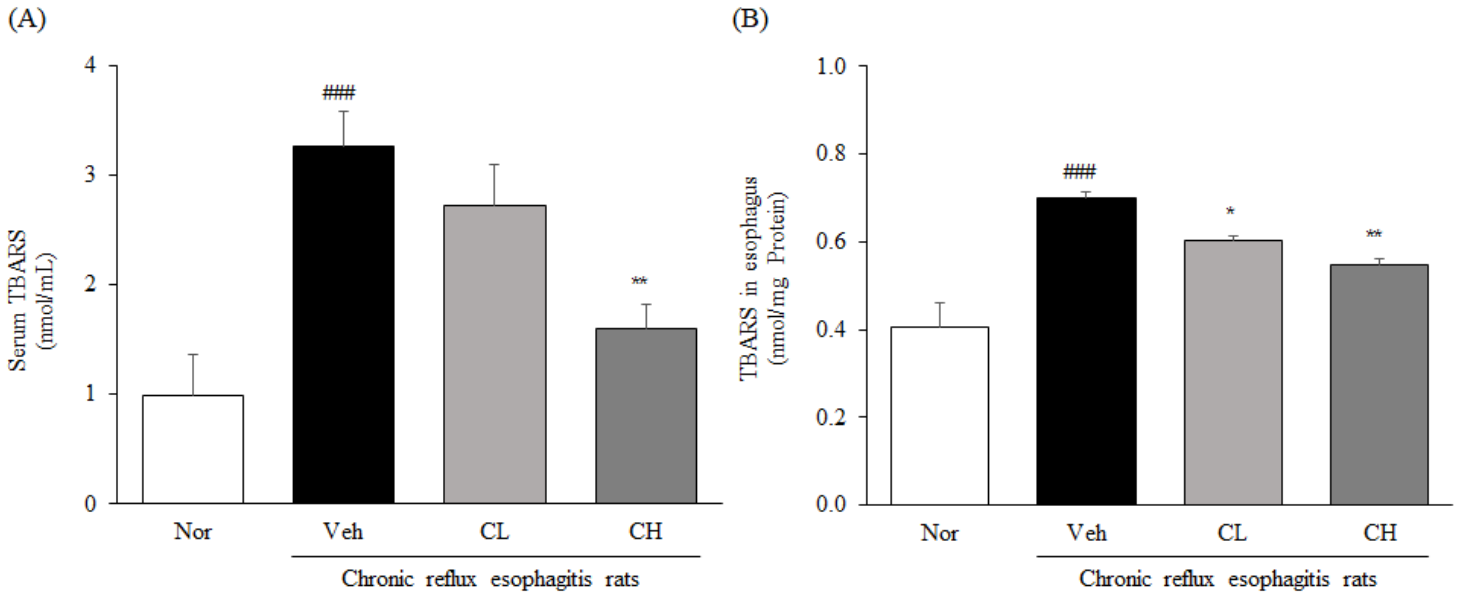

Figure 6

Serum and tissue TBARS levels. Thiobarbituric acid reactive substances (TBARS) in serum; (A), TBARS in esophagus tissue; (B). Nor; normal group, Veh; water administered and chronic acid reflux esophagitis rats, CL; CLR 100 mg/kg body weight administered and chronic acid reflux esophagitis rats, CH; CLR 200 mg/kg body weight administered and chronic acid reflux esophagitis rats. Data are mean±SD (n=8). Significance: ###p < 0.001 vs. Nor group, *p < 0.05, **p < 0.01 vs. Veh group.

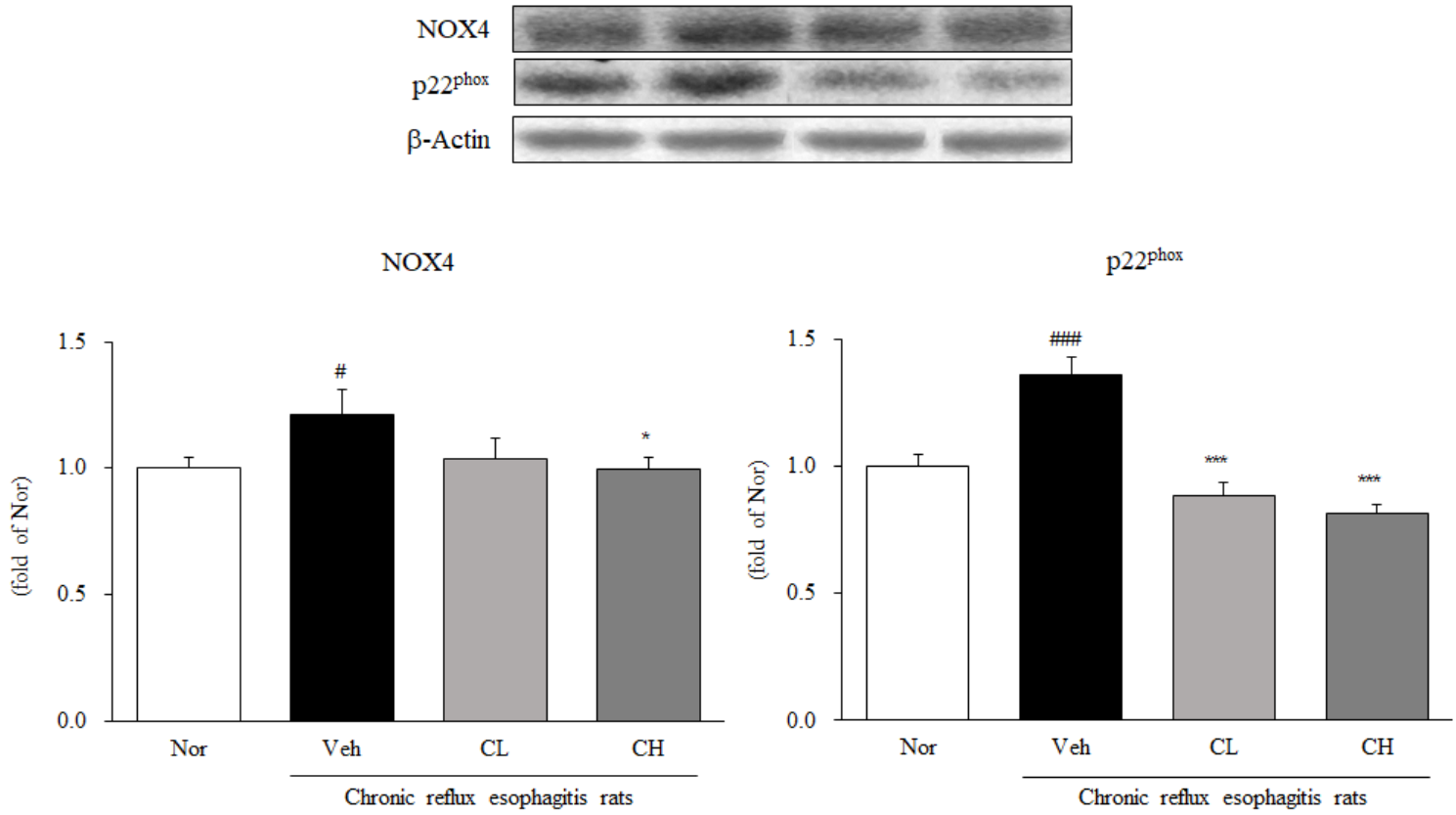

Figure 7

Expressions of NADPH oxidase proteins. NOX4 and p22phox protein expressions. Nor; normal group, Veh; water administered and chronic acid reflux esophagitis rats, CL; CLR 100 mg/kg body weight administered and chronic acid reflux esophagitis rats, CH; CLR 200 mg/kg body weight administered and chronic acid reflux esophagitis rats. Data are mean±SD (n=8). Significance: #p < 0.05, ###p < 0.001 vs. Nor group, *p < 0.05, ***p < 0.001 vs. Veh group.

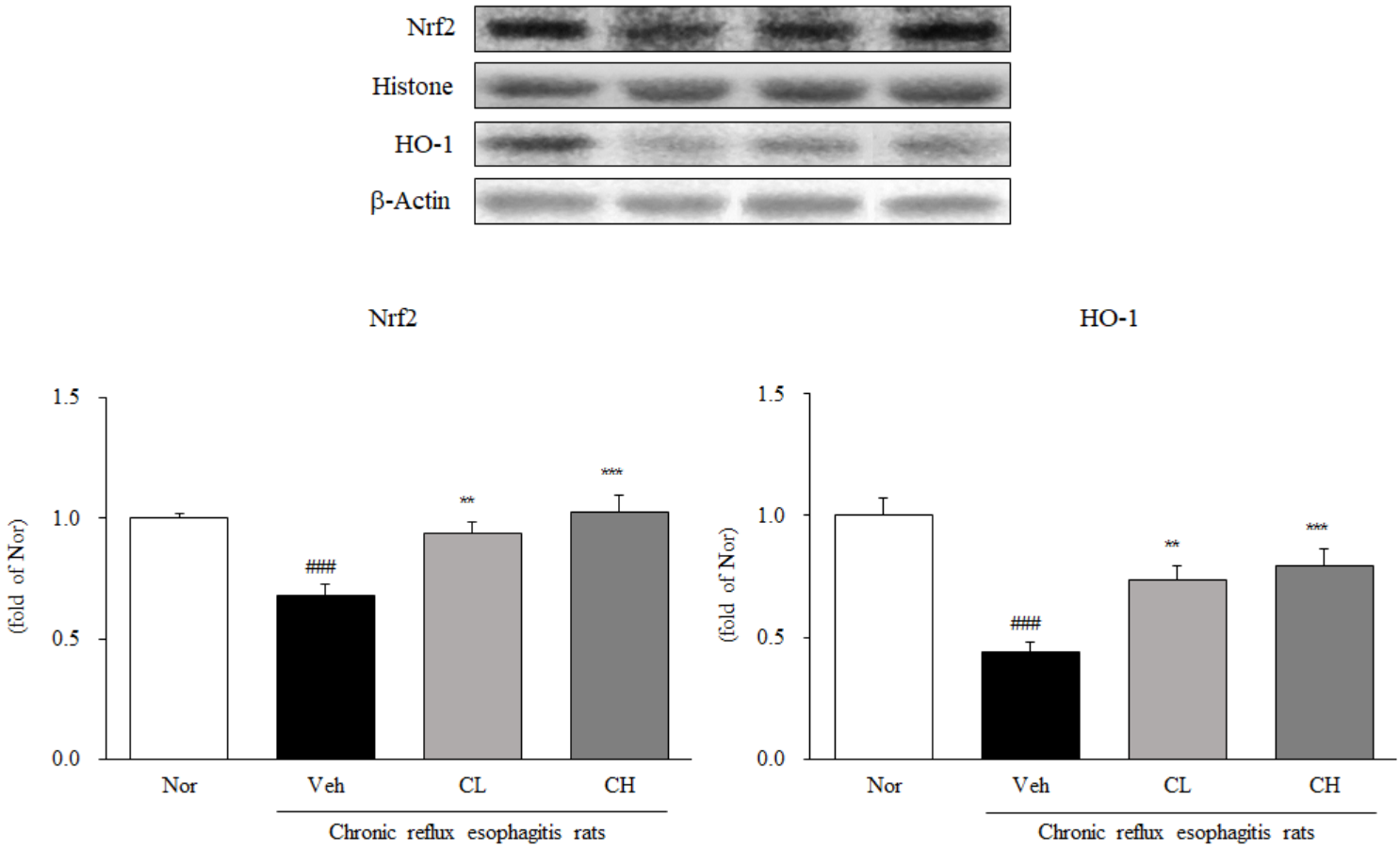

Figure 8

Expressions of anti-oxidation related proteins. Nrf2 and HO-1 protein expressions. Nor; normal group, Veh; water administered and chronic acid reflux esophagitis rats, CL; CLR 100 mg/kg body weight administered and chronic acid reflux esophagitis rats, CH; CLR 200 mg/kg body weight administered and chronic acid reflux esophagitis rats. Data are mean±SD (n=8). Significance: ###p < 0.001 vs. Nor group, **p < 0.01, ***p < 0.001 vs. Veh group.

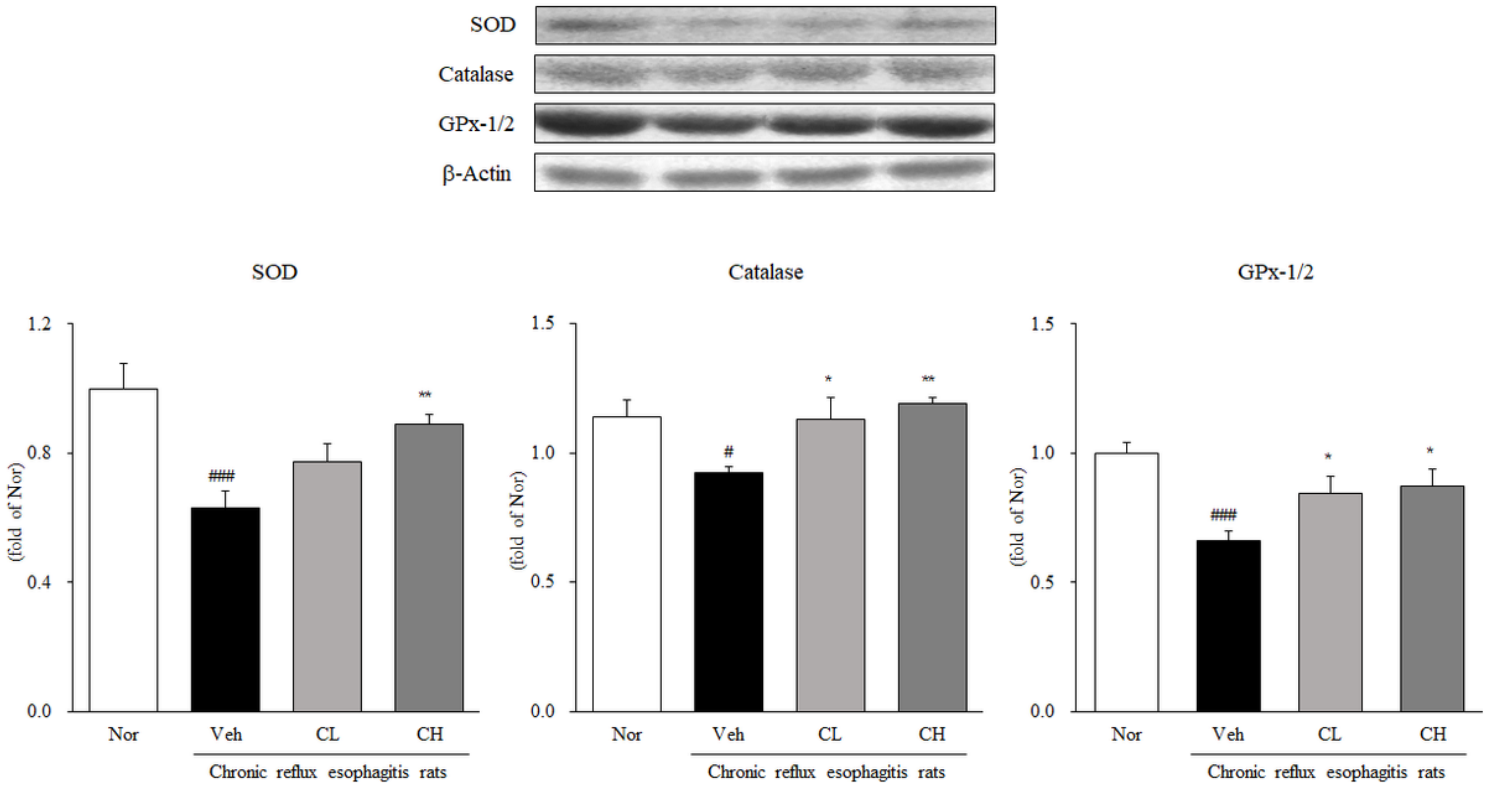

Figure 9

Expressions of anti-oxidant enzymes. SOD, Catalase, and GPx-1/2 protein expressions. Nor; normal group, Veh; water administered and chronic acid reflux esophagitis rats, CL; CLR 100 mg/kg body weight administered and chronic acid reflux esophagitis rats, CH; CLR 200 mg/kg body weight administered and chronic acid reflux esophagitis rats. Data are mean±SD (n=8). Significance: #p < 0.05, ###p < 0.001 vs. Nor group, *p < 0.05, **p < 0.01 vs. Veh group.

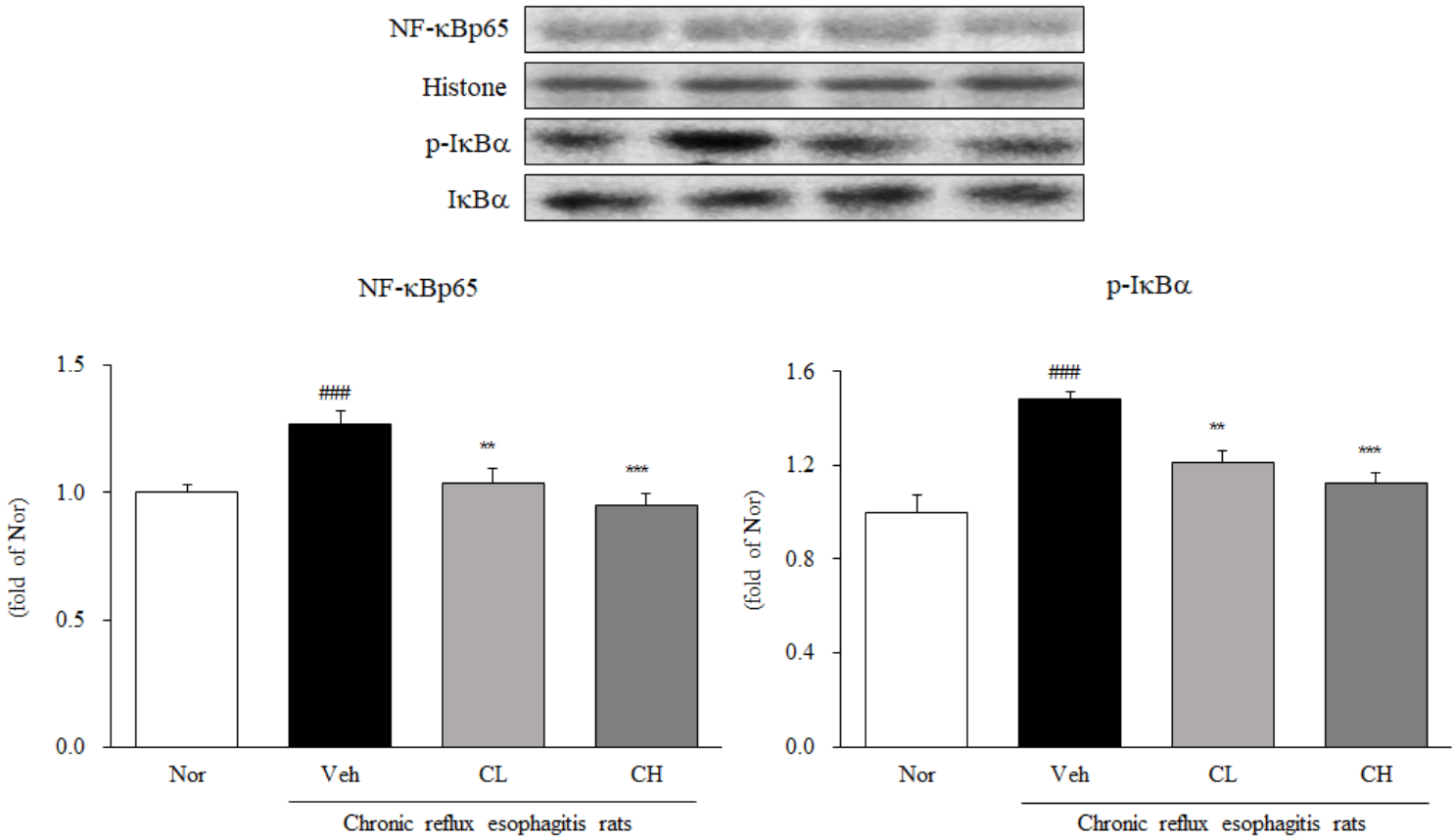

Figure 10

Expressions of inflammation related proteins. NF-κBp65 and p-IκBα protein expressions. Nor; normal group, Veh; water administered and chronic acid reflux esophagitis rats, CL; CLR 100 mg/kg body weight administered and chronic acid reflux esophagitis rats, CH; CLR 200 mg/kg body weight administered and chronic acid reflux esophagitis rats. Data are mean±SD (n=8). Significance: ###p < 0.001 vs. Nor group, **p < 0.01, ***p < 0.001 vs. Veh group.

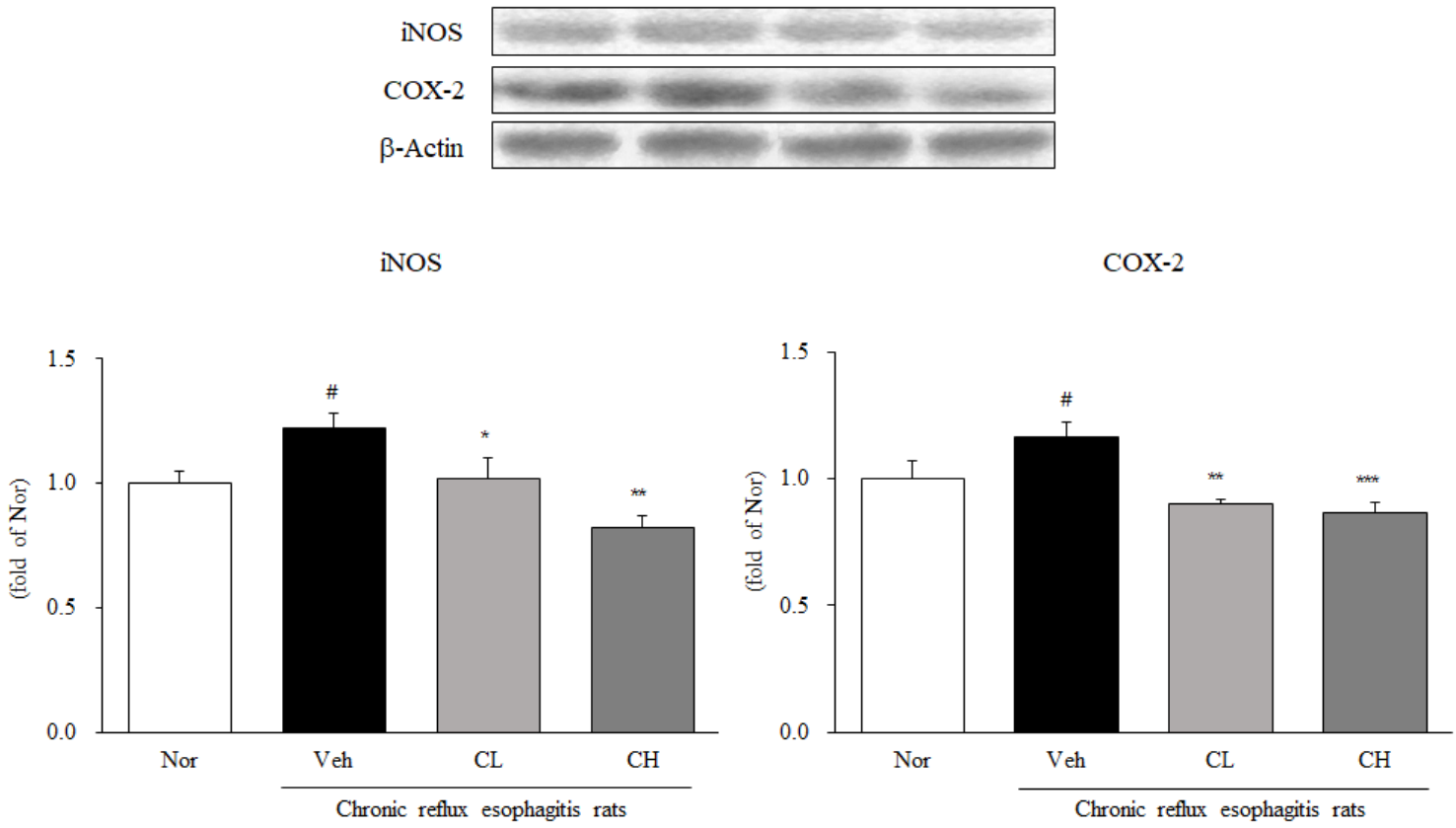

Figure 11

Expressions of proinflammatory enzymes. iNOS and COX-2 protein expressions. Nor; normal group, Veh; water administered and chronic acid reflux esophagitis rats, CL; CLR 100 mg/kg body weight administered and chronic acid reflux esophagitis rats, CH; CLR 200 mg/kg body weight administered and chronic acid reflux esophagitis rats. Data are mean±SD (n=8). Significance: #p < 0.05 vs. Nor group, *p < 0.05, **p < 0.01, ***p < 0.001 vs. Veh group.

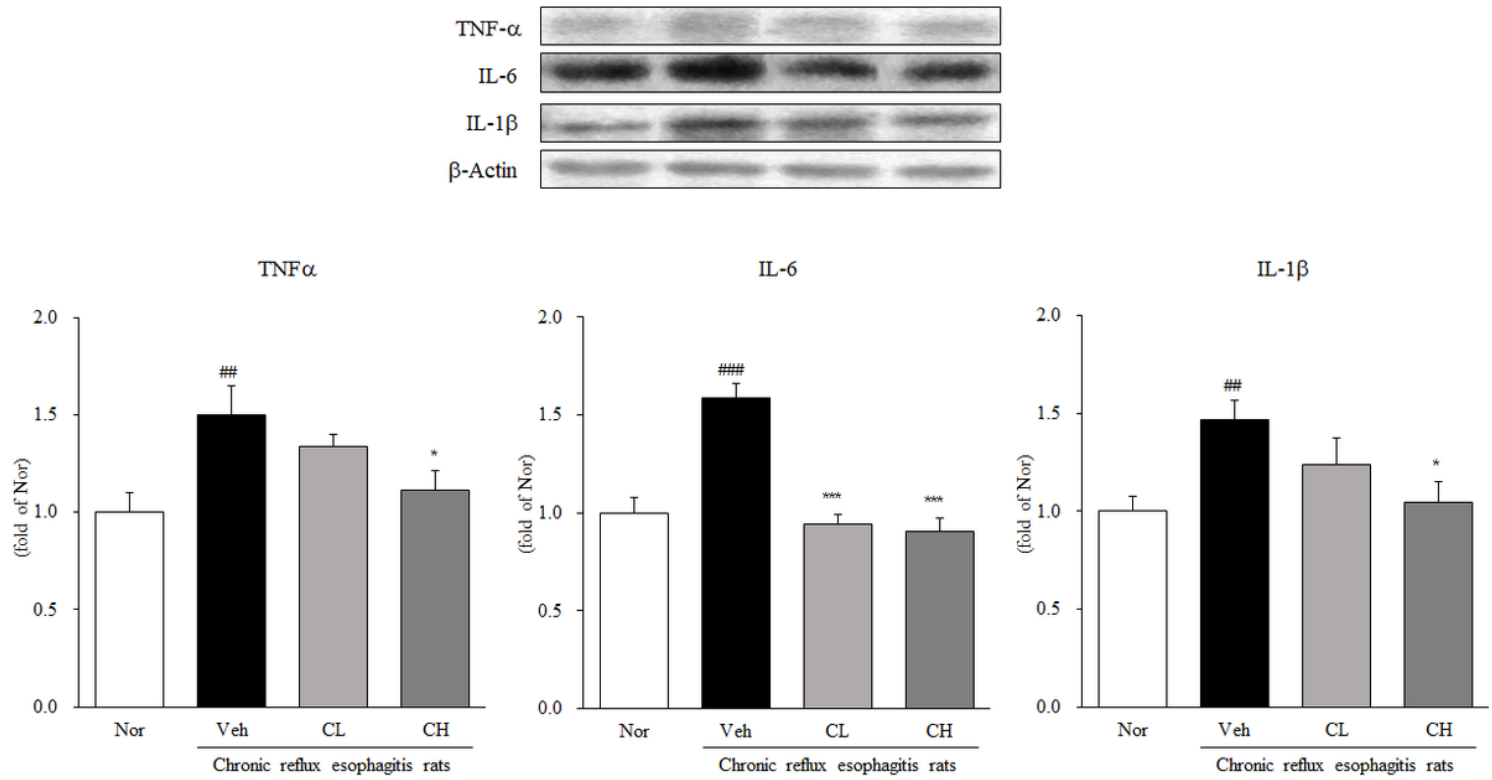

Figure 12

Expressions of inflammatory cytokines. TNF-α and IL-6, and IL-1β protein expressions. Nor; normal group, Veh; water administered and chronic acid reflux esophagitis rats, CL; CLR 100 mg/kg body weight administered and chronic acid reflux esophagitis rats, CH; CLR 200 mg/kg body weight administered and chronic acid reflux esophagitis rats. Data are mean±SD (n=8). Significance: ##p < 0.01, ###p < 0.001 vs. Nor group, *p < 0.05, ***p < 0.001 vs. Veh group.

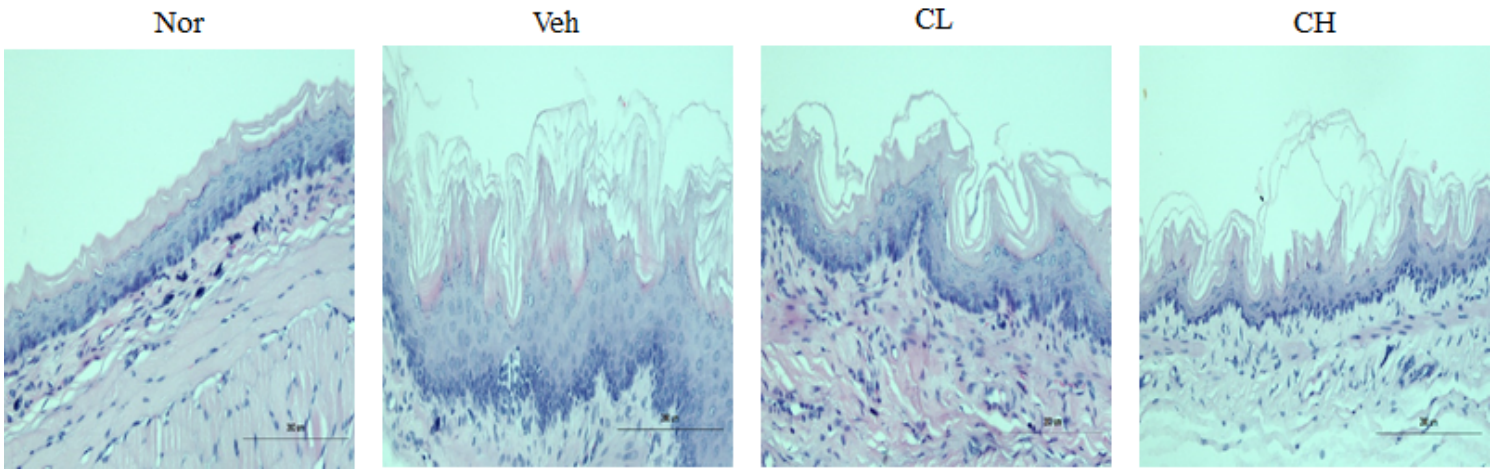

Figure 13

Esophagus histological examination through H&E staining. Magnification×200. Nor; normal group, Veh; water administered and chronic acid reflux esophagitis rats, CL; CLR 100 mg/kg body weight

administered and chronic acid reflux esophagitis rats, CH; CLR 200 mg/kg body weight administered and chronic acid reflux esophagitis rats.