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Therapeutic potential and mechanism of thymol action against ethanol-induced gastric mucosal injury in rat model

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In this study, we investigated the potential of thymol and its mode of action to protect against the gastric mucosal injury induced by ethanol consumption in an *in vivo* model. Moreover, we determined the role of thymol in regulation of matrix metalloproteinase-9 (MMP-9), an enzyme belonging to the metalloproteinase group, which is responsible for the remodeling of injured tissues. Sprague–Dawley rats pretreated with thymol (10 mg/kg body weight) or normal saline were subjected to intragastric administration of 95% ethanol (5 mL/kg body weight). Morphological examination included ulcer index as a measurement of hemorrhages, and hematoxylin and eosin (H&E) staining was performed to analyze severity of gastric mucosal damage. Gelatinase zymography of tissue extract and *in situ* zymography were performed to demonstrate MMP-9 activity. Results of macroscopic examination suggested that thymol significantly protected gastric mucosa from damage induced by alcohol, which was severe in the case of alcohol-only treatment. H&E data demonstrated necrosis of the corpus region in alcohol-treated rats, which was abrogated in rats pretreated with thymol. Further, thymol protected against the constriction of small arteries and neutrophil infiltration in lymphatic vessels. Expression of antioxidant enzymes increased in the thymol-pretreated group, and downregulation of MMP-9 protein expression was observed by gelatin zymography as well as *in situ* zymography. The results of this study suggest that thymol protects against gastric mucosa injury induced by ethanol consumption by upregulating the secretion of antioxidant enzymes and downregulating the expression of the MMP-9 protein.

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Introduction

Gastric ulcer is a clinical condition characterized by ulceration of the surface of the gastric mucosa. Symptoms of gastric ulcer include abdominal discomfort, severe pain, nausea, and a burning sensation in the stomach. Occurrence of ulcer is the most common disorder of the gastrointestinal tract and has multiple etiologies. There are various aggressive factors such as hydrochloric acid, pepsin, and bile salts, which cause gastric mucosal injury, and there are also gastro-protective factors such as mucus bicarbonate layers to defend the mucosa. These factors act in a balanced manner under normal physiological conditions, but any imbalance in these factors can cause gastric mucosal damage resulting in gastric ulcer (Allen & Flemström, 2005; Wallace, 1992).

Beside aggressive factors, consumption of alcohol is found to be a major contributor to gastric ulceration (Franke, Teyssen, & Singer, 2005). Alcohol has been used for centuries in social, medical, cultural, and religious settings worldwide. Currently, it is also considered to be one of the most commonly abused drugs, related to a wide range of physical, mental, and social damage, and is responsible for 3.8% of deaths and 4.6% of disability-adjusted life years lost worldwide (Rehm et al., 2009). Among the various organ systems that mediate alcohol's effects on the human body, the gastrointestinal tract plays a particularly important role. Alcohol absorption into the bloodstream occurs throughout the gastrointestinal tract, and its direct contact with the mucosa can induce numerous metabolic and functional changes. These alterations may lead to marked mucosal damage, which can result in a broad spectrum of acute and chronic diseases, such as gastrointestinal bleeding and ulcer (Bode & Bode, 1997). There are various reports suggesting that induction of oxidative stress and depletion of antioxidant enzymes are crucial steps in ethanol-induced gastric mucosal damage (La Casa, Villegas, Alarcón de la Lastra, Motilva, & Martín Calero, 2000; Pan et al., 2008).

Thymol is a natural phenolic compound grouped in monoterpenes and found abundantly in many medicinal plants (Baser, 2008; Sánchez, Turina, García, Nolan, & Perillo, 2004). It has been

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widely accepted as an antimicrobial agent that can be effectively used against both Gram-positive as well as Gram-negative bacteria (Dorman & Deans, 2000). We previously reported that thymol has the potential to kill *Salmonella* Typhimurium by damaging its membrane integrity (Chauhan & Kang, 2014). Further, thymol is a potent immunostimulator that can enhance the membrane fluidity of macrophages to increase phagocytosis, and it downregulates secretion of pro-inflammatory cytokines to induce suppression of inflammatory responses (Chauhan, Jakhar, Paul, & Kang, 2014).

In the present study, we evaluated the potential of thymol to prevent gastric mucosal injury and determined its role in regulation of MMP-9 activity during gastric injury. We also demonstrated that thymol can upregulate the production of antioxidant enzymes, thus protecting the gastric mucosa from oxidative stress.

Materials and methods

Chemicals and biological reagents

All chemicals, unless otherwise stated, were of the highest quality and used as supplied. Thymol, omeprazole, hematoxylin, eosin, dimethyl sulfoxide (DMSO), and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Animals

Male Wistar rats weighing 180–220 g were purchased from Orient BioInc. (Busan, South Korea). The animals were housed under standard conditions (temperature 24 $\pm$ 1 °C, relative humidity 55 $\pm$ 1%, and 12-h/12-h light/dark cycle) and were fed standard pellet diet and water *ad libitum*. Animals described as fasted were deprived of food for 24 h but had free access to water.

Induction of gastric mucosal damage (gastric erosions)

Gastric erosions were induced according to a previously described model (Nwafor, Okwuasaba, & Binda, 2000). The rats were deprived of food but had *ad libitum* access to tap water for 24 h before induction of erosions. Animals were randomized into four groups ($n = 8$): control, ethanol (5 mL/kg body weight), omeprazole (20 mg/kg body weight), and thymol (10 mg/kg body weight). An oral gavage of 5.0 mL/kg body weight of ethanol (95%) was given to conscious rats. Both drugs (thymol and omeprazole) were administered together, and control rats were given 1 mL of normal saline containing 5% DMSO as a vehicle control (hereafter referred to as normal saline) 60 min prior to ethanol administration. Animals were sacrificed 1 h later and their stomachs were excised.

Assessment of gastric mucosal erosions

To assess gastric mucosal erosions, an incision was made through the wall of each stomach along the entire length of the greater curvature, after which the anterior wall of the stomach was retracted and rinsed thoroughly with normal saline. Each stomach was examined under a microscope to observe erosions, and ulcer index was calculated as described by Nwafor et al. (2000).

Histopathological examination

For pathological examination, all samples were fixed in 10% formalin buffer, dehydrated with graded alcohol. The tissues were then embedded in paraffin blocks, and pathological sections were sliced along the longitudinal axis. From each sample, 5-μm thick sections were obtained, and staining with hematoxylin and eosin (H&E) was performed to evaluate gastric morphology.

Determination of superoxide dismutase, glutathione, and lipid peroxidation

Stomach tissue homogenates were prepared in phosphate-buffered saline (0.1 M, pH 7.4) and then centrifuged at 10,000 $\times$ *g* for 15 min. Supernatant of the homogenates was collected carefully and used for determination of superoxide dismutase (SOD), glutathione (GSH), and lipid peroxidation (LPO). The tests were carried out using respective test kits (Sigma, USA) according to the manufacturer's instructions.

Myeloperoxidase activity assay

Myeloperoxidase (MPO) activity was determined through a method previously described by Pulli et al. (2013). Supernatant of the stomach tissue homogenates was used to analyze MPO activity. The assay was based on the 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma) colorimetric determination method. Briefly, 10 μL of sample was added to 80 μL of 0.75 mM H_2O_2 in a 96-well microtiter plate, followed by the addition of 110 μL of TMB solution. The sample was then incubated for 5 min at 37 °C. After the incubation period, 50 μL of 2 M H_2SO_4 was added to stop the reaction, and the optical density was recorded at 450 nm using a microplate reader (Bio-tek, WA, USA). The results were expressed as OD values.

Gelatin zymography

Gelatin zymography was done as previously described (Ganguly & Swarnakar, 2012) with slight modifications. Briefly, tissue homogenate was prepared by using phosphate-buffered saline (PBS). Electrophoresis of tissue homogenate was done on 8% SDS-polyacrylamide gel containing 1 mg/mL of gelatin. The gels were then washed with 2.5% Triton-X-100 and kept in developing buffer (40 mM Tris–HCl, pH 7.4, 0.2 M NaCl, 10 mM $CaCl_2$) for 18 h at 37 °C. After the incubation period, the gel was stained with 0.1% coomassie brilliant blue stain for 20 min. The gel was then de-stained with de-staining solution, and an image of gelatinolytic bands was taken.

In situ zymography

To determine the localization of gelatinolytic activity, *in situ* zymography was performed as previously described (Hadler-Olsen et al., 2010; Miller et al., 2005) with slight modifications. DQ gelatin was used to carry out *in situ* zymography, which is a highly quenched, fluorescein-labeled gelatin that upon proteolytic digestion can be visualized by its bright, green fluorescence and can be used to measure gelatinase enzymatic activity. Paraffin-embedded tissue slides were prepared by sectioning the tissue (7 μm thick) on a cryostat microtome and kept overnight at 60 °C. After heating overnight, sections were de-paraffinized in xylene and rehydrated with alcohol. The sections were then incubated with 40 μg/mL of DQ gelatin (Invitrogen, USA) for 2 h at 37 °C in a CO_2 incubator. After the incubation period, sections were washed with PBS three times and counterstained with 4′, 6–diamidino-2–phenylindole DAPI nuclear stain. Slides were then mounted and observed under an Epi-fluorescence microscope (Nikon Eclipse, TS 100, Japan) with respective filters.

Statistical analysis

Data are presented as means $\pm$ SD. Multiple comparisons were performed using one-way ANOVA followed by Duncan test for *post*

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