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DIETARY THREONINE PREVENTED STRESS-RELATED MUCOSAL DISEASES IN RATS

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Stress-related mucosal disease (SRMD), or stress ulceration, is a group of conditions ranging from stress-related superficial gastric mucosal damage to deep gastric ulcers that are primarily correlated with mucosal ischemia, and pharmacologic interventions that optimize tissue perfusion or preserve defensive mucus aim to decrease the occurrence of conditions, such as gastric acidity, or enhance gastric defenses. However, the identification of multifactorial pathogenesis may be effective in preventing SMRD, and the use of stress prophylaxis is generally preferred. Since threonine is a component in the polymerization and synthesis of gastric mucin and possibly enhanced defense actions and lignin may provide structural support for defense and antioxidative function, we hypothesized that dietary intake of threonine and/or lignin can enhance defense against SRMD. The water immersion-restraint stress (WIRS) was used in rats and additional groups were pretreated with threonine alone or the combination of threonine and lignin. Based on gross and microscopic evaluations, threonine alone or the combination of threonine and lignin, a natural antioxidant, significantly reduced the development of SRMD ($P < 0.05$). According to molecular explorations, the levels of inflammatory mediators, such as interleukin (IL)-8, IL-6, IL-1β, inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ), all of which are mediators that play a significant role in controlling WIRS, significantly decreased in the groups pretreated with either threonine alone or the combination of threonine and lignin ($P < 0.01$). WIRS significantly increased apoptosis in the stomach. However, the apoptotic index significantly decreased with threonine pretreatment. According to periodic acid Schiff staining results, the expression of gastric mucin was significantly preserved in groups pretreated with threonine but remarkedly decreased in the WIRS group. The gastric heme oxygenase-1 levels significantly increased in the group treated with threonine. In conclusion, the dietary intake of threonine or the combination of threonine and lignin is effective in preventing SRMD.

Key words: *stress-related mucosal disease, threonine, lignin, gastric defense, mucin, cytoprotection, heme oxygenase-1*

INTRODUCTION

Stress-related mucosal disease (SRMD) is a common clinical finding that encompasses the continuum from stress-related mucosal damage to stress ulceration in humans as well as animals. On endoscopy, SRMD is shown as diffuse and superficial mucosal erosions to deep submucosal ulcers, some of which present with overt gastrointestinal (GI) bleeding, leading to hemodynamic instability and hypovolemic shock in some cases. The incidence of such condition was as high as 6 – 100% in critically ill human patients presenting with superficial and erosive changes in the stomach and overt ulcers who were admitted in the intensive care unit (ICU) (1-4). The incidence of SRMD is not high among patients in the ICU. However, in patients who develop such condition, the morbidity and mortality are extremely high. Furthermore, in the field of veterinary, SRMD is troublesome since animals are at extremely high risk for stress, and such condition is a common cause of mortality; thus, a fundamental treatment is required (5-6).

Although the exact pathogenesis of SRMD is not completely elucidated, it is known to be multifactorial and complex and may be primarily correlated with mucosal ischemia, decreased host defenses, and subsequent enormous gastric acid attack (2-4, 7, 8). Therefore, therapy aims to enhance the defense system, which is composed of mucus barrier, such as unstirred layer of mucus gel, bicarbonate, and surfactant phospholipids, epithelial integrity composed of rapid epithelial cell restitution and renewal of damaged mucosa, and adequate mucosal blood flow along with the prevention of conditions, including strong acid suppression (9, 10). Among the implications of gastric defenses, gastrointestinal hypoperfusion and subsequent ischemia are the primary conditions leading to SRMD. After ischemia/reperfusion, proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and inducible nitric oxide synthase (iNOS), released after leukocyte activation, cause endothelial cell swelling and vascular leakage, which further aggravate and compromise gastrointestinal perfusion (3-4, 7).

The frontline and immediate mechanism among the defensive barriers include maintenance of a neutral pH at the surface of the gastric epithelium *via* HCO_3^- retention, prevention of pepsin proteolysis, and hydrophobic maintenance of surfactant phospholipids, in which the final efficacy is

dependent on the mucus gel structure and thickness. Mucin is produced within mucus-producing surface or neck cells in which mucus are composed of glycoprotein subunits joined by disulfide bridges that form polymers. Each glycoprotein subunit comprised a central peptide core with closely packed sugar residues, two large functional regions composed of glycosylated regions that protect mucin from proteolytic attack, and other non-glycosylated regions accessible to proteolysis that facilitates the formation of the polymeric mucin structure. In this process, with the use of serine or threonine in the polypeptide core synthesized in the ribosomes, sugars are transferred one after the other in the golgi to complete glycosylation that is essential in mucin synthesis. Therefore, providing optimal threonine amino acid in the pellet diet can protect the stomach from SRMD *via* enhanced defense systems, such as strengthened gastric mucin. Thus, threonine-enriched diet can help prevent stress ulceration in animals that are at risk.

In this study, based on the hypothesis that threonine and/or lignin can protect against SRMD after water immersion-restraint stress (WIRS), we performed a study on animal model to measure the efficacy of threonine and/or lignin and to elucidate the underlying molecular mechanisms. Lignins are complex phenolic polymers extracted from vegetal resources, such as trees and crops, and are amorphous polymers composed of methoxylated phenylpropane structures. The current study used lignins to dilute threonine with additional anticipation of antioxidative action (11-13). Our study aimed to identify the possible application of dietary threonine to prevent SRMD in the future, particularly in the field of veterinary since threonine is abundant in dietary food for animals.

MATERIALS AND METHODS

Water immersion restraint stress model to induce stress related gastric disease

A total of 110 Sprague-Dawley (SD) rats were purchased from Charles River (Osaka, Japan) and kept in an animal facility. Animals were handled in an accredited animal facility in accordance with theAAALAC International Animal Care Policies of CHA Bio Complex (CHA University, Pangyo, Korea) after IRB approving (# 2018-0411). The animals were deprived of food, but allowed free access to water 24 hours before exposure of WIRS. Ten rats in each group were placed in strained cages and immersed in water (WIRS) for 6 hours. Animals were killed immediately after the end of 6-h of WIRS. As shown in *Fig. 1*, 60 rats were subjected to WIRS and gross lesion index were determined in 6 groups (n = 10/group). After this preliminary study, animals were repeatedly divided into four groups as follows: normal rats without any intervention except oral administration of normal saline, WIRS group as applying WIRS for 6 hours. Threonine group as threonine 30 mg/kg per oral (p.o.) pretreatment 8 hours before applying WIRS, and the combination of threonine and lignin group p.o. 8 hours before WIRS. Animals were killed with high dose anesthesia (thiopental sodium, 50 mg/kg, intraperitoneally). The stomachs of rats were removed and opened along the greater curvature, and then washed with iced cold PBS solutions. The number and size of either erosions or ulcers were determined under the magnified photographs, after which half of each dissected. Stomach was spread onto a plastic sheet, fixed in 10% buffered formalin for 4 hours, and prepared for paraffin tissue slidesand the remaining half was kept in a liquid nitrogen tank for further molecular study. The mucosal homogenates were pooled together.

Macroscopic and microscopic evaluation

The stomach isolated from the rat was placed in 10% buffered formalin and embedded in paraffin, and sections were cut. With the modification of the criteria of Yamamoto *et al.* (14), the bleeding index was evaluated on formalin-fixed sections and the bleeding rate (%) was calculated. Simply stated, index 0 means no bleeding at all, index 1 is mild bleeding showing the presence of small amounts of coagula in the stomach, index 2 is moderate bleeding showing intermediate state between 1 and 3 points, and index 3 is severe bleeding that contents of the stomach were filled with bleedings including coagula.

Preparation of threonine and the combination of threonine and lignin

The threonine samples used in this experiment were prepared in one powder type (Threonine ca. ≥ 99.9%), one granule type (Threonine cont. ca. 75%) and three granular-type threonine containing calcium-lignosulfonate (threonine 70%, 65% and 60%). The powder type threonine was produced through refining and crystallizing the *Corynebacterium glutarium* fermentation broth, and the granule-type threonine (threonine ca. 75%) was produced by directly drying the *C. glutarium* fermentation broth without any eradication process. Lastly, the granular-type threonine samples containing lignosulfonate were prepared by mixing Ca-lignosulfonate (Aladdin industrial Co., Shanghai, China) with granule threonine for making different threonine level of samples. The content ratios threonine samples are shown in the *Table 1*. Before the

Table 1. Composition of powder and granular threonine samples.

Thr content (%)	Fermentation broth (%)	*Corynebacterium* in fermentation broth (%)[1]	Calcium lignosulfonate (%)
Powder Thr 99.9%	–	–	–
Granule Thr 75.0%	25.0	4.0	0.0
Granule Thr 70.0%	23.3	3.8	6.7
Granule Thr 65.0%	21.7	3.5	13.3
Granule Thr 60.0%	20.0	3.2	20.0

[1]Estimated value according to the number of colony in the fermentation broth.

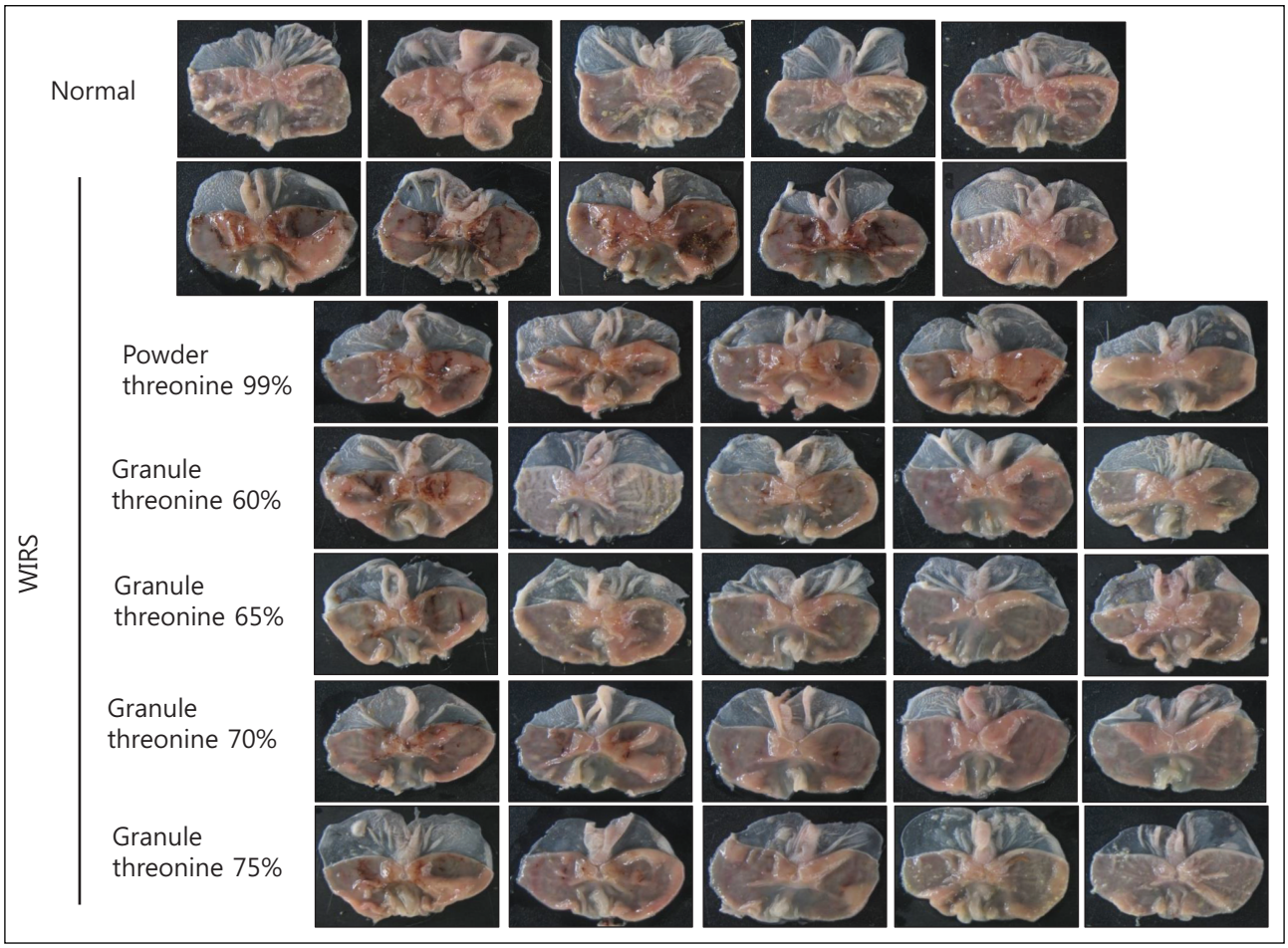

Fig. 1. Based on the previous study used to determine the group, as shown *Fig. 2*, we have included seven experimental groups (normal, WIRS control, threonine 99%, granule threonine 60%, granule threonine 65%, granule threonine 70%, and granule threonine 75% groups), after which four groups were selected.

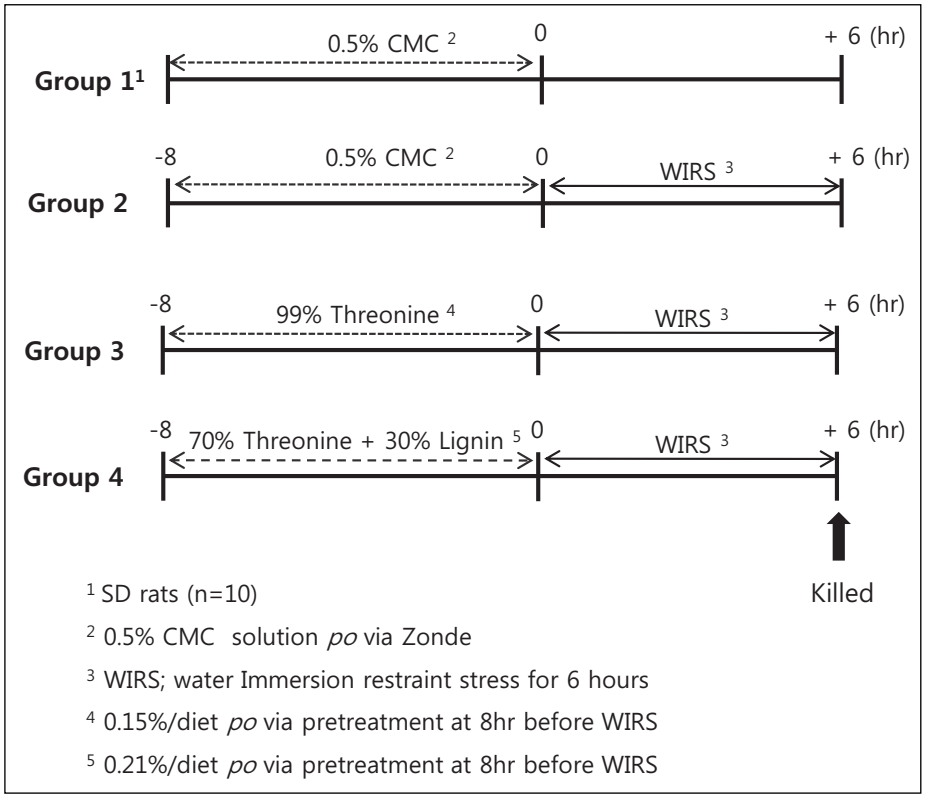

Fig. 2. Preventive action of threonine in WIRS-induced gastric mucosal damage. WIRS and experimental protocol. To identify the preventive effects of either threonine alone or the combination of threonine and lignin in pellet diets, pretreatment was carried out 8 h before the 6-h WIRS.

Table 2. Primers used in RT-PCR.

Gene	Forward Primer(5'→3')	Reverse Primer(5'→3')
IL-8	CACTCCCAGCATCGTAGAGC	CAGTGTACTTGTGGCGTGGA
iNOS	TTTTCCCAGGCAACCAGACG	GTAGCGGGGTTCAGAATGG
TNF-α	CCCTCACACTCAGATCATCTTCTCAA	TCTAAGGTACTTGGGCAGGTTGACCTC
IFN-γ	ATCCATGAGTGCTACACGCC	TCTGTGGGTTGTTCACCTCG
IL-6	CTTCCAGCCAGTTGCCTTCT	GAGAGCATTGGAAGTTGGGG
HIF-1α	TATCACTGGACTTCGGCAGC	GCTGCCGAAGTCCAGTGATA
PDGF	AGGAAGCCATTCCCGCAGTT	CTAACCTCACCTGGACCTCT
VEGF	CAATGATGAAGCCCTGGAGT	GATTTCTTGCGCTTTCGTTT
GAPDH	GGTGCTGAGTATGTCGTGGA	TTCAGCTCTGGGATGACCTT

current study, we have pretreated with five different formula as shown in *Table 1* before WIRS and according to gross lesion index as shown in *Fig. 1*, we proceed in detailed molecular exploration in group preated with threonine 99% and 70% threonine plus 30% lignin (*Fig. 2*).

RT-PCR

Total RNA was extracted using an RNeasy Mini kit (Qiagen Korea, Seoul, Korea). Primers used for inflammatory cytokines and mediators were shown in *Table 2*. The amplifications were done in 50-mL reaction volumes containing 10 × reaction buffer (Promega Korea, Seoul, Korea), 1.5 mM/L $MgCl_2$, 200 mM/L deoxynucleotide triphosphates (dNTP), 1 mM/L of each primer, and 2.5 units of *Taq* DNA polymerase (Promega) using a Perkin-Elmer GeneAmp PCR System 2400. Each cycle consists of denaturationat 95ºC for 1 min, annealing at 55ºC for 45 s, and amplification at 72ºC for 45 s.

Western blotting

Gastric mucosa was homogenized in ice-cold 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM ethylene diamine tetraacetic acid (EDTA), 0.5 mM ethylene glycol tetraacetic acid (EGTA), 300 mM sucrose, and 2 mM phenylmethylsulfonyl fluoride with a tissue homogenator. 30 μg of the protein was subjected to electrophoresison an 8% sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene fluoride (PVDF) membrane using a semidry transfer system (Hoefer, Holliston, MA). Non-specific bindings were blocked by incubation with 5% non-fat dry milk. The membranes were incubated overnight at 4ºC with a 1:500 dilution of each primary antibody in blocking solution, followed by incubation with 1:1000 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody. The immunocomplexes were detected using an ECL detection kit (Amersham Biosciences Korea, Seoul, Korea) and auto-radiographed onto X-ray film. The gastric mucosal homogenates were pooled together in order to compare. The antibodies used for Western blot are as follow; β-actin, iNOS, cyclooxygenase (COX-2), phosphor-JNK (p-JNK), phosphor-ERK (p-ERK), B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-2-associated X-protein (Bax) and cyclin D1 antibodies were purchased from Santa Cruz Biotechnology(Santa Cruz, CA), phosphorylated inhibitor of kappa B alpha (p-IκBα), phosphorylated signal transducer and activator of transcription 3 (p-STAT3), poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, cleaved caspase-8, cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 2 (CDK2) were from Cell Signaling Technology (Beverly, MA). A heme oxygenase-1 (HO-1) antibody was obtained from Enzo Life Sciences (Farmingdale, NY).

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL)

Apoptosis was visualized with terminal *TdT* FragEL DNA fragmentation detection kit (Oncogene Research Products, Cambridge, MA). To determine the apoptotic index (AI) in each group, we first scanned terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL)-immunostained sections under 100 power magnification to locate the apoptotic hot spots. Then, AI at 400 field was scored by counting the number of TUNEL-positive cells. At least five hot spots in a section containing erosive or ulcerative lesions were randomly selected and average count was determined. Data were expressed as a meanpercentage of total cell numbers.

Periodic acid-Schiff staining

Mucin contents were determined by periodic acid-Schiff (PAS) staining in the stomach tissues.

Statistical analysis

Data are expressed as the mean ± SD. Statistical significance was examined with the Mann-Whitney test or Fisher's exact test. A P value < 0.05 was considered to indicate statistical significance. All statistical analyses were conducted using GraphPad Prism (GraphPad, La Jolla, CA) and SPSS version 18.0 (SPSS Inc., Chicago, IL).

RESULTS

Pretreatment of threonine or the combination of threonine and lignin decreased the development of stress-related mucosal disease in rats

In a preliminary study presented in *Fig. 1*, WIRS for 6 h significantly induced the development of SRMD in SD rats. Based on this SRMD model, to identify the preventive effects of either threonine alone or the combination of threonine and lignin in pellet diets, pretreatment was conducted 8 h before performing the 6-h WIRS (*Fig. 2*). As shown in *Fig. 3*, the development of SRMD, such as erosions, hemorrhages, and ulcers, was noted in all rats in the control group. However, pretreatment of either threonine alone or the combination of 70% threonine and 30% lignin significantly decreased the development of SRMD ($P < 0.05$, *Fig. 4A-4D*). *Fig. 4B*, *4C*, and

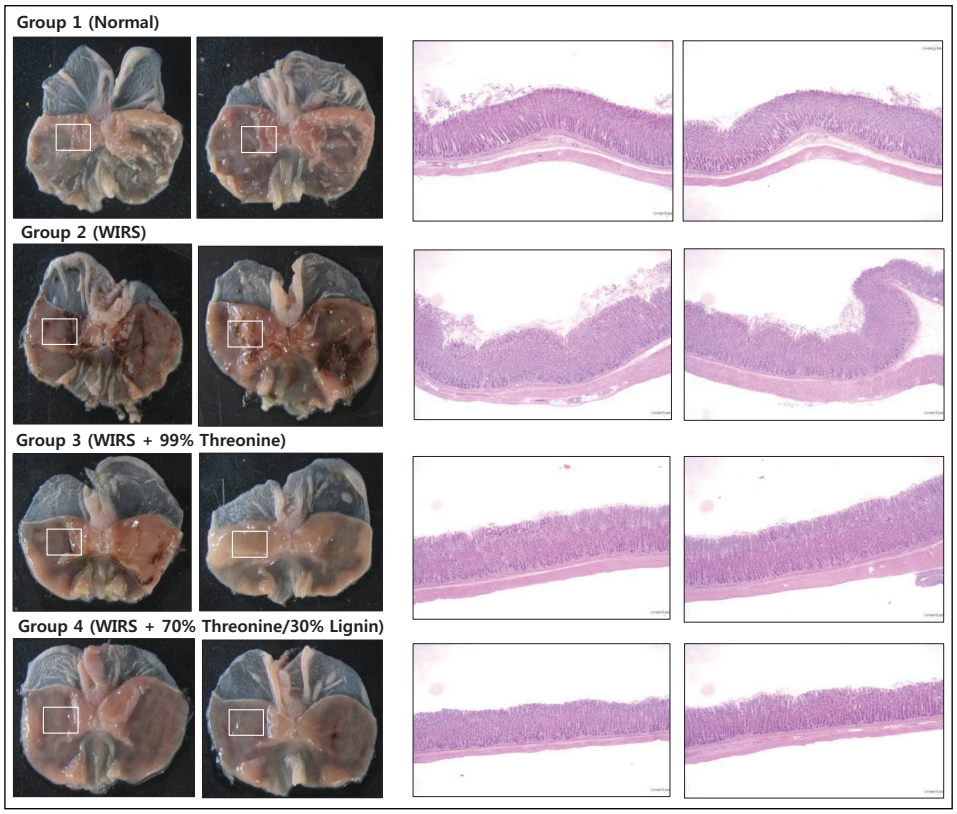

Fig. 3. Preventive action of threonine in WIRS-induced gastric mucosal damage. Gross and pathological images. Two images per group were presented (× 40 magnification on pathology images showing multiple ulcers/erosions in group 2.

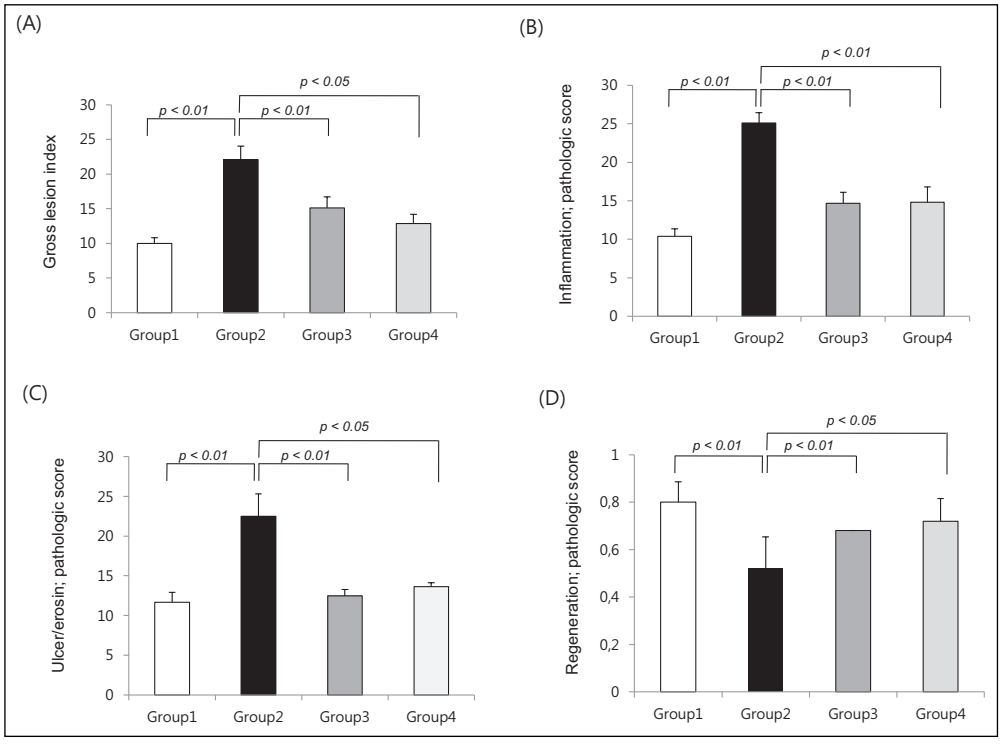

Fig. 4. Preventive action of threonine in WIRS-induced gastric mucosal damage. (A-D): Mean gross and pathological score for the lesion. Pretreatment of either threonine alone or the combination of 70% threonine and 30% lignin significantly ameliorated the SRMD scores, gross pathological score, (A) and each pathological scores (B: inflammation, C: ulcerations, D: regeneration, respectively) separately scored for inflammation, mucosal ulceration, and regenerative activities, respectively ($P < 0.05$).

4D showed the individual pathological scores for the assessment of inflammation, ulcers, and regeneration, and statistically significant changes were observed after the 6-h WIRS. However, all scores were significantly ameliorated by pretreatment with threonine ($P < 0.05$).

Anti-inflammatory actions of dietary threonine in the treatment of stress-related mucosal disease in rats

The expressions of IL-8, iNOS, TNF-α, interferon gamma (IFN-γ), and IL-6 mRNA were measured in all animal groups, of

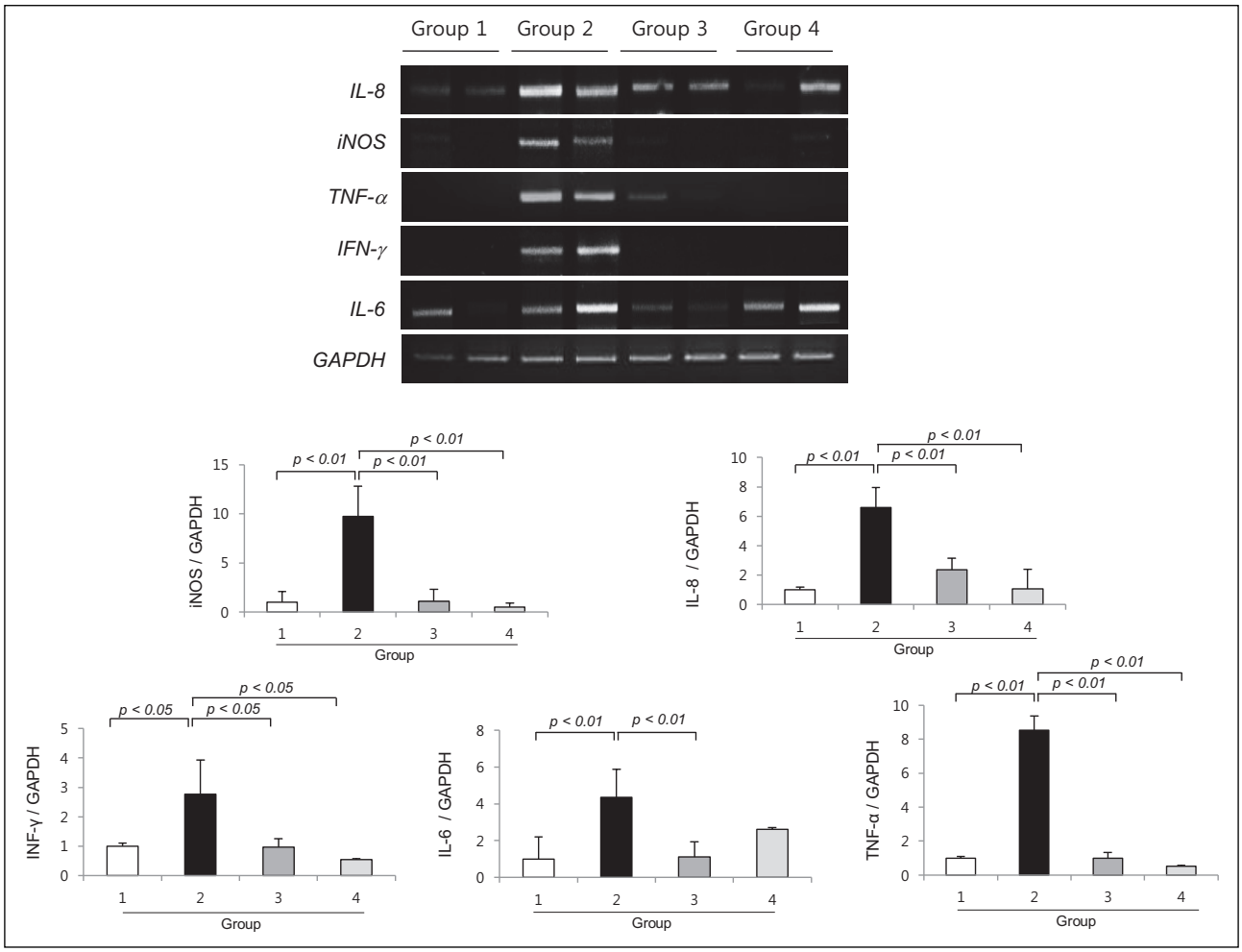

Fig. 5. RT-PCR for inflammatory mediators. The expressions of IL-8, *iNOS*, TNF-α, IFN-γ, and IL-6 mRNA were measured in all animal groups, of which all expressions significantly increased in WIRS-induced SRMD ($P < 0.01$).

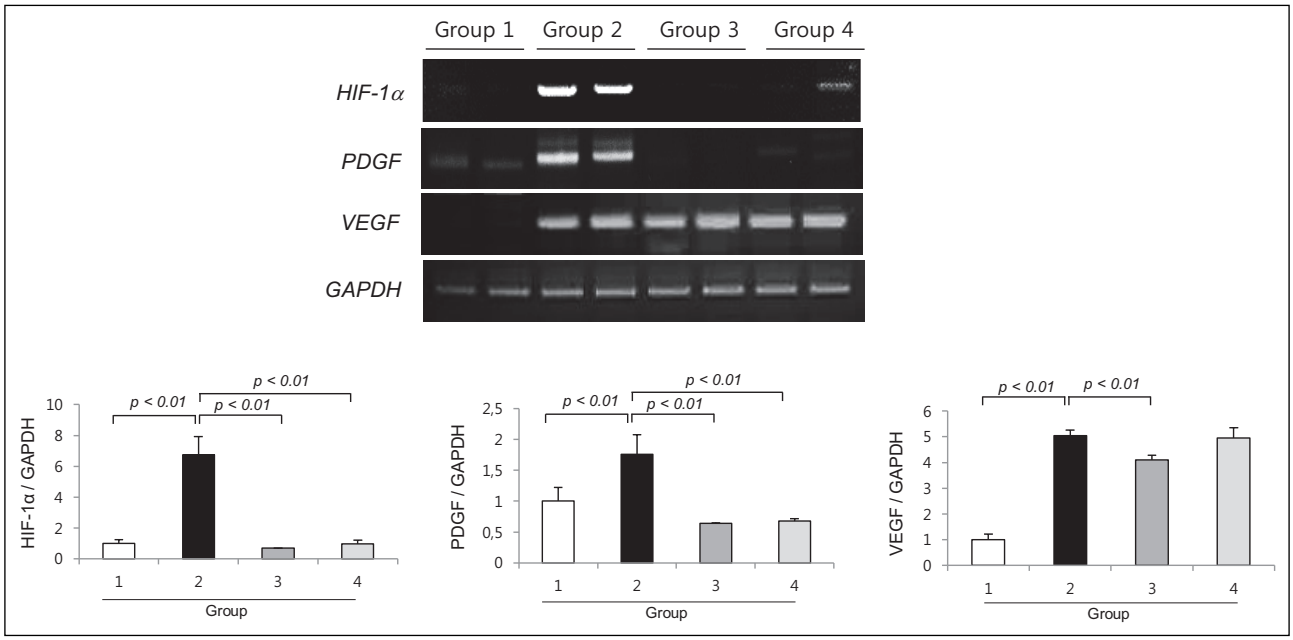

Fig. 6. RT-PCR for angiogenic growth factors. Angiogenic growth factors, including HIF-1α, PDGF, and VEGF mRNA, were measured. The expressions of HIF-1α, PDGF, and VEGF significantly increased in the SRMD group ($P < 0.01$), whereas these expressions significantly decreased in groups 3 or 4 ($P < 0.01$).

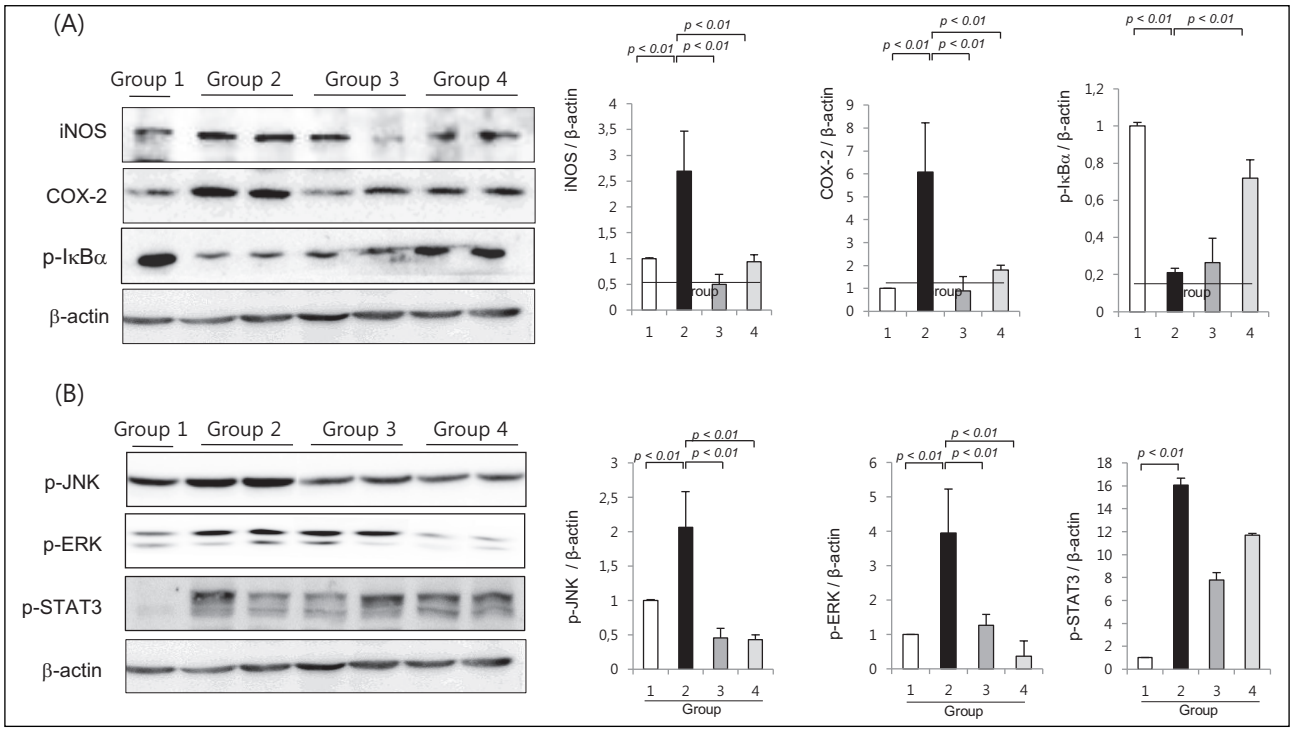

Fig. 7. (A): Western blot analysis of iNOS, COX-2, and p-IκBα. The SRMD group had significantly increased expressions of iNOS and COX-2. However, these levels significantly decreased with threonine pretreatment ($P < 0.05$). (B): Signal transduction correlated with mucosal damages. Signal transduction correlated with ischemia and inflammation was measured via western blot analysis of p-JNK, p-ERK, and p-STAT3.

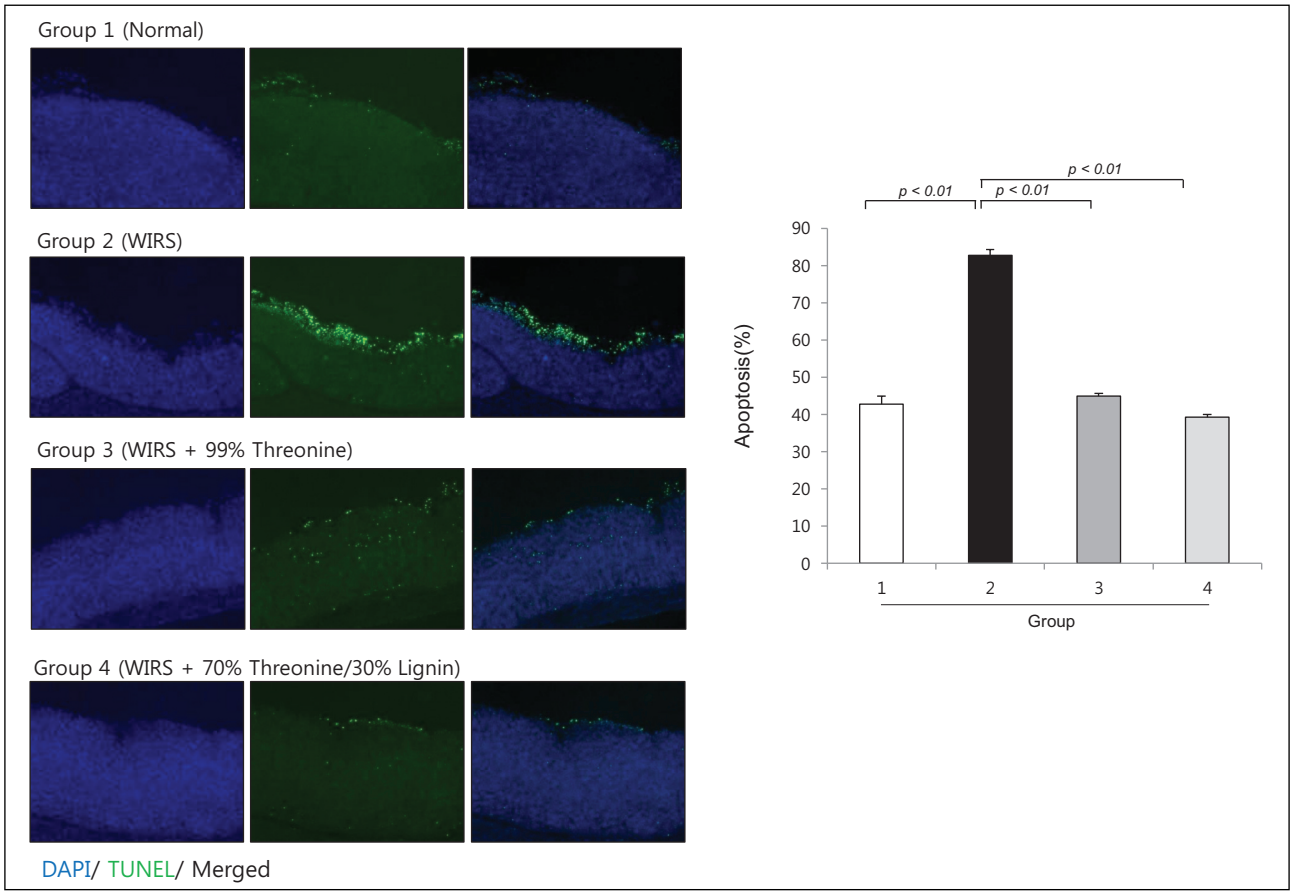

Fig. 8. TUNEL study. In group 2, SRMD was associated with increased apoptotic event in eroded or ulcerative SRMD area. The apoptotic index significantly increased in the SRMD group. However, threonine pretreatment significantly decreased apoptosis even in the erosive gastric mucosa ($P < 0.01$, × 100 magnification).

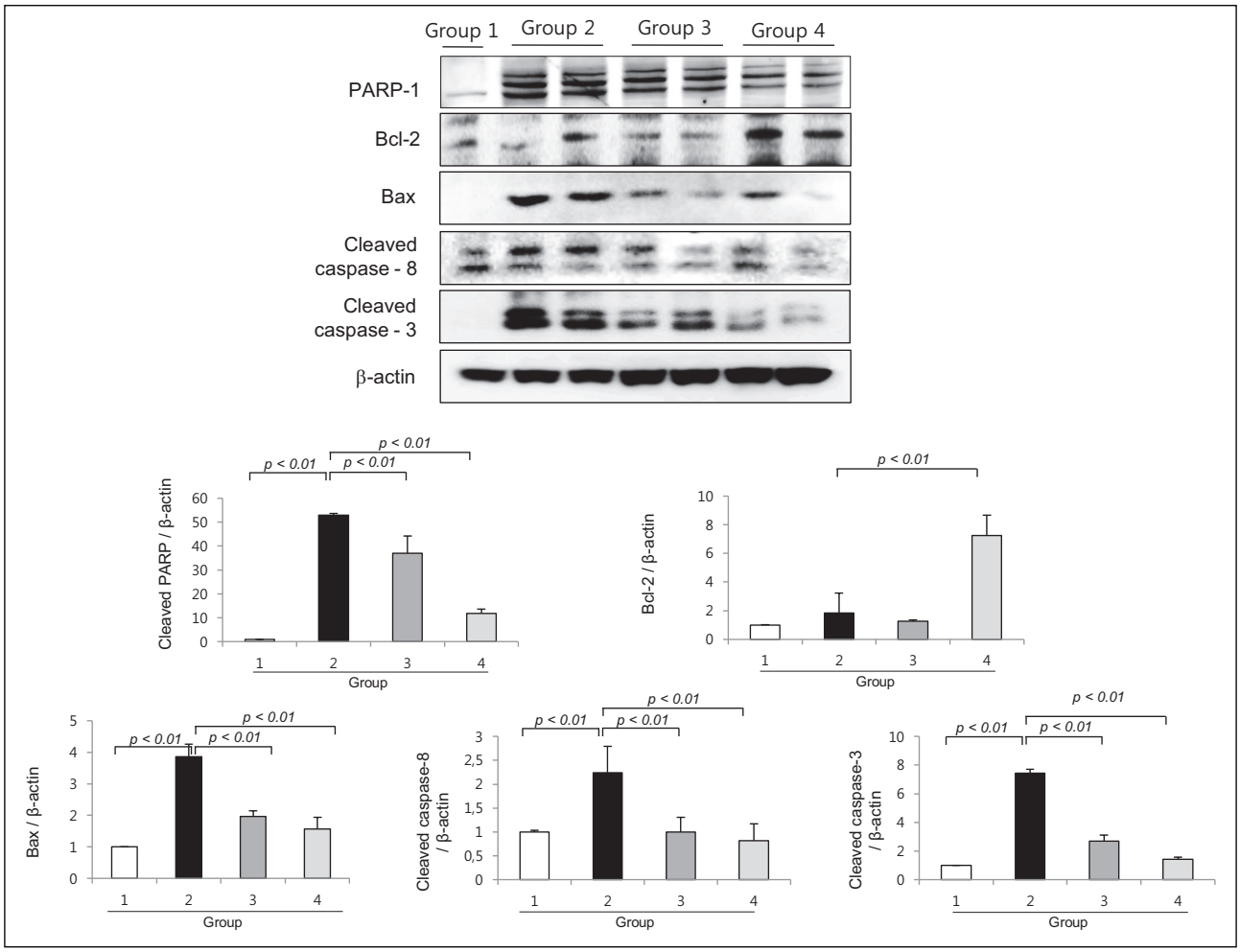

Fig. 9. Western blot analysis of PARP, Bcl-2, Bax, and caspase. To confirm the TUNEL findings, Western blot analysis of PARP cleavage was carried out. The SRMD group had significantly increased PARP cleavage ($P < 0.001$), whereas the combination of threonine and lignin pretreatment significantly decreased PARP cleavage ($P < 0.001$).

which all expressions significantly increased in WIRS-induced SRMD ($P < 0.01$, *Fig. 5*). However, the expressions of all inflammatory mediators implicated in the model of WIRS-induced SRMD significantly decreased in the group treated with either 99% threonine or the combination of 70% threonine and lignin ($P < 0.01$, *Fig. 5*). Since SRMD is pathophysiologically associated with ischemic/reperfusion damages (4-6), we have identified the changes in angiogenic growth factors, including hypoxia inducible factor (HIF)-1α, platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) mRNA (*Fig. 6*). As expected, the expressions of HIF-1α, PDGF, and VEGF significantly increased in the SRMD group ($P < 0.01$). However, these expressions significantly decreased in either group 3 or 4 ($P < 0.01$, *Fig. 6*). Accordingly, we have measured the expressions of inflammatory mediators, such as iNOS, COX-2, and p-IκBα and the results presented in *Fig. 7A* showed that the expressions of iNOS and COX-2 in the SRMD group significantly increased. However, these levels significantly decreased with threonine ($P < 0.05$). All these changes were associated with the transcriptional activation of NF-κB since p-IκBα significantly decreased with WIRS, whereas p-IκBα significantly increased, leading to the redox inhibition of NF-κB with the administration of threonine ($P < 0.05$, *Fig. 7A*). Since the signal transduction correlated with ischemia and inflammation with WIRS was curious, we have checked MAPK according to group. As observed in *Fig. 7B*, ERK and JNK activations were observed in SRMD, whereas threonine pretreatment partially inactivated these signal transductions. STAT3 activation was not related ($P < 0.05$, *Fig. 7B*).

Antiapoptotic actions mitigated the action of threonine against stress-related mucosal disease in rats

SRMD develops with robust apoptosis (15). As observed in *Fig. 8*, the apoptotic index significantly increased in the SRMD group ($P < 0.05$). However, threonine pretreatment significantly decreased apoptosis even in the erosive gastric mucosa ($P < 0.01$). To validate the TUNEL findings, Western blot analysis of the PARP cleavage was carried out. The SRMD group had significantly increased PARP cleavage ($P < 0.001$), whereas the group that received the combination of threonine and lignin pretreatment had significantly decreased PARP cleavage ($P < 0.001$). Conversely, Bcl-2 expression significantly increased in the group pretreated with the combination of threonine and lignin ($P < 0.05$). The expressions of cleaved capspase-3 significantly increased in the group pretreated with threonine alone, and the expressions of cleaved capspase-8 did not differ between the groups, indicating that executive pathways in each group were different. However, the PARP and Bcl-2 pathways reflect the antiapoptotic mechanisms of the combination of threonine and lignin (*Fig. 9*). Therefore, we assessed the changes in cyclin

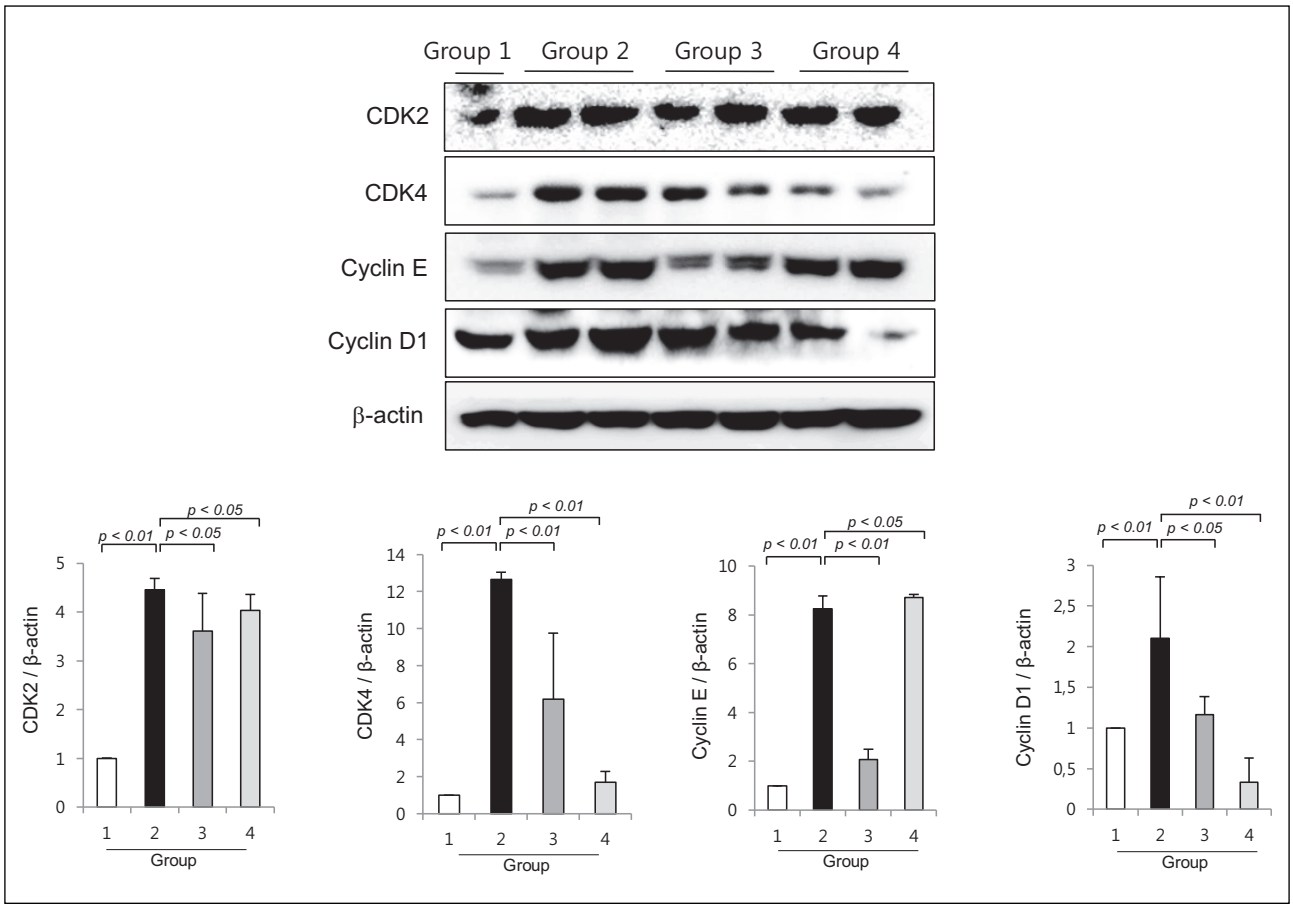

Fig. 10. Western blot analysis of cyclins and CDKs. The expressions of CDK4 and cyclin D1 significantly increased in the SRMD control groups ($P < 0.05$), whereas the expressions of CDK4 and cyclin D1 significantly decreased in the group pretreated with either threonine alone or the combination of threonine and lignin ($P < 0.05$).

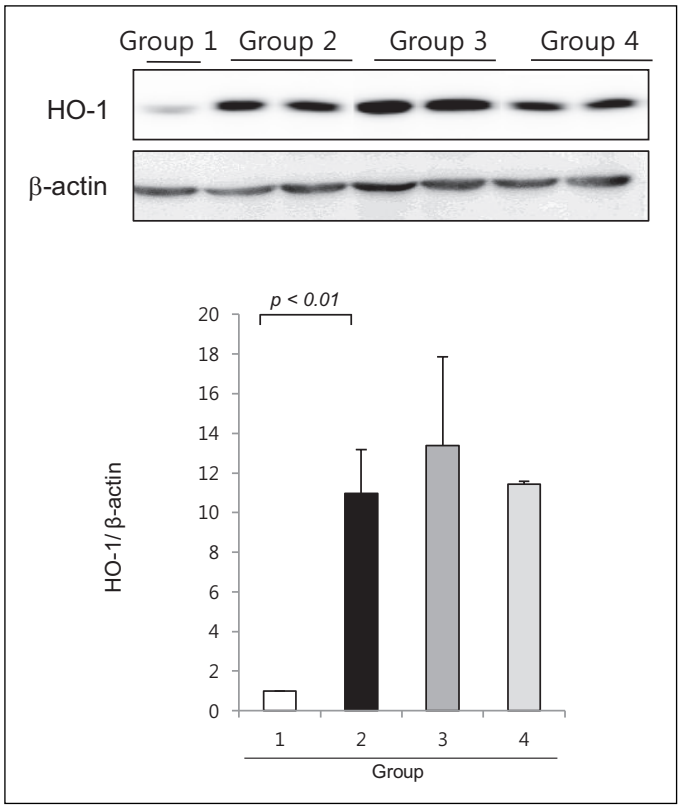

Fig. 11. Western blot analysis of HO-1. WIRS led to a significant increase in the expressions of HO-1 as part of the host defense response ($P < 0.001$). However, the expressions of HO-1 in group 3 were significantly higher than those in group 2, indicating that threonine led to increased host cytoprotection under SRMD in rats ($P < 0.05$).

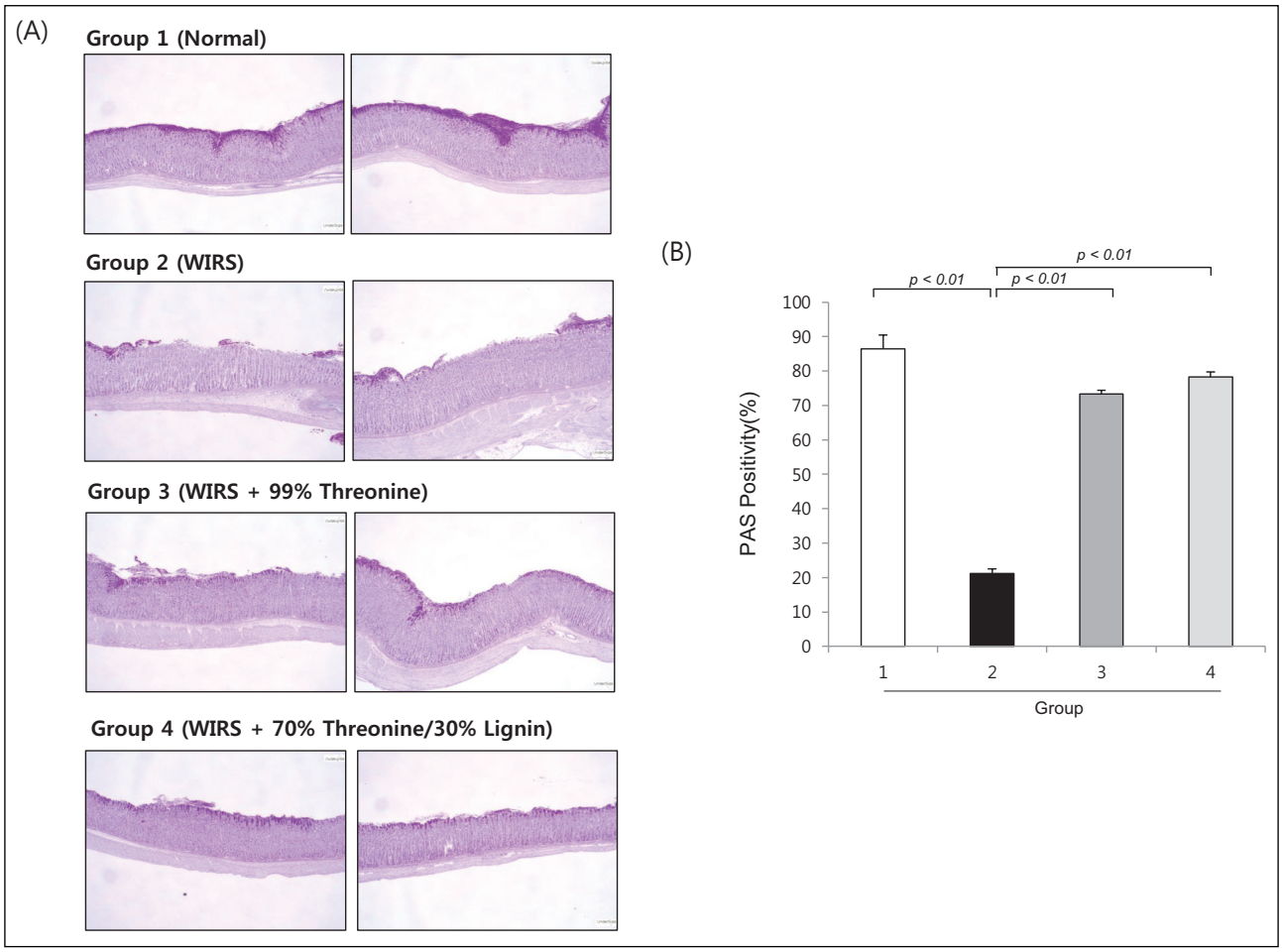

Fig. 12. PAS staining according to group. Significantly decreased levels of gastric mucin were observed in group 2 ($P < 0.01$), whereas the expressions of gastric mucin were significantly preserved in the group pretreated with either threonine or the combination of threonine and lignin ($P < 0.05$; × 40 magnification of PAS staining).

expressions to explain the uncertain results of apoptosis. As observed in *Fig. 10*, the expressions of CDK4 and cyclin D1 significantly increased in the SRMD control groups ($P < 0.05$), whereas the expressions of CDK4 and cyclin D1 significantly decreased in the group pretreated with either threonine alone or the combination of threonine and lignin ($P < 0.05$). Taken together, WIRS significantly threatened the mucosal integrity. However, it was significantly mitigated with the pretreatment of threonine.

Preservation of gastric mucin with the administration of threonine prevented stress-related mucosal disease in rats

As observed in *Fig. 11*, WIRS alone increased the host defense system *via* the induction of HO-1 ($P < 0.001$). However, its expressions significantly increased in the group treated with threonine ($P < 0.05$, *Fig. 11*), indicating that WIRS-induced oxidative stress led to a significant HO-1 expression in control rats (16, 17). However, its levels further increased in the group pretreated with threonine, thereby strengthening cytoprotection *via* phase 2 enzyme response under SRMD. Finally, since threonine is a core amino acid that is a prerequisite for mucin synthesis, we considered the hypothesis that the mechanism rescuing from WIRS in the threonine pretreatment group might preserve the expression of gastric mucin. As can be seen in *Fig. 12*, significantly lower levels of gastric mucin were observed in group 2 ($P < 0.01$), whereas the expressions of gastric mucin were significantly preserved in the group pretreated with either threonine or the combination of threonine and lignin ($P < 0.05$). Lignin has antioxidative action, by which we assessed HO-1, an antioxidative gene implicated in cytoprotection.

DISCUSSION

Based on the current study, the dietary intake of threonine alone or the combination of threonine and lignin could significantly prevent SRMD, in which the concerted actions of cytoprotection were triggered in this prevention, including significant anti-inflammatory, anti-apoptotic, anti-oxidative, and mucin-preserving actions. Threonine alone or the combination of threonine and lignin had similar preventive effects. However, the combination of threonine and lignin had better outcomes. Considering that the etiopathogenesis of SRMD is multifactorial, the use of prophylaxis is preferred. In relation to this, the dietary intake of threonine or the combination of threonine and lignin in the field of veterinary might be considered.

The incidence of SRMD in both veterinary and human medicine has not been fully elucidated due to similar reasons. That is, multiple etiologies for gastroduodenal ulcerative disease exist, which include the frequent use of nonsteroidal anti-inflammatory drugs and corticosteroids and the presence of infiltrative GI diseases, such as cancer and toxin exposure, and exercise-induced gastric lesions (14, 18, 19). Of these conditions, exercise-induced gastric lesions are frequently observed in performing animals.

Although the presentation slightly differs in humans, this exercise-induced gastric lesion observed in animals is a kind of stress ulcer commonly observed in patients admitted in the intensive care units. The terminology of SRMD is often confused with stress ulcer, stress gastritis, stress erosions, and stress lesions, and such terms are used interchangeably (2-4). However, such conditions had the same pathogenesis, starting from small superficial erosions to overt gastric ulcers, which are all correlated with stress response (20). In non-stress conditions, mucosal defense is closely correlated with adequate microcirculation, and an efficient circulation provides nutrients. However, it simultaneously removes waste products, free radicals, waste secretion, and any irritants. However, in stress conditions, inefficient perfusion, rather than increased number of irritants, leads to the formation of gastric lesions (21).

Although the improvement in GI mucosal hemodynamics *via* the aggressive treatment of the underlying disease is of paramount importance in the treatment of SRMD and the removal of mucosal irritants, such as gastric acid, is crucial, a more adequate and ideal agent would be preferred, particularly one that can enhance the gastric defense systems and secure perfusion. Particularly in the field of veterinary, an aggressive treatment for either gastric acidity or prophylaxis is not available. Although exercise-induced gastric damages are frequently observed in veterinary medicine, an agent that is effective in treating such condition is not available. Thus, an agent that can be effectively used for the treatment must be urgently developed. Based on this information, we consider threonine or lignin as potential dietary ingredients for the treatment of SRMD based on the following experimental results.

Faure *et al.* (22) have shown that the utilization of threonine significantly increased in septic rats to synthesize intestinal mucins, and another study has shown that oral threonine was quite important in the production of mucin in neonatal piglets (23, 24). These studies have consistently shown that adequate dietary threonine was quite important to produce mucus. In addition to oral threonine, parenteral administration of threonine was extremely helpful in ameliorating the symptoms of oral threonine deficiency. In addition, Faure *et al.* (25) have provided more evidence that the use of threonine, serine, proline, and cysteine as dietary supplements significantly attenuated dextran sulfate sodium-triggered colitis *via* the promotion of mucin synthesis and gut microbiota equilibration.

Our study has also validated the beneficiary outcome of the combination of threonine and lignin. Then, the notion regarding how threonine increases mucin production was raised. However, studies about lignin are limited. Lignins, which are abundant in plants, accounting for about 15 – 25% (weight/weight) of herbaceous biomass, are obtained as a by-product of lignocellulose treatments performed during pulp and paper processing (26). Lignins are most commonly used in energy production, with only a small fraction used for medicinal applications due to its antioxidative properties (12, 13, 27). However, with recent advances in medicinal chemistry, lignins were recently found to have biological actions, which were as follows: it reduces serum cholesterol by binding to bile acids in the intestine (28) and has anti-gastrointestinal cancer effects (29), antioxidative action (12, 13, 30), and anti-pancreatic action (31). In the current study, the combination of threonine and lignin significantly prevented SRMD, and it also had mucin-preserving effects and antioxidant actions. Similar with the results of lignin, Kwiecien *et al.* (32) have proven the efficacy of pentoxyfilline, an inhibitor of TNF-α activity against WIRS-induced gastric lesions, as well as olive leaf extract (33), and prickly pear cactus (34) were also shown to attenuate these gastric lesions. That is, it significantly scavenges the action of free radicals (3).

Mucin is biosynthesized within the mucus-producing cells and is secreted by such cells, largely two kinds of cells, including surface and gland mucus cells. The respective mucins differ in terms of their peptide sequence and chemical composition of carbohydrate moieties. However, the core peptides are characterized as MUC5AC and MUC6, respectively. In this synthesis, serine or threonine contributed to the polypeptide core in ribosomes. A portion of an *N*-glycoside sugar chain is connected to the end of the peptide in the rough endoplasmic reticulum and is required for the efficient oligomerization of the precursor. Each glycoprotein subunit consists of a central peptide core with carbohydrate side chains. In this structure, amino acid-like threonine and serine are used in the formation of a core peptide (35). Based on biosynthesis in mucus-producing cells, mucin accumulates as mucus granules in the cells and is subsequently secreted through exocytosis (36, 37). In our study, the number of PAS-positive mucus cells significantly decreased in group 2, whereas the number of PAS-positive mucus cells was significantly preserved in groups 3 and 4. Further immunohistochemical stainings with MUC6 and TFF antibody showed that the expression of gastric mucins significantly increased in the group pretreated with threonine or the combination of threonine and lignin. However, the data were not presented. Similar to gastric mucin synthesis and preservation, threonine significantly preserved the cytoprotective mechanisms of HO-1 (16, 17).

Considering the limitation of treatment targeting SRMD, the current study showed that threonine-containing formula may prevent SRMD, particularly in veterinary medicine because it is abundant in animal food. Since the exact mechanism by which SRMD develops in animals is unknown, the role of physiologic stress is considered, and the results obtained from our study might contribute to this knowledge after an extensive clinical trial. In practice, considering that the incidence rates of SRMD in Alaskan sled dogs and racehorses are 48.5% and 93%, respectively (7), and the unexpected death of swine, our formula, which is essential in animal foods, must be developed.

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