

Protective effect of *Pyropia yezoensis* glycoprotein on chronic ethanol consumption-induced hepatotoxicity in rats

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Abstract. The present study investigated the protective effect of *Pyropia yezoensis* glycoprotein (PYGP) against chronic ethanol consumption-mediated hepatotoxicity in rats. Male Sprague-Dawley rats (n=20; 6 weeks old) were randomly divided into four groups. The rats in each group were treated for 30 days with the following: i) CON group, distilled water only; ii) EtOH group, 20% ethanol 3.7 g/kg/BW; iii) EtOH+150 group, 20% ethanol 3.7 g/kg/BW+PYGP 150 mg/kg/BW; iv) EtOH+300 group, 20% ethanol 3.7 g/kg/BW+PYGP 300 mg/kg/BW. EtOH, PYGP and water were orally administered. The rats were sacrificed after 30 days, and blood and liver samples were collected for analysis. Treatment with ethanol caused significant elevation of serum levels of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). Furthermore, inhibition of the antioxidant defense system in the liver, including glutathione (GSH), glutathione peroxidase (GSH-px) and catalase (CAT) was observed. However, co-administration with PYGP recovered the antioxidant defense system, and the serum levels of GOT and GPT. PYGP was shown to attenuate ethanol toxicity via the inactivation of mitogen-activated protein kinases (MAPKs). PYGP suppressed the overexpression of cytochrome P450 2E1 (CYP2E1), inducible nitric oxide synthase and cyclooxygenase-2. These results suggested that the protective effect of PYGP was associated with antioxidant activities, MAPKs and the CYP2E1 signaling pathway.

Introduction

Chronic ethanol consumption-induced alcoholic liver disease is one of the most common causes of liver cancer and

cancer-associated mortality (1). Ethanol generates several harmful products, including reactive oxygen species (ROS), acetaldehyde (ADH) and cytochrome P450 2E1 (CYP2E1) during metabolism (2).

The CYP2E1 generated during ethanol metabolism generates reactive oxygen species and ADH (3). Ethanol consumption-induced liver pathology is correlated with the expression of CYP2E1 (4). The overexpression of CYP2E1 promotes lipid oxidation and oxidative stress in the liver (5).

ROS have been reported to affect protein oxidation, lipid oxidation, damage to DNA, enzyme inactivation and the depletion of various antioxidant enzymes (6-8). These results can lead early stages of the liver disease and dysfunction (9).

Glutathione (GSH), glutathione peroxidase (GSH-px) and catalase (CAT) are antioxidants, which affect the anti-oxidative system (10). The enzymatic antioxidant system includes superoxide dismutase (SOD), CAT and GSH-px (11). Nonenzymatic antioxidants consist of GSH, and vitamins A, C and E (12). The antioxidant system can assist in eliminating ROS and oxidative stress (13).

To date, several studies have shown that the mitogen-activated protein kinase (MAPK) family is crucial in cellular systems, including cell proliferation, cell differentiation, development, apoptosis and inflammatory responses (14-17). MAPKs consist of c-jun N-terminal kinase (JNK), p38 MAP kinase and extracellular signal-regulated kinase (ERK). Ethanol affects MAPKs in various cells and organ systems, which consequently show different pathologic consequences (14).

Chronic ethanol consumption upregulates the levels of inducible nitric oxide (NO) synthase (iNOS) and the protein expression levels of cyclooxygenase (COX)-2 in liver tissues (18). This protein leads to induction of the inflammatory response and oxidative stress (19). The increased expression of iNOS promotes the production of NO and the COX-2 protein, which subsequently leads to the release of pro-inflammatory cytokines (20).

Pyropia yezoensis, a species of marine algae, has long been consumed in Korea, Japan and China. It has a range of biological activities, including cell proliferation (21), antioxidant (22) and antiinflammatory effects (23). In the present study, the *in vivo* protective effect of *P. yezoensis* on chronic ethanol consumption-induced liver injury was investigated in mice.

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Materials and methods

Preparation of *P. yezoensis* glycoprotein (PYGP). Dried *P. yezoensis* was purchased in the Republic of Korea in 2014 (Suhyp, South Korea) and was homogenized using a blender. The *P. yezoensis* powder (40 g) was diluted 1:1 with distilled water and stirred for 4 h at room temperature. The solution was centrifuged (3,000 \times g at 4°C for 20 min) and vacuum filtered, following which triple volumes of ethanol (extract:ethanol, 1:3) were added. After 24 h at 4°C, the solution was filtered and concentrated using rotary evaporation at 40°C. The concentrated solution was divided into 1.5 ml tubes, freeze-dried, and stored at -70°C until use.

Experimental animals. Male Sprague-Dawley rats (n=20; 6 weeks old) were purchased from Samtaco (Osan, South Korea). The rats were allowed to adapt to laboratory conditions for 1 week (temperature: 23 \pm 3°C, 12 h light/12 h dark cycle, 50% humidity) with free access to water and food. Animal studies were conducted in accordance with the Animal Ethics Committee of Pukyong National University (Busan, South Korea).

Experimental design. The animals were randomly divided into four groups of five rats, as follows: Control rats, which received distilled water only (CON group); rats administered with 20% ethanol (3.7 g/kg/BW; EtOH group); rats administered with 20% ethanol (3.7 g/kg/BW)+PYGP (150 mg/kg/BW; EtOH+150 group); and rats administered with 20% ethanol (3.7 g/kg/BW)+PYGP (300 mg/kg/BW; EtOH+300 group). The PYGP and ethanol were administered orally once per day for 30 days. The animals in all groups were sacrificed for blood and liver collection at the end of experimental period (day 31). Blood was collected immediately, and the livers were frozen in liquid nitrogen and stored at -70°C until use.

Biochemical indicators of liver function. Blood samples were centrifuged at 3,000 \times g for 20 min at 4°C to collect serum, which was stored at -20°C until further analysis. Chronic hepatic damage was measured by detecting the serum levels of GOT and GPT using an enzymatic analysis kit (Asan Pharmaceuticals Co., Ltd., Hwasung, South Korea) according to the manufacturer's protocol. The absorbance was measured using a UV spectrometer (Ultrospec 2100 pro; GE Healthcare Life Sciences, Cambridge, UK).

Antioxidant enzyme measurement. The activities of antioxidant enzymes, including CAT, GSH and GSH-px, in the homogenized liver samples were measured using a CAT assay kit, GSH assay kit and GSH-px assay kit, respectively, according to the manufacturer's protocols (Cayman Chemical Company, Ann Arbor, MI, USA). The absorbance was measured using a microplate reader (Benchmark Plus 10730; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. The liver tissue protein was homogenized in lysis buffer containing 150 mM sodium chloride, 50 mM Tris-HCl (pH 7.5), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% triton X-100 and 2 mM

ethylenediaminetetra-acetic acid (Intron Biotechnology, Inc., Seoul, South Korea) with inhibitors (1 mM Na₃VO₄, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A and 1 mM PMSF; (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). The protein levels were determined using a Bichinchomonic Acid Assay kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal amounts of protein (20 μ g) were separated via 10-15% SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The transferred membrane was blocked with 1% bovine serum albumin (BSA) in TBS-T containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20 (USB, Cleveland, OH, USA). Following blocking, the membranes were incubated for 4 h at room temperature with the following primary antibodies: Rabbit anti-rat ERK IgG polyclonal antibody (diluted 1:1,000 with BSA/TBS-T; cat. no. sc-94), rabbit anti-rat phosphorylated (p)-ERK IgG polyclonal antibody (diluted 1:1,000 with BSA/TBS-T; cat. no. sc-7383), mouse anti-rat JNK IgG monoclonal antibody (diluted 1:1,000 with BSA/TBS-T; cat. no. sc-7345), mouse anti-rat p-JNK IgG monoclonal antibody (diluted 1:1,000 with BSA/TBS-T; cat. no. sc-6254), rabbit anti-rat p38 IgG polyclonal antibody (diluted 1:1,000 with BSA/TBS-T; cat. no. sc-7149), mouse anti-rat p-p38 IgG monoclonal antibody (diluted 1:1,000 with BSA/TBS-T; cat. no. sc-7973), mouse anti-rat iNOS IgG polyclonal antibody (diluted 1:1,000 with BSA/TBS-T; cat. no. sc-650), goat anti-rat COX-2 IgG polyclonal antibody (diluted 1:1,000 with BSA/TBS-T; cat. no. sc-1745), rabbit anti-rat CYP2E1 IgG polyclonal antibody (diluted 1:1,000 with BSA/TBS-T; cat. no. sc-133491) and rabbit anti-rat GAPDH IgG polyclonal antibody (diluted 1:1,000 with BSA/TBS-T; cat. no. sc-25778), all from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The membranes were then incubated with peroxidase-conjugated goat (cat. no. A50-101P), mouse (cat. no. A90-116P) and rabbit (cat. no. A120-101P) secondary antibodies (1:10,000; GE Healthcare Life Sciences, Little Chalfont, UK) for 1 h at room temperature. Antibody binding was visualized using Super Signal West Pico Stable Peroxide solution and Super Signal West Pico Luminol/Enhancer solution (Thermo Fisher Scientific, Inc.). The signal was monitored using X-ray film (Kodak, Rochester, NY, USA), and a developer and fixer twin pack (Kodak).

Statistical analysis. Values are presented as the mean + standard deviation and data were analyzed using SPSS version 10.0 software (SPSS, Inc., Chicago, IL, USA) using one-way analysis of variance followed by a Duncan's multiple range test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Hepatoprotective effect of PYGP against chronic ethanol consumption. In hepatotoxicity, serum levels of GOT and GPT are increased by liver injury or liver cell destruction (24). The results of the present study revealed that the levels of GOT and GPT were significantly increased in the ethanol group, compared with those of the control group. However, the groups co-administered with PYGP showed decreased levels of GOT and GPT (Fig. 1A and B).

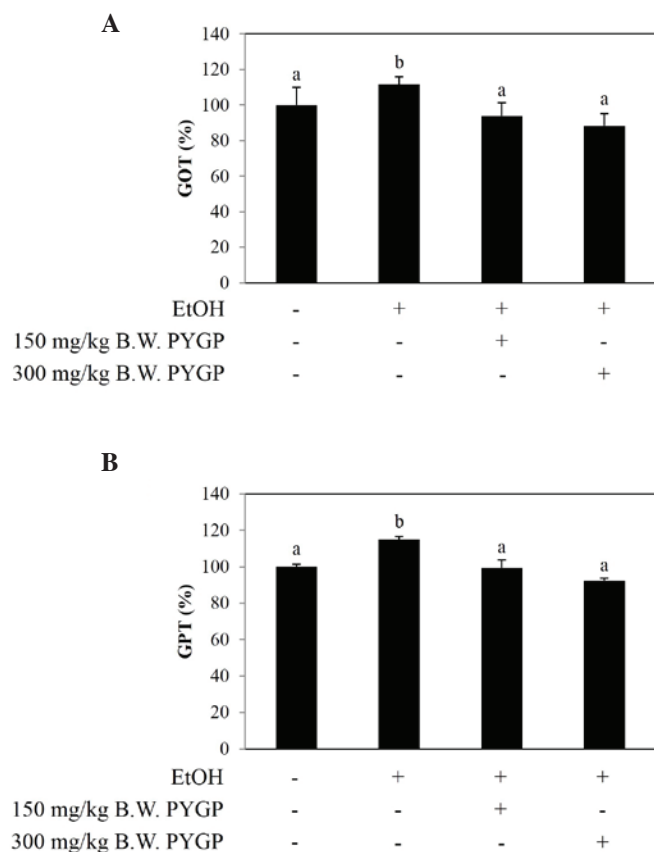


Figure 1. Levels of (A) GOT and (B) GPT in the serum of control and experimental rat groups. Values are presented as the mean + standard deviation. Groups with different letters (a and b) are significantly different ($P < 0.05$). EtOH, ethanol; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; CON, control; 150, 150 mg/kg *Pyropia yezoensis* glycoprotein, 300, 300 mg/kg *Pyropia yezoensis* glycoprotein.

Antioxidant enzyme activity in the rat liver. The activities of the CAT, GSH and GSH-px antioxidant enzymes were markedly decreased in the ethanol-only group, compared with the control group. By contrast, the activities of GSH and GSH-px were restored in the group co-administered with ethanol and PYGP (300 mg/kg; Fig. 2A and B). In addition, the activity of CAT was significantly increased by co-administration with PYGP in a concentration-dependent manner (Fig. 2C).

Ethanol-induced phosphorylation of MAPK is inhibited by PYGP. To examine whether PYGP can inhibit the phosphorylation of MAPK, the present study used western blot assays. The results revealed that ethanol induced the phosphorylation of ERK, JNK and p38, compared with the control group. PYGP was effective at inhibiting the ethanol-induced protein phosphorylation of ERK, JNK and p38. By contrast, ethanol and PYGP had no effect on the total protein expression of ERK, JNK or p38. These results suggested that PYGP inhibited the ethanol-induced phosphorylation of MAPK (Fig. 3).

Effects of PYGP on the expression of COX-2, iNOS and CYP2E1. Chronic ethanol consumption is known to increase the protein expression levels of iNOS, COX-2 and CYP2E1. These proteins are associated with liver inflammation and cell injury (19,20). In the present study, chronic ethanol consumption

upregulated the protein expression levels of iNOS, COX-2 and CYP2E1. By contrast, when the rats were co-administered with ethanol and PYGP, the protein expression levels of iNOS, COX-2 and CYP2E1 were markedly downregulated. These results confirmed that PYGP was important in the suppression of chronic ethanol-induced protein expression of iNOS, COX-2 and CYP2E1 (Fig. 4).

Discussion

Ethanol consumption-induced pathogenesis is complicated. It is associated with oxidative stress, ROS generation and alterations in the innate immune response via ethanol metabolism (25-27). Chronic ethanol consumption in humans leads to serious liver problems, including fibrosis, cirrhosis and hepatocellular carcinoma (28). The increased levels of GOT and GPT as a result of liver injury are commonly used as a measure of hepatotoxicity. In the present study, chronic ethanol consumption increased the serum levels of GOT and GPT, whereas co-administration with PYGP attenuated this increase, resulting in levels similar to those measured in the control group.

Chronic ethanol consumption induces the loss of antioxidant or the diminution of enzyme activities, including those of GSH, GSH-px and CAT (29). GSH is a tripeptide and effectively scavenges ROS and free radicals (30). GSH-px acts as a catalyst in the reduction of H_2O_2 and diverse hydroperoxides, with GSH acting as an electron donor (31). CAT is important in the decomposition of H_2O_2 and the formation of H_2O and O_2 (32). In the present study, the activities of the antioxidant enzymes, GSH, GSH-px and CAT, were significantly decreased by chronic ethanol consumption, whereas co-administration with PYGP increased the activities of these enzymes, compared with the ethanol-only treatment group.

MAPKs are serine-threonine kinases, which are essential in intracellular signaling, including cell proliferation, differentiation, transformation, survival and death (33). Ethanol consumption activates the MAPK cascade via protein phosphorylation (34). In particular, these proteins regulate oxidative stress in ethanol-induced hepatotoxicity (35). In the present study, the results showed that ethanol consumption caused the phosphorylation of ERK, JNK and p38 in the rat liver, whereas co-administration with PYGP attenuated the levels of phosphorylation.

Chronic ethanol consumption increases the protein expression levels of COX-2 and iNOS in the liver (36). These proteins are associated with the ethanol-induced liver inflammatory response (37). NO is a reactive oxidizing agent, and the synthesis of NO is associated with the expression of iNOS (38). Although predominantly involved in the protective effect against bacteria, parasites and tumor cells, the overexpression of NO causes damage to organs (39). COX-2 is associated with several biological responses, including inflammation, carcinogenesis and hepatic fibrogenesis (40). In alcoholic liver disease, the expression of COX-2 is increased in Kupffer cells (41). The increased expression of COX-2 promotes lipid peroxidation, endotoxins, synthesis of tumor necrosis factor- α and thromboxane B_2 (TXB $_2$). TXB $_2$, in particular, is associated with serious alcoholic liver disease (42). In the present study, the protein expression levels of iNOS and COX-2 were increased by chronic ethanol

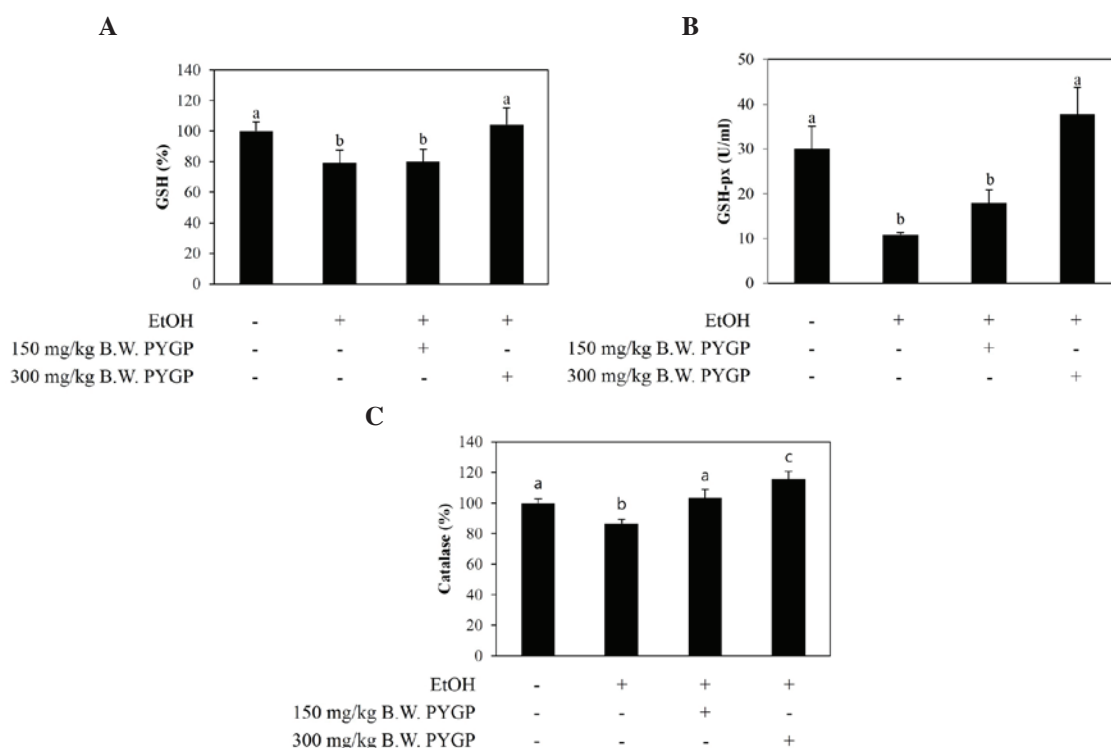


Figure 2. Levels of the antioxidant enzymes, GSH, GSH-px and CAT, in the livers of rats in control and experimental groups. (A) GSH, (B) GSH-px and (C) CAT. Values are presented as the mean + standard deviation. Groups with different letters (a, b and c) are significantly different from each other ($P < 0.05$). EtOH, ethanol; GSH, glutathione; GSH-px, glutathione peroxidase; CAT, catalase; CON, control; 150, 150 mg/kg *Pyropia yezeensis* glycoprotein, 300, 300 mg/kg *Pyropia yezeensis* glycoprotein.

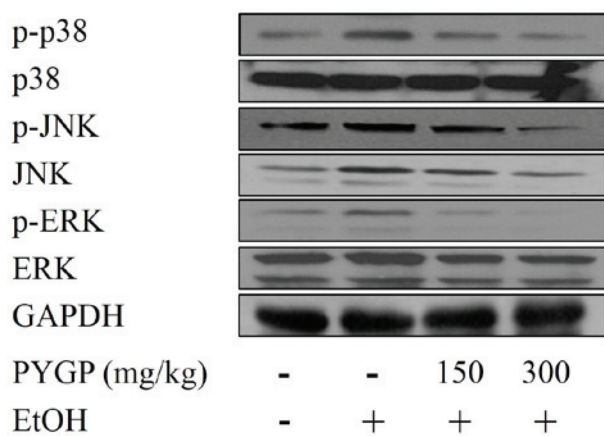


Figure 3. Expression of proteins of the mitogen-activated protein kinase family in the livers of rats in the control and experimental groups. Livers were collected and lysed, and western blot analysis was performed to determine the total protein expression and phosphorylation levels of ERK, JNK and p38. PYGP, *Pyropia yezeensis* glycoprotein; EtOH, ethanol; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; p-, phosphorylated.

consumption, whereas co-administration with PYGP attenuated the levels of expression, compared with the ethanol only group.

Chronic ethanol consumption promotes the production of CYP2E1 and generates increased levels of ROS, including H_2O_2 and O_2 (43,44). CYP2E1 catalyzes the oxidation of

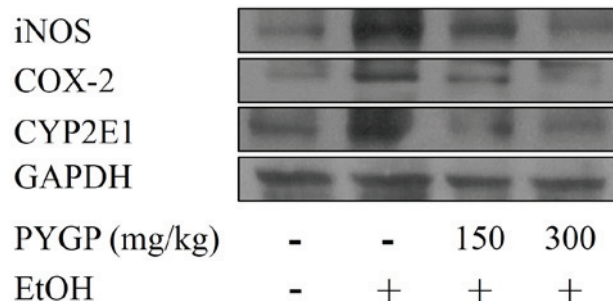


Figure 4. Protein expression of iNOS, COX-2 and CYP2E1 in the livers of rats in the control and experimental groups. Livers were collected and lysed, and western blot analysis was performed to determine the total protein expression of iNOS, COX-2 and CYP2E1. PYGP, *Pyropia yezeensis* glycoprotein; EtOH, ethanol; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; CYP2E1, cytochrome P450 2E1.

small quantities of ethanol (~10%) into ADH (45), and ADH is considered to be a major toxin in ethanol-induced liver injury, inflammation and extracellular matrix (46). In the present study, the rats exposed to chronic ethanol consumption showed higher levels of CYP2E1 enzyme production in the liver, compared with the rats in the control group. The co-administration of ethanol with PYGP showed inhibition in the production of CYP2E1.

In conclusion, the present study demonstrated that chronic ethanol consumption induced hepatotoxicity and inhibited the levels of antioxidants, including GSH,

GSH-px and CAT, in the liver. In addition, chronic ethanol consumption promoted the overexpression of iNOS, COX-2 and CYP2E1, and the overactivation of ERK, JNK and p38. PYGP prevented chronic ethanol consumption-induced hepatotoxicity in the rats via the downregulation of MAPKs, iNOS, COX-2 and CYP2E1. These results suggested that PYGP may offer potential for use as a novel treatment against chronic ethanol hepatotoxicity.

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