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ORIGINAL ARTICLE

Rhei Rhizoma and Citri Pericarpium Mixture Regulates Oxidative Stress and Tight Junction Proteins on Acute Reflux Esophagitis

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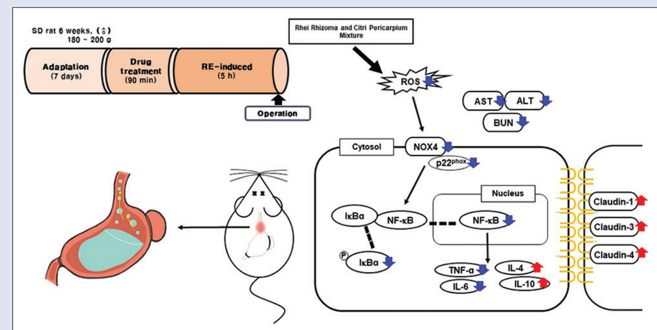
ABSTRACT

The aim of the present study was to assess the impact of a Rhei Rhizoma and Citri Pericarpium (RC) mixture on esophageal mucosal injury in rats with acute reflux esophagitis (ARE). An experimental model of ARE was established surgically. RC mixture was administered at a dose of 100 or 200 mg/kg body weight 90 min prior to induction of ARE, and its effects were compared with those of the Control group, which underwent the ARE procedure without treatment. Administration of the RC mixture significantly ameliorated the severity of the histopathological changes compared with the ARE control group. Elevated levels of reactive oxygen species (ROS) in the esophageal tissues of reflux esophagitis-induced rats was significantly decreased by the RC mixture. Moreover, the increased ROS production induced by the increase in NADPH oxidase 4 (NOX4) and $p22^{phox}$ was down-regulated by the RC mixture. The protein expression of inflammatory mediators and cytokines together with nuclear factor kappa B (NF-κB) through the degradation of inhibitor of NF-κB was modulated by the RC mixture treatment. The levels of the anti-inflammatory cytokines, such as interleukin (IL)-4 and IL-10, were significantly increased. Additionally, RC mixture supplementation improved esophageal barrier function through upregulating claudin −1, −3 and −4 protein expression levels. Taken together, these results suggest that RC mixture treatment may attenuate the esophageal mucosal injury, possibly through suppression of ROS formation, inhibiting NF-κB inflammatory signaling, and up-regulating the expression of proteins associated with tight junction formation, including claudin −1, −3 and −4.

Key words: Acute reflux esophagitis, citri pericarpium, inflammation, rhei rhizoma, tight junctions

SUMMARY

- RC mixture alleviated acute reflux esophagitis through both the inhibition of oxidative stress and the up-regulation of TJ proteins, such as claudins. Moreover, RC mixture reduced significantly the pro-inflammatory cytokines via the suppression of NF-κB activation and also elevated dramatically anti-inflammatory cytokines. Therefore, RC mixture could be a clinical remedy for patients suffering acute reflux esophagitis in the future.



Abbreviations used: GERD: Gastroesophageal reflux disease; ARE: Acute reflux esophagitis; ROS: Reactive oxygen species; TJ: Tight junction; NOX4: NADPH oxidase 4; NF-κB: Nuclear factor kappa B.

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INTRODUCTION

Gastroesophageal Reflux Disease (GERD) is one of the most prevalent gastrointestinal tract disorders, and it is characterized by regurgitation of gastric contents into the esophagus. The prevalence of GERD is ~20% in adults in western contries; however, the underlying pathophysiology incompletely understood.[1] The prevalence of GERD is predicted to increase with time, due to increasing consumption of alcohol, tobacco and high-fat diets.[2] Additionally, the high prevalence of GERD has a significant socio-economic burden due to the cost of prescription drugs, hospitalization, work absence and loss of productivity, whilst also adversely affects the quality of life, which is associated with the typical and clinical presentation of GERD including heartburn and regurgitation.[3,4] Proton pump inhibitors are commonly used to manage GERD; however, long term use can lead to serious complications.[5]

In recent years, significant progress has been made in explaining the esophageal barrier structure and function. This may allow easier identification and provide an improved understanding of potential therapeutic target that can help in the control of these diseases. The esophageal mucosa comprises of various constituents that serve as a defense barrier.[6,7] This defensive barrier can be collapsed by prolonged and repetitive exposure to the refluxate, which consists of acidic gastric contents such as hydrochloric acid and pepsin leading to mucosal damage. Namely, disruption of this epithelial defense is shown

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as a common phenomenon in GERD.[8] Tight junctions (TJ) form a paracellular barrier protecting them from the external environment by the expression of tissue-specific transport proteins or channels. Of the TJ proteins, the claudin family, comprised up to 27 members, is responsible for the formation of TJ strands and plays an important role in general epithelial transport physiology. Therefore, the dysfunction of TJ led to GERD, which is verified as damage to esophageal epithelium.[9-11] During gastro-esophageal reflux, gastric contents penetrated into the intercellular spaces led to inflammation, erosion, and necrosis.

The role of increased oxidative stress in the development of GERD has been well established.[12,13] Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-) and hydroxyl radicals (−OH), as well as reactive nitrogen species, such as peroxynitrite ($OONO^-$) and nitric oxide (NO) are released excessively into the inflammatory tissues, and this leads to their accumulation in the gastrointestinal tract. Therefore, oxidative stress is implicated in inflammation-based GERD, and this serves a key role in its pathogenesis.[14]

Rhei Rhizoma (Dahuang in Chinese) is a purgative and anti-inflammatory agent widely used in Korean, Chinese and Japanese pharmacopoeias.[15] It is known to exert several beneficial effects, including anti-inflammatory, antipyretic, anti-angiogenic and antineoplastic properties.[16-18] Moreover, in our previous study, the protective effect of Rhei Rhizoma in ARE was shown.[19] Citri Pericarpium has been widely used in Southeastern and Eastern Asia for treating respiratory and indigestion associated conditions.[20] Additionally, other pharmaceutical effects of Citri Pericarpium include anti-inflammatory, antioxidant, anticancer, anti-adipogenic, antiviral, anti-microbial and anti-allergenic properties, as well as neuroprotective effects.[21-23]

Accordingly, the aim of the present study was to determine whether a Rhei Rhizoma and Citri Pericarpium (RC) mixture could alleviate esophageal mucosal injury as a result of acute reflux esophagitis (ARE). In addition, the underlying mechanism by which the RC treatment exerted its effects on ARE was determined.

MATERIALS AND METHODS

Materials

Vitamin E and Phenyl methyl sulfonyl fluoride (PMSF) were provided by Sigma-Aldrich; Merck KGaA. The protease inhibitor mixture solution and ethylenediaminetetraacetic acid (EDTA) were provided from Wako Pure Chemical Industries, Ltd. Sodium carbonate was provided by Daejung Chemicals and Metals Co., Ltd. Sodium hydroxide was provided by OCI Company Ltd. Phosphoric acid was provided from Duksan company. 2',7'-Dichloro fluorescein diacetate (DCF-DA) was provided by Molecular Probes (Thermo Fisher Scientific, Inc.). The Pierce BCA protein assay kit was provided from Thermo Fisher Scientific, Inc. Enhanced chemiluminescence reagent (ECL), western blotting detection reagents and pure nitrocellulose membranes were obtained from GE Healthcare. The following antibodies were purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal NADPH oxidase 4 (NOX4), inhibitor of nuclear factor κBα (IκBα) and $p22^{phox}$; mouse monoclonal interleukin (IL)-4 and IL-10; goat polyclonal tumor necrosis factor-α (TNF-α) and IL-6; mouse polyclonal NF-κB p65 (NF-κBp65), phospho-inhibitor of NF-κBα (p-IκBα), claudin-1, claudin-3, claudin-4, histone and β-actin. 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. All other chemicals and reagents were purchased from Sigma-Aldrich; Merck KgaA, unless otherwise stated. Zoletil®50 was purchased from Virbac Laboratory. Isotroy was purchased from Troikaa Pharmaceuticals, Ltd.

Preparation of the plant material

RC were purchased from Ominherb Co. Extracts of the dried Rhei Rhizoma (30 g) and Citri Pericarpium (30 g) were obtained by addition of ×10 the volume of boiled water at room temperature (2 h for each extraction), and the solvent was evaporated *in vacuo* to obtain an extract with a yield of 16.33% (Rhei Rhizoma) and 35% (Citri Pericarpium) by weight. The two prepared powders were stored at −80°C and dissolved in water when required.

Experimental animals and treatment

All animal experimental protocols were performed in accordance with the Animal Care and Use Committee of Daegu Haany University (approval no.DHU2020-072). The 6-week-old male Sprague-Dawley rats (body weight, 180–200 g) were obtained from DaehanBioLink and allowed to acclimatize for 1 week. Rats were with a 12 h light/dark cycle, at a controlled humidity (50% ± 5%) and temperature (22°C ± 2°C). During the adaptation period, we looked at them once a day. The rats were constantly monitored on the day of the experiment. After 1 week of adaptation, 40 rats were randomly divided into 5 groups (n = 8 per group) as follows:

(i) Normal, normal group; (ii) Control, ARE-induced rats were treated with distilled water; (iii) Vitamin E, ARE-induced rats were treated with Vitamin E (30 mg/kg body weight); (iv) RC100, ARE-induced rats were treated with RC (100 mg/kg body weight); and (v) RC200, ARE-induced rats were treated with RC (200 mg/kg body weight).

Herein, RC100 and RC200 were used by mixing RC (1:1) just before drug treatment. We previously reported the protective effect of a mixture containing Rhei Rhizoma on reflux esophagitis.[24] Since the mixture exhibited a significant effect at 200 mg/kg body weight, the maximum concentration was set to 200 mg/kg body weight. Rats were fasted for 18 h prior to surgery, maintained with a raised mesh-bottom cage to prevent co-propagation, and water was supplied until surgery. A total of 90 min prior to surgery, rats were orally administered distilled water, vitamin E or RC. The general anesthesia of rats was performed using a modified protocol described by Ferrari *et al*.[25] A anesthesia was induced using tiletamine and zolazepam (Zoletil®50; 37.5 mg/kg), after which, the gastric gland was exposed and a midline laparotomy was performed, and both the pylorus and the transitional junction between the corpus and the forestomach was ligated with a 2-0 silk thread as described previously by Pawlik *et al*.[26] A total of 5 h after surgery, rats were anesthetized by Isotroy inhalation anesthesia (induction, 4% isoflurane; maintenance, 2% isoflurane) for 5–7 min. Subsequently, blood was drawn from the abdominal vein, and centrifuged at 4800 × g for 20 min at 4°C. After collecting blood samples of 2 mL, 40 rats were euthanized by cervical dislocation and the death of rats was confirmed through the absence of reflexes. Accordingly, total duration of this experiment is 8 days. The esophageal tissues were immediately stored at −80°C.

Esophageal mucosal damage ratio

After sacrificing animals, the rat esophagus was cut from the gastroesophageal junction to the pharynx. The dissected esophagus was imaged using an optical digital camera and then analyzed using the i-Solution Lite software program (Innerview Co.).

The gross mucosal damage ratio as a percentage was calculated as follows: (Width of area with esophageal mucosal damage [mm^2]/width of the total area of esophagus [mm^2]) × 100.

Measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) *levels in serum.* Hepatic functional parameters, AST and ALT, were measured using specific assay kits and a microplate

fluorescence reader (Transaminase CII-Test; Wako Pure Chemical Industries Ltd.).

Measurement of reactive oxygen species levels in the serum

ROS, a marker of oxidative stress biomarker, was measured as described by Ali *et al.*[27] After mixing serum with 1 mM EDTA-50 mM sodium phosphate buffer (pH 7.4), 25 mM DCF-DA was added, and incubated for 30 min. The changes in fluorescence values were determined at an emission spectra of 535 nm and an excitation spectra of 485 nm using a ultraviolet-visible spectrophotometer (Infinite M200 Pro; Tecan) every 5 min for 30 min.

Preparation of cytosol and nuclear fractions

We used 7 out of 8 rats for western blotting. Protein extraction was performed as described by Komatsu,[28] with minor modifications. Briefly, the cytosolic fractions from the esophageal tissues were obtained by homogenization of the tissues in ice-cold lysis buffer (250 ml; 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). After incubation for 20 min at 4˚C, the solution was centrifuged on a 5415R Centrifuge (Eppendorf). The supernatant (cytosolic fraction) was collected and placed in a clean e-tube and the homogenate was incubated for 20 min at 4°C with 10% NP-40, ensuring thorough mixing. After centrifugation (19,200 × g for 2 min at 4°C, the pellets were washed twice with the lysis buffer and the supernatant was discarded. Next, the pellets were resuspended in 20 ml ice cold lysis solution consisting of 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 for 30 min at 4°C. After centrifugation (19,200 × g for 10 min at 4°C), the nuclear fraction was prepared to collect the supernatant. Both the cytosolic and nuclear fractions were stored at −80°C prior to the analysis.

Immunoblotting analysis

To determine the protein expression levels of NF-κBp65 (SC-8008; 1:1000) and histone (SC-8030; 1:1000), 12 μg protein from each nuclear fraction was loaded on a 10% SDS-PAGE, resolved using SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in 5% (w/v) skimmed milk solution for 1 h, then incubated with primary antibodies against NF-κBp65 or histone overnight at 4°C. After the blots were washed, they were incubated with anti-rabbit or anti-mouse IgG HRP-conjugated secondary antibody for 1 h at room temperature. In addition, 8 μg protein from each cytosolic fraction was resolved using 8%–15% SDS-PAGE to analyze NOX4 (SC-518092; 1:1000), $p22^{phox}$ (SC-271968; 1:1000), claudin-1 (SC-166338; 1:1000), claudin-3 (SC-517546; 1:1000), claudin-4 (SC-376643; 1:1000), IL-4 (SC-53084; 1:1000), IL-10 (SC-365858, 1:1000), TNF-α (SC-133192; 1:1000), IL-6 (SC-5735; 1:1000) and β-actin (SC-4778; 1:1000) expression. Each antigen-antibody complex was developed using ECL Western Blotting Detection Reagent, and visualized based on the chemiluminescence with a Sensi-Q 2000 Chemidoc (Lugen Sci Co., Ltd.). Densitometry analysis was measured using the ATTO Densitograph software (ATTO Corporation) and quantified relative to the expression of histone or β-actin. Protein expression levels in each group is expressed relative to that of the Normal rats (which was considered as 1).

Histological examination

For microscopic evaluation, esophageal tissue was fixed in 10% neutral-buffered formalin and after embedding in paraffin, cut into 2-μm sections and stained using hematoxylin and eosin, for microscopic evaluation. The stained slices were observed under an optical microscope and analyzed using the i-Solution Lite software program (InnerView Co.).

Statistical analysis

Data are presented as the mean ± standard deviation. Data were compared using a one-way analysis of variance followed by Tukey's *post hoc* test in SPSS version 26.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Rhei Rhizoma and Citri Pericarpium mixture improves gross mucosal damage and histological changes in the esophagus

The gross observation of the esophagus was first used to determine the efficacy of RC mixture on the surgically induced ARE lesions. As shown in Figure 1a, the Normal group did not exhibit any detectable and definite damage of the esophageal mucosa, whereas the esophagus in the Control group showed notable changes in the morphology, including erosions and hyperemia. Additionally, gross mucosal damage in the RC-treated groups was notably lower compared with the Control group [Figure 1b]. The histology of the esophagus in the Normal group exhibited a regular epithelium without infiltration of inflammatory cells. However, the esophagus in the ARE control group exhibited considerable inflammatory cell infiltration and the elimination of the mucosa [Figure 1c]. The esophageal mucosal damage was reduced in the rats treated with the RC mixture compared with that of the Control rats.

Rhei Rhizoma and Citri Pericarpium mixture significantly reduces serum reactive oxygen species levels

Serum analysis was used to detect the ROS levels, as an indicator of oxidative stress and inflammation. As shown in Figure 2, ROS levels in the Control group were markedly higher compared with the Normal group (10,457 ± 1287 vs. 20,829 ± 872 fluorescence/min/ml, $P < 0.001$), whereas the levels were significantly reduced in the RC mixture.

Rhei Rhizoma and Citri Pericarpium mixture significantly regulates the expression of markes of liver and kidney function

Figure 3 shows the effects of RC mixture on general biochemical serum parameters. The Control group showed significantly higher AST and ALT levels compared with the Normal group. Specifically, their levels were 1.51-and 6.34-fold higher in the Control group, respectively sompared with the Normal group. The increase in these parameters of hepatic function were significantly reduced in the RC200 group. In addition, BUN levels, which are used a parameter of kidney function were increased by administration of the RC mixture.

Esophageal NADPH oxidase expression is reduced by the Rhei Rhizoma and Citri Pericarpium mixture

NOX4 and $p22^{phox}$ protein expression was quantified using western blotting [Figure 4]. The protein expression levels of ROS-generating NADPH oxidases in the Control group were significantly increased

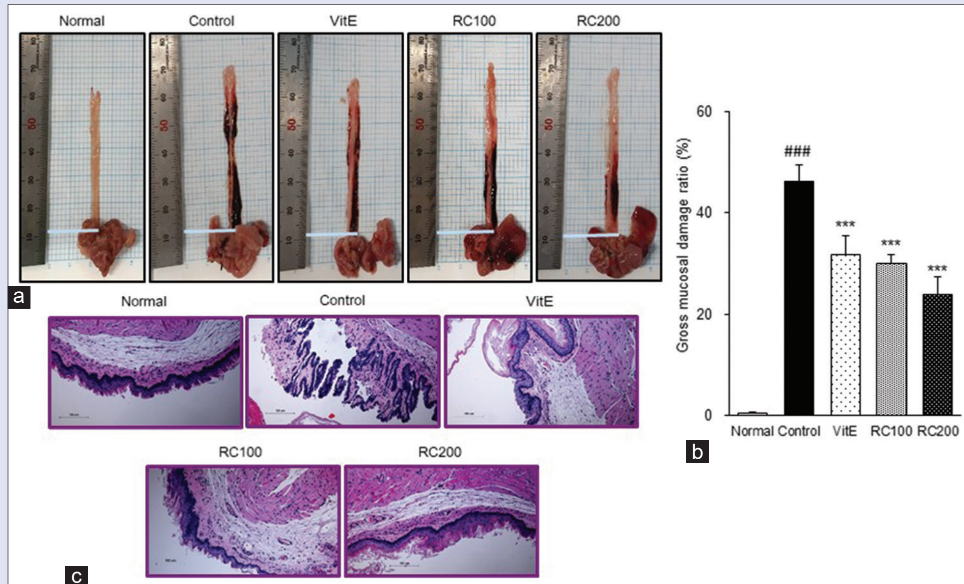

Figure 1: Rhizoma and Citri Pericarpium mixture reduces gross mucosal damage and histological changes in the esophagus. Representative images are shown. (a) Gross images of esophageal mucosal damage, (b) gross mucosal damage ratio and (c) histological images of esophageal mucosal damage. Representative gross appearance and hematoxylin and eosin staining of acute reflux esophagitis-induced esophageal mucosal injury. Injury was notably ameliorated by Rhizoma and Citri Pericarpium mixture treatment. Magnification, x100; scale bar, 100 μm. $^{###}P < 0.001$ versus Normal, $^{***}P < 0.001$ versus Control. acute reflux esophagitis, acute reflux esophagitis; VitE: vitamin E; RC: Rhei Rhizoma and Citri Pericarpium; Normal, normal group; Control, acute reflux esophagitis control group; VitE, VitE 30 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 100, Rhei Rhizoma and Citri Pericarpium 100 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 200, Rhei Rhizoma and Citri Pericarpium 200 mg/kg body weight/day-treated acute reflux esophagitis group

compared with the Normal group (NOX4, 1.43 ± 0.15; $p22^{phox}$, 1.24 ± 0.08). Conversely, RC200 supplementation significantly decreased their levels.

Esophageal phospho-inhibitor of nuclear factor kappa B α and nuclear factor kappa B p65 protein expression is reduced by the Rhei Rhizoma and Citri Pericarpium mixture

The expression of inflammation-related proteins, including p-IκBα and NF-κBp65 were examined. As shown in Figure 5, the protein expression levels of p-IκBα and NF-κBp65 were up-regulated by 0.32-and 0.25-fold in the Control compared with the Normal group, whereas the increase in their expression was significantly reduced in the RC mixture treated ARE rats. In particular, treatment with RC200 reduced their levels to that observed in the Normal group.

Levels of inflammatory cytokines, such as tumor necrosis factor-α and interleukin-6, are reduced by the Rhei Rhizoma and Citri Pericarpium mixture

The protein expression levels of inflammatory cytokines, including TNF-α and IL-6, were examined. As shown in Figure 6, both TNF-α and IL-6 expression was significantly increased in the esophagus of the Control group compared with the Normal group, whereas these elevated levels were significantly reduced by treatment with the RC mixture. Here, TNF-α levels were reduced to below that of the Normal group by the RC mixture.

Levels of anti-inflammatory cytokines, such as interleukin-4 and interleukin-10, are upregulated by the Rhei Rhizoma and Citri Pericarpium mixture

The protein expression levels of anti-inflammatory cytokines, such as IL-4 and IL-10, were examined. As shown in Figure 7, the protein levels of IL-4 and IL-10 decreased in the esophagus of the Control group. Here, IL-4 was significantly downregulated by 21.2% and IL-10 was significantly downregulated by 16.3%. However, these reduced levels were significantly increased in the RC mixture treated ARE rats compared with the Control group.

Expression of tight junction-related proteins is increased by the Rhei Rhizoma and Citri Pericarpium mixture

Expression of TJ-related proteins was quantified, as they function to ensure the esophageal epithelium is adequately sealed to prevent leakage [Figure 8]. In the Control group, claudin-1,-3 and-4 protein expression was significantly downregulated compared with the Normal group. RC200 significantly increased the expression both claudin-3 and-4, except for claudin-1 compared with the Control group. RC200 resulted in a greater increase in the expression of these TJ proteins.

DISCUSSION

Both RC are well documented for their anti-inflammatory effects; however, the effects and the underlying mechanisms of the combination of these two treatments has not been assessed, to the best of our knowedge.[29,30] In the present study, the effects of the RC mixture on

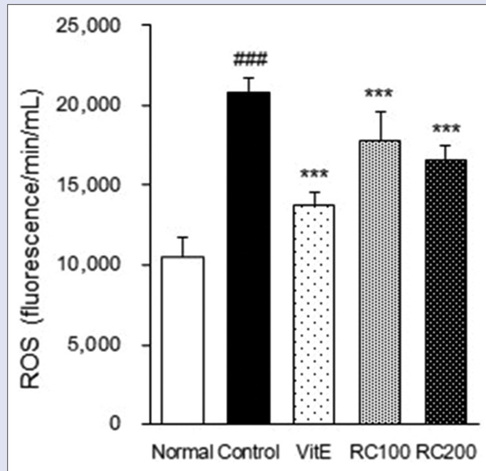

Figure 2: Rhei Rhizoma and Citri Pericarpium mixture significantly reduces serum reactive oxygen species levels. Effects of the Rhei Rhizoma and Citri Pericarpium mixture on the reactive oxygen species levels in rats with surgically induced acute reflux esophagitis. The Rhei Rhizoma and Citri Pericarpium mixture (100 or 200 mg/kg) was administered 90 min before the surgical procedure. The increased reactive oxygen species levels in the control group were significantly decreased by administration of the Rhei Rhizoma and Citri Pericarpium mixture. Data are expressed as the mean ± standard deviation (n = 8). ###P < 0.001 versus Normal group, ***P < 0.01 versus Control. reactive oxygen species, reactive oxygen species; acute reflux esophagitis, acute reflux esophagitis; VitE: Vitamin E; RC: Rhei Rhei Rhizoma and Citri Pericarpium; Normal, normal group; Control, acute reflux esophagitis control group; Vitamin E, Vitamin E 30 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 100, Rhei Rhizoma and Citri Pericarpium 100 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 200, Rhei Rhizoma and Citri Pericarpium 200 mg/kg body weight/day-treated acute reflux esophagitis group

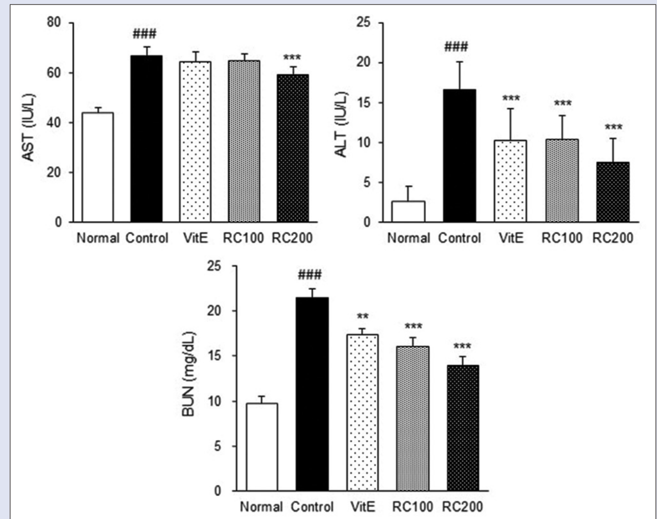

Figure 3: Rhei Rhizoma and Citri Pericarpium mixture significantly regulates the expression of functional markers of liver and kidney function. The increases in the levels of functional markers in the Control group were significantly decreased by Rhei Rhizoma and Citri Pericarpium supplementation. Data are expressed as the mean ± standard deviation (n = 8). ###P < 0.001 versus Normal group; **P < 0.01, ***P < 0.01 versus Control. acute reflux esophagitis, acute reflux esophagitis; VitE: vitamin E; RC: Rhei Rhei Rhizoma and Citri Pericarpium; Normal, normal group; Control, acute reflux esophagitis control group; Vitamin E, Vitamin E 30 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 100, Rhei Rhizoma and Citri Pericarpium 100 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 200, Rhei Rhizoma and Citri Pericarpium 200 mg/kg body weight/day-treated acute reflux esophagitis group

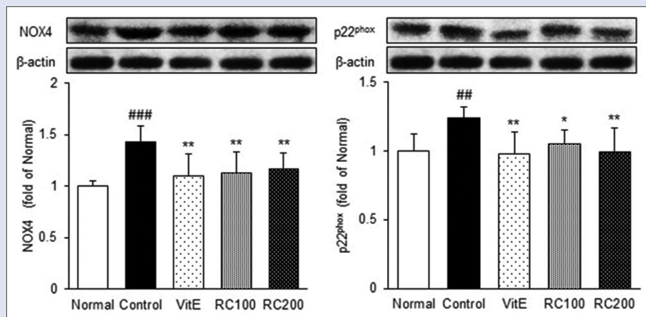

Figure 4: Esophageal NADPH oxidase expression is reduced by the Rhei Rhizoma and Citri Pericarpium mixture. The increased expression of NOX4 and $p22^{phox}$ in the Control group were significantly decreased by Rhei Rhizoma and Citri Pericarpium treatment. Data are expressed as the mean ± standard deviation (n = 7). ###P < 0.001 versus Normal group; *P < 0.05, **P < 0.01, ***P < 0.01 versus Control. Acute reflux esophagitis, acute reflux esophagitis; VitE: Vitamin E; RC: Rhei Rhei Rhizoma and Citri Pericarpium; Normal, normal group; Control, acute reflux esophagitis control group; Vitamin E, Vitamin E 30 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 100, Rhei Rhizoma and Citri Pericarpium 100 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 200, Rhei Rhizoma and Citri Pericarpium 200 mg/kg body weight/day-treated acute reflux esophagitis group; NOX4, NADPH oxidase 4

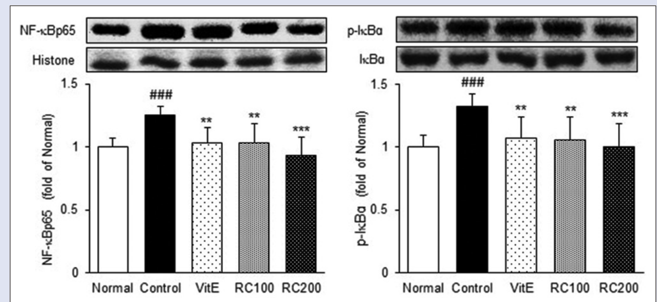

Figure 5: Esophageal p-IκBα and nuclear factor-κBp65 protein expression is reduced by the Rhei Rhizoma and Citri Pericarpium mixture. Expression of p-IκBα and nuclear factor-κBp65 in the Control group was significantly decreased by Rhei Rhizoma and Citri Pericarpium treatment. Data are expressed as the mean ± standard deviation (n = 7). ###P < 0.001 versus Normal group; **P < 0.01, ***P < 0.01 versus Control. acute reflux esophagitis, acute reflux esophagitis; VitE: Vitamin E; RC: Rhei Rhei Rhizoma and Citri Pericarpium; Normal, normal group; Control, acute reflux esophagitis control group; Vitamin E, Vitamin E 30 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 100, Rhei Rhizoma and Citri Pericarpium 100 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 200, Rhei Rhizoma and Citri Pericarpium 200 mg/kg body weight/day-treated acute reflux esophagitis group; nuclear factor-κBp65, nuclear factor κB p65; IκBα, inhibitor of nuclear factor-κBα; p-, phospho-

surgically induced ARE was assessed. Here, the highest concentration of RC mixture was set 200 mg/kg body weight based on the previously

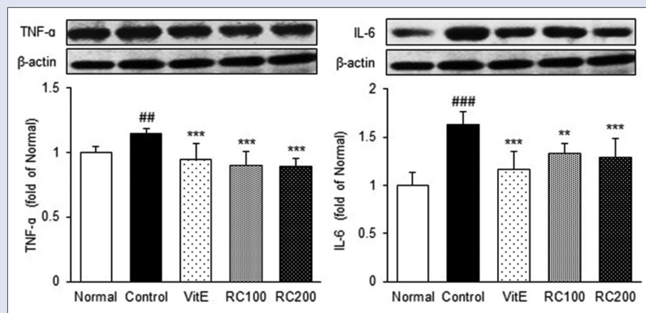

Figure 6: Expression of the inflammatory cytokines tumor necrosis factor-α and interleukin-6 is reduced by the Rhei Rhizoma and Citri Pericarpium mixture. The increased levels of tumor necrosis factor-α and interleukin-6 in the Control group were significantly decreased by Rhei Rhizoma and Citri Pericarpium supplementation. Data are expressed as the mean ± standard deviation (n = 7). ###P < 0.001 versus Normal group; **P < 0.01, ***P < 0.01 versus Control. acute reflux esophagitis, acute reflux esophagitis; VitE: Vitamin E; RC: Rhei Rhei Rhizoma and Citri Pericarpium; Normal, normal group; Control, acute reflux esophagitis control group; Vitamin E, Vitamin E 30 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 100, Rhei Rhizoma and Citri Pericarpium 100 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 200, Rhei Rhizoma and Citri Pericarpium 200 mg/kg body weight/day-treated acute reflux esophagitis group; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6

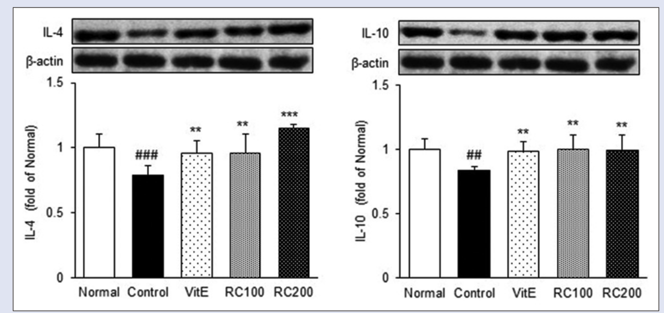

Figure 7: The expression of inflammatory cytokines, such as interleukin-4 and interleukin-10, is reduced by the Rhei Rhizoma and Citri Pericarpium mixture. The increased expression of interleukin-4 and interleukin-10 in the Control group compared with the Normal group was significantly reduced by treatment with the Rhei Rhizoma and Citri Pericarpium mixture. Data are expressed as the mean ± standard deviation (n = 7). ##P < 0.01, ###P < 0.001 versus Normal group; **P < 0.01, ***P < 0.01 versus Control. ARE: Acute reflux esophagitis; VitE: Vitamin E; RC: Rhei Rhei Rhizoma and Citri Pericarpium; Normal, normal group; Control, acute reflux esophagitis control group; Vitamin E, Vitamin E 30 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 100, Rhei Rhizoma and Citri Pericarpium 100 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhizoma and Citri Pericarpium 200, Rhei Rhizoma and Citri Pericarpium 200 mg/kg body weight/day-treated acute reflux esophagitis group; IL-4: Interleukin-4; IL-10: Interleukin-10

reported experiment. RC treatment significantly reduced the degree of esophageal mucosal damage in rats, reduced ROS production, inhibited activation of the NF-κB inflammatory pathway, and restored the expression of TJ proteins. In addition, based on the levels of anti-inflammatory cytokines, RC mixture significantly exerted protective effects on the inflammatory response.

ARE, which is considered as the early stage of GERD, is known to be regulated by numerous inflammatory mediators and other factors. These inflammatory mediators can decrease the lower esophageal sphincter pressure, degrade the mucosal barrier function, and reduce the esophageal peristalsis.[31,32] Recently, several studies showed that the severity of the inflammatory disorders in patients with reflux esophagitis is closely associated with oxidative stress.[33] ROS production results from the metabolic reactions, such as those that occur during the mitochondrial electron-transport chain, which use oxygen, or through excessive activity of NAD(P)H. Additionally, it can arise through an imbalance in pro-oxidant/anti-oxidant reactions in living organisms.[34] Excess ROS can be affect physiological function through damage to basic structures of cells, including membranes, lipids, proteins and DNA. Thus, oxidative stress has been implicated in several human diseases, including reflux-induced esophageal damage.[35,36] ROS scavengers were shown to prevent DNA damage and alleviate the various symptoms of esophageal disorders.[37] Kwon *et al*.[24] showed that the severity of esophageal mucosal damage on reflux esophagitis was increased when ROS levels were increased. In the present study, excessive stimulation of NAD(P)H isoforms, such as NOX4 and p22^{phox}, or the increase in serum ROS in the Control group resulted in oxidative stress. Whereas, the increased ROS levels were significantly reduced by treatment with the RC mixture. This suggests that the RC mixture may effectively augment elimination of ROS.

Increased production of ROS can trigger the proinflammatory pathways of NF-κB. NF-κB is a ubiquitous transcription factor of inflammatory mediators that controls transcription of a diverse set of gene targets. It is involved in the cellular responses to various stimuli, such as free radicals, ROS, stress and cytokines. Under physical conditions, NF-κB is bound to IκB in the cytoplasm, where IκB is an inhibitor of NF-κB. However, the translocation of NF-κB to the nucleus results in its activation, where it regulates gene expression.[38] NF-κB activation results in increased production of inflammatory mediators, including enzymes, cytokines, cell adhesion molecules and chemokines.[39] NF-κB stimulation promotes the expressions of pro-inflammatory cytokines, such as TNF-α and IL-6. A series of processes can exacerbate the inflammatory response.[40] However, anti-inflammatory cytokines, such as IL-4 and IL-10, serve an antagonistic role, attenuating the inflammatory response and reducing the secretion of pro-inflammatory cytokines.[41] The present study demonstrated that the RC mixture downregulated gene expression levels of TNF-α, and IL-6 whereas, IL-4 and IL-10 were upregulated, compared with the Control group. The RC mixture not only suppressed proinflammatory factors, but also increased anti-inflammatory cytokines, via the inactivation of the NF-κB pathway by inhibiting the phosphorylation of IκB.

The human epithelium is formed of epithelial cells, and the space between these cells is closely sealed by TJs. The failure of the TJ barrier function by detrimental inflammatory mechanisms is an important concept in gastrointestinal pathophysiology.[42] TJs consist of members of the claudin family of proteins, occludin, and junctional adhesion molecules. Claudins, which are TJ integral membrane proteins, help to maintain the specificity of TJ permeability and act as a key regulator of the paracellular pathway. Among the claudins, claudins-1 and-3 exhibit sealing functions, and H_2O_2-mediated degradation of claudin-3 results in the increased epithelial permeability.[42,43] Kojima *et al*.[44] reported that claudin-4 was directly regulated by p63 in normal epithelial cells or diseases. Accordingly, the regulation of claudin-4 in human epithelial cells is considered an important factor in the development of drug delivery systems. Additionally, Chen *et al*.[7] demonstrated that acid, pepsin, and bile acids disrupt epithelial barrier function partly by regulating TJ proteins, such as claudin-1 and-4. In the present

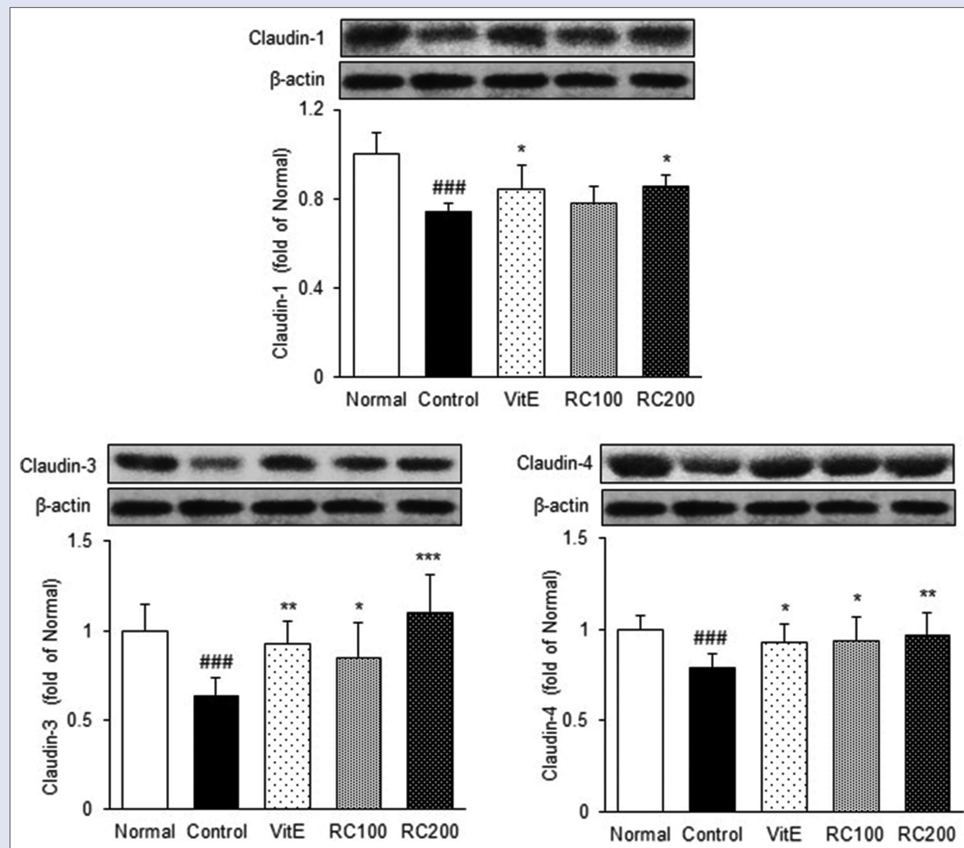

Figure 8: Expression of Tight junction-related proteins is increased by the Rhei Rhizoma and Citri Pericarpium mixture. The decreased expression of claudin-1, 3 and 4 in the Control group compared with the Normal group was significantly increased by Rhei Rhizoma and Citri Pericarpium treatment. Data are expressed as the mean ± standard deviation ($n = 7$). $^{###}P < 0.001$ versus Normal group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$ versus Control. acute reflux esophagitis, acute reflux esophagitis; VitE: Vitamin E; RC: Rhei Rhei Rhizoma and Citri Pericarpium; Normal, normal group; Control, acute reflux esophagitis control group; Vitamin E, Vitamin E 30 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhei Rhizoma and Citri Pericarpium 100, Rhei Rhei Rhizoma and Citri Pericarpium 100 mg/kg body weight/day-treated acute reflux esophagitis group; Rhei Rhei Rhizoma and Citri Pericarpium 200, Rhei Rhizoma and Citri Pericarpium 200 mg/kg body weight/day-treated acute reflux esophagitis group

study, claudin-1,-3 and-4 were shown to be down-regulated in the esophageal epithelium in the Control group, whereas RC mixture treatment significantly up-regulated the expression of TJ proteins. These results suggest that the RC mixture reduced the disturbance to the esophageal barrier function by modulating TJ proteins in the ARE rat models.

CONCLUSION

Recent studies have shown that both oxidative stress and epithelial barrier dysfunction are damaging factors in esophageal mucosal damage, including in ARE. In the present study, administration of the RC mixture significantly reduced the levels of oxidative stress, and up-regulated the expression of TJ proteins, such as claudin-1,-3 and-4. Furthermore, the anti-inflammatory effects of the RC mixture suggested that the inhibition of NF-κB nuclear translocation resulted in reduced production of pro-inflammatory enzymes and cytokines. Consequently, the RC mixture significantly ameliorated ARE-induced esophageal mucosal damage. These results improve our understanding of effect and the underlying mechanism of the RC mixture in the management of ARE.

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Ethics approval and consent to participate

Animal experiments were carried out according to the “Guidelines for Animal Experimentation” approved by the Ethics Committee of the Daegu Haany University with certificate number DHU2020-072.

Authors' Contributions

Mi-Rae Shin and Jin A Lee contributed equally to this work.

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