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Korean red ginseng prevents ethanol-induced hepatotoxicity in isolated perfused rat liver

Hye-Min Park[1,2,†], Shang-Jin Kim [1,3,†], Hyeon-Kyu Go[1,2], Gi-Beum Kim[2,3], Sung-Zoo Kim[2], Jin-Shang Kim[1,3], Hyung-Sub Kang[1,2,*]

[1]College of Veterinary Medicine, [2]Center for Healthcare Technology Development, and [3]Korean Zoonoses Research Institute, Chonbuk National University, Jeonju 561-756, Korea

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Abstract : Alcohol abuse and its medical and social consequences are a major health problem in many areas of the world. Korean red ginseng (KRG) has been traditionally used for the treatment of liver disease. This study was conducted to evaluate the hepatoprotective effects of KRG against hepatotoxicity in Sprague-Dawley rats treated with ethanol (EtOH). Administration of EtOH for 20 days induced significant changes in serum biochemical parameters (aspartate aminotransferase, alanine transaminase, and glucose) accompanied by histological changes in the liver tissue. Treatment with KRG prior to administration of EtOH inhibited the EtOH-induced biochemical and histological changes of the liver. In perfused rat livers, administration of EtOH caused an increase in lactate dehydrogenase (LDH) release into the perfusate and activated the pro-apoptotic Bax protein but inhibited the anti-apoptotic Bcl-2 protein. Pretreatment with KRG prior to administration of EtOH decreased the EtOH-induced LDH release and inhibition of Bcl-2 protein. These results suggest that KRG exerts anti-apoptotic effects and alleviated EtOH-induced liver injury in rats.

Keywords : ethanol, ginseng, liver, liver perfusion, rat

Introduction

Alcoholism, as the major cause of liver cirrhosis, fatty liver, and alcoholic hepatitis, is a leading cause of morbidity and mortality throughout the world [18, 22]. The pathogenesis of these hepatic diseases is known to be related to programmed cell death, or apoptosis [17]. It has been shown that an increase in the number of apoptotic cells in the liver correlates with the development of ethanol (EtOH)-induced pathologic liver injury [15].

Some phytodrugs and extracts prepared from medicinal plants have proven hepatoprotective properties when used in the treatment of liver diseases such as hepatitis, cirrhosis, steatosis, and chemical-induced liver injury [3]. Korean ginseng (*Panax ginseng,* C.A. Meyer) is one of the most widely used medicinal plants and has a wide range of pharmacological and physiological actions [9]. Red ginseng (RG) is *Panax ginseng* that has been heated, either through steaming or sun-drying. During the production process of RG chemical transformations of active physiological properties occur involving ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids [19]. Korean RG (KRG) has been traditionally used for the treatment of various maladies including liver disease. In animal experiments, KRG was able to partially reverse the hepatotoxicity induced by carbon tetrachloride in rats [7] and inhibit increases in serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase levels in a rat model of acute hepatitis [14].

The present study investigated the protective effect of KRG on EtOH-induced hepatotoxicity in an *ex vivo* isolated liver model and an acute EtOH-induced liver injury model in rats.

Materials and Methods

Animals and drug administration

KRG extract obtained from red ginseng manufactured from the fresh roots of six-year-old *Panax ginseng* plants

*Corresponding author
Tel: +82-63-270-3883, Fax: +82-63-270-3780
E-mail: kang-hs@jbnu.ac.kr
†The first two authors equally contributed to this study.

whose botanical identity had been verified was provided by the Korea Ginseng Corporation (Korea). Red ginseng was extracted seven times with 10 volumes of distilled water at 85°C for 8 h followed by cooling. The aqueous extract was combined, and then concentrated twice under vacuum at 40°C.

Male Sprague-Dawley rats weighing 180~220 g were used for the *in vivo* experiment. The animals were purchased from Koatech (Korea) and housed for one week under controlled humidity (60~70%) and temperature (25 ± 1°C) conditions prior to experimentation. All experimental protocols were approved by the Committee on the Care of Laboratory Animal Resources, Jeonbuk National University (Korea), and conducted in accordance with the Guide [16]. The rats had free access to a standard rat pellet diet and tap water. The animals were randomly assigned to three equal groups (n = 8, per group) as follows: normal control, EtOH, or KRG + EtOH. The daily dose of KRG was 100 mg/kg and the other groups received an equal volume of vehicle. The selected dose of KRG was based on previous work [1, 8, 20]. EtOH groups were administered EtOH (3 g/kg body weight, 30% v/v) orally one hour after KRG or vehicle over 20 consecutive days. Six hours after the final EtOH treatment, animals were anesthetized by intraperitoneal injection of tiletamine/zolazepam (Zoletile, 40 mg/kg). Blood was collected in tubes containing the anticoagulant sodium heparin (5 IU/mL). Serum was separated by centrifugation at 3,000 rpm for 20 min and analyzed for aspartate aminotransferase (AST), alanine transaminase (ALT), and glucose levels using an autoanalyzer Model 7020 (Hitachi, Japan).

Histopathology

The liver was excised for histopathological examination. Portions of the same lobe of liver from each animal were immediately fixed in 10% formaldehyde, embedded in paraffin, cut into 5~6 μm sections, stained with hematoxylin-eosin (H&E), and observed under a light microscope (×400).

Lactate dehydrogenase (LDH) measurement and liver perfusion

Male Sprague-Dawley rats weighing 250~280 g were used for the *ex-vivo* experiment. The animals were purchased from Koatech (Korea) and had free access to a standard rat pellet diet and tap water until 16 h before surgery at which time the food but not the water was removed. Excision of the liver and its connection to a recirculating perfusion system was performed as previously described [23]. After removal of the livers, rats were euthanized by exsanguination. The albumin- and serum-free perfusion medium consisted of oxygenated modified Krebs-Henseleit (KH) buffer containing 120 mM NaCl, 3.5 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 1.2 mM KH_2PO_4, 12 mM $NaHCO_3$, 10 mM glucose, and 2 mM Na-pyruvate; pH was adjusted to 7.4 ± 0.02 using 10 mM Tris-base and 10 mM Tris-acid. The buffer was gassed with 95% O_2 and 5% CO_2 and the temperature was maintained at 36.5 ± 0.5°C. Perfusion was performed under conditions of constant pressure (240 mm H_2O) throughout the experiment and the perfusion flow rate was regulated at 10 mL/min using a peristaltic pump. For LDH measurements, samples of 1 mL were taken from the perfusate every minute. After 20 min of perfusion to stabilize the liver, treatment was initiated. Perfusion with KH buffer for an additional 10 min was performed as a control. Livers were perfused with KH buffer containing 1% EtOH for 10 min. KRG (100 μg/mL) was added to KH buffer, which was perfused 5 min before EtOH administration. The concentration of EtOH used was selected based on previously reports which showed that it were capable of inducing hepatotoxic injury [24]. Samples from the perfusate were used to measure LDH activity by the established chemical-based colorimetric method, in which generated pyruvic acid is quantified using a spectrophotometer (SpectraMax fluorometer with a SoftMax Programme; Molecular Probes, USA) at a wavelength of 490 nm. At the end of the experiments liver samples were frozen in liquid nitrogen until further analysis.

Western blot analysis of Bcl-2 and Bax

Frozen liver samples (100 mg) were suspended in 1 mL of an ice-cold solution containing 250 mM sucrose, 30 mM histidine, 1 mM EDTA, 1 mM phenylsulfonyl fluoride, 23 mg aprotinin, 50 mg leupeptin, 0.25 mM dithiothreitol, and 1% Triton X-100 (pH 6.8). The tissue was immediately homogenized and the extract transferred to a 1.5 mL microfuge tube on ice. The sample was then sonicated using an ultrasonic processor (Sonics & Materials, USA) and 0.5 mL was microcentrifuged at 14,000 rpm for 30 min. The protein in 30 μL of the resulting supernatant was resolved with 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved protein was electrotransferred to a Hybond-ECL nitrocellulose membrane blocked with

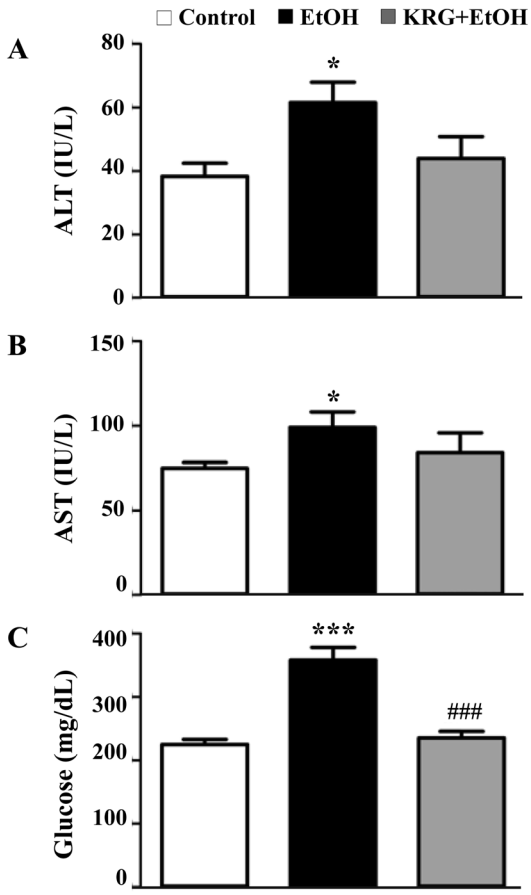

Fig. 1. Effects of Korean red ginseng (KRG) on serum biochemical levels in rats with ethanol (EtOH)-induced liver injury. The data are reported as mean ± SE (n = 8) for each group. $^{*}p < 0.5$, $^{***}p < 0.001$ *vs.* control, $^{###}p < 0.001$ *vs.* EtOH.

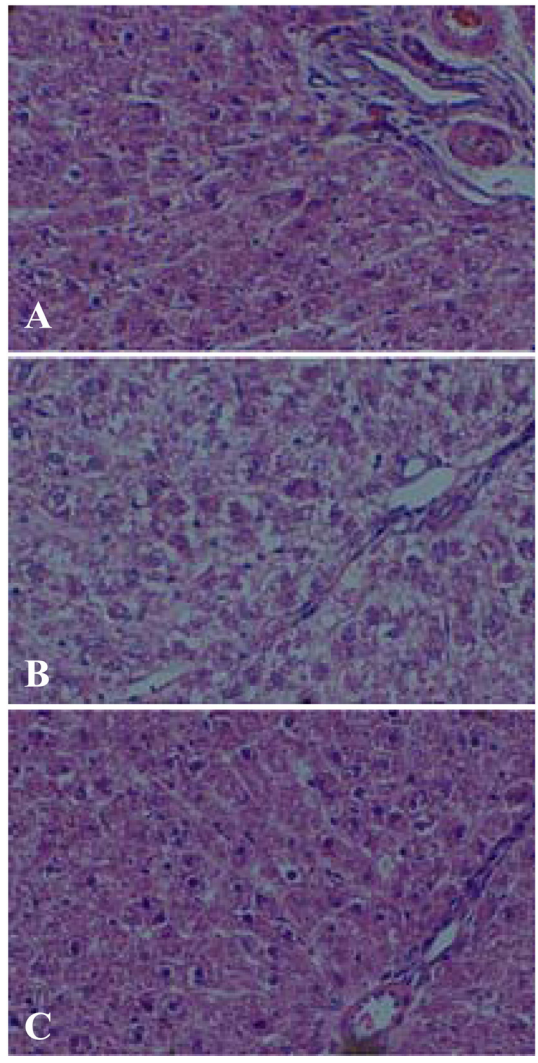

Fig. 2. Light microscopic analysis of liver sections of normal and EtOH-treated rats with or without KRG administration. (a) Control, (b) EtOH, (c) KRG+EtOH.

Tris-buffered saline (20 mM Tris and 140 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBST) and 5% milk at room temperature for 2 h. The membrane was then incubated overnight with a monoclonal antibody (primary antibody; Bcl-2, Bax, β-actin; Cell Signaling Technology, USA) at a 1 : 1000 dilution in TBST with 5% milk at 4℃. The blots were washed in TBST three times for 5 min each time, and the bands were detected using enhanced chemiluminescence. Blots were quantified using laser scanning densitometry (Bio-Rad Laboratories, USA).

Statistical analysis

The data were analyzed using *t* tests and repeated measures of analysis of variance (ANOVA) followed by a Bonferroni correction. The results are expressed as the mean ± SE of the mean. *P*-values < 0.05 were considered significant.

Results

Serum enzyme parameters in EtOH-induced rat liver injury

The serum levels of ALT, AST, and glucose were used as biochemical markers for hepatic damage. Compared to the control group, the EtOH treated group showed a significant increase in serum AST, ALT, and glucose levels (AST: 75.0 ± 3.4 IU/L *vs.* 99.0 ± 9.2 IU/L, ALT:

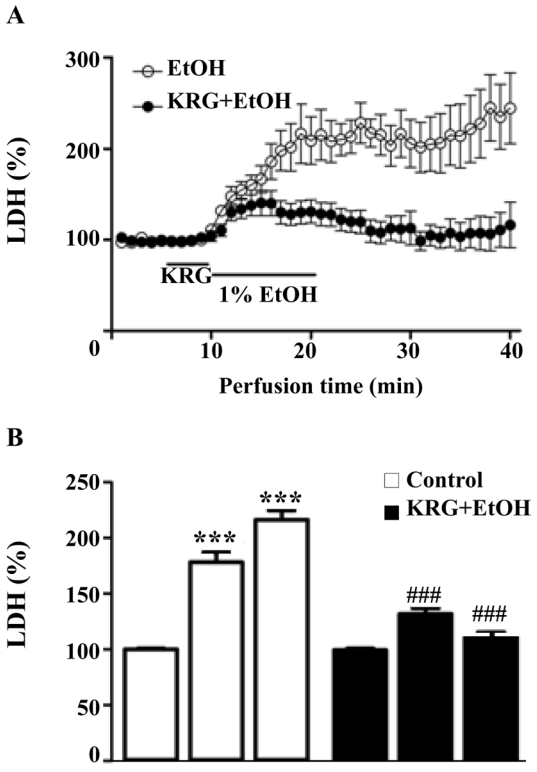

Fig. 3. Lactate dehydrogenase (LDH) release into the perfusate. (a) Time course of LDH release from isolated rat livers as influenced by the addition of EtOH with and without KRG. (b) Levels of LDH released into the perfusate after addition of 1% EtOH without □ and with ■ KRG pretreatment (100 g/mL). The data is reported as the mean ± SE of five different preparations. $^{***}p < 0.001$ vs. control, $^{###}p < 0.001$ vs. KRG + EtOH group.

38.4 ± 4.1 IU/L *vs*. 61.6 ± 6.4 IU/L, glucose: 225.0 ± 8.1 mg/dL *vs*. 358.3 ± 19.7 mg/dL). Co-administration of KRG tended to reduce serum AST, ALT, and glucose to nearly control levels (AST: 75.0 ± 5.2 IU/L, ALT: 40 ± 4.2 IU/L, glucose: 235.4 ± 10.5 mg/dL).

Histologic analysis

Microscopic examination of the liver sections of the control animals showed normal architecture of hepatic lobules in the form of hepatocytes arranged from the portal vein (Fig. 2a). Examination of rat livers from the group treated with EtOH showed swelling, slight hydropic degeneration, and lipid changes of hepatocytes (Fig. 2b). Histopathological changes induced by EtOH were remarkably improved by co-administration of KRG (Fig. 2c).

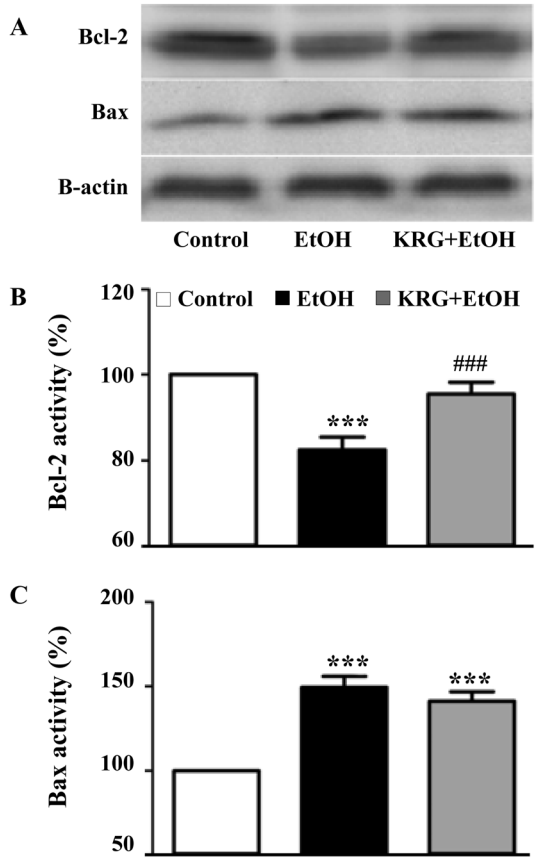

Fig. 4. Effects of EtOH and KRG on Bcl-2 family proteins in isolated perfused rat livers. Immunoprecipitation of Bcl-2 and Bax under EtOH loading in perfused rat livers. Livers were perfused with 1% EtOH for 30 min. Prior to EtOH loading, livers were pretreated with or without 100 μg/mL KRG. (a) The amount of Bcl-2 and Bax were measured by Western blot analysis. This picture shows the typical changes in Bcl-2 and Bax in a perfused rat liver. (b) Blots were quantified by laser scanning densitometry. The data are reported as mean ± SE of five different preparations. $^{***}p < 0.001$ *vs*. control, $^{###}p < 0.001$ *vs*. EtOH.

Effects of EtOH and KRG on LDH release in perfused rat liver

We measured LDH release from isolated rat liver in the presence of 1% EtOH with and without KRG (100 mg/kg body weight). As shown as Fig. 3, administration of EtOH for 10 min resulted in significant increase in LDH release (during 10 min of EtOH administration; $p < 0.001$ compared to control, during 20 min of wash-out; $p < 0.001$, Bonferroni post hoc test following one-way ANOVA). Pretreatment with KRG 5 min prior to administration of EtOH produced a remarkable attenuation

in elevated levels of LDH (during 10 min of EtOH administration; $p < 0.001$ compared to the administration of EtOH per se, during 20 min of wash-out; $p < 0.001$, Bonferroni *post hoc* test following two-way ANOVA).

Effects of EtOH and KRG on Bcl-2 family proteins in perfused rat livers

To evaluate the effects of EtOH on the production of apoptotic proteins, we measured activity of Bcl-2 and Bax in the perfused livers following the LDH release experiments. The administration of 1% EtOH induced significant Bax activation and Bcl-2 inhibition (Fig. 4). Pretreatment with KRG (100 mg/kg body weight) 5 min prior to administration of EtOH counteracted EtOH-induced Bcl-2 inhibition. Using densitometry, the percent variations in the EtOH treated group (n = 4) compared to the control group (100%) were determined to be 149.4 ± 6.5% (Bax protein) and 82.5 ± 2.9% (Bcl-2 protein), and those of the KRG+EtOH group were 141.2 ± 5.6% (Bax protein) and 95.5 ± 2.7% (Bcl-2 protein).

Discussion

Chronic alcohol consumption has been associated with excessive blood glucose levels. It can reduce the body's responsiveness to insulin and cause glucose intolerance in healthy individuals [21] and alcoholics with liver cirrhosis [13]. In this study, rats treated with EtOH for 20 days had significantly increased blood glucose levels and pathological changes were observed in their livers. The obvious indicator of liver injury is leakage of cellular enzymes into plasma [2]. We confirmed an increase in serum enzymes levels associated with ALT and AST in EtOH-treated rats, indicating enhanced permeability, injury, and necrosis of hepatocytes [4].

Korean ginseng is one of the most widely used medicinal plants, particularly in traditional oriental medicine. Previous studies have suggested that ginseng extracts bestow a pronounced hepatoprotective effect, assessed through the activity of transaminases (AST, ALT) following hepatotoxicity in rats treated with chromium VI [1]. Also, RG has been shown to accelerate the rate of liver regeneration and ameliorate liver injury after partial hepatectomy in dogs [12]. In the present study, co-administration of KRG alleviated liver damage and inhibited the increases in glucose, ALT, and AST levels caused by EtOH.

In perfused rat livers, administration of EtOH caused liver cell membrane damage as evidenced by an unreversible significant increase in LDH release into the perfusate. Pretreatment with KRG prior to administration of EtOH reduced LDH release. Thus, KRG inhibited the cell membrane damage induced by EtOH.

Short-term EtOH intoxication causes mitochondrial dysfunction [10], and apoptosis [6, 11] in primary cultures of rat hepatocytes. During apoptosis, pro-apoptotic proteins of the Bcl-2 family, including Bax, Bim, and Bid, can translocate to the outer membrane of mitochondria and induce apoptosis [26]. In contrast, anti-apoptotic proteins in this family, including Bcl-2, sequester in the mitochondria and prevent apoptosis [5, 25]. The present results indicate that the administration of EtOH to perfused rat livers activates the pro-apoptotic Bax protein but inhibits the anti-apoptotic Bcl-2 protein. Pretreatment with KRG prior to the administration of EtOH reduced the EtOH-induced inhibition of Bcl-2 protein. Although pretreatment with KRG reduced activation of Bax protein, there was no significant difference. Therefore, it appears that the protective effect of KRG against EtOH-induced hepatic injury may be regulated by mitochondrial intracellular stress signaling.

Conclusion

These results suggest that KRG has potent hepatoprotective activity against EtOH-induced hepatic injury in rats. Since KRG may possess anti-apoptotic activity and ameliorate hepatic injury induced by EtOH, the current data may be useful in the development of therapeutic strategies to ameliorate or prevent the toxic effects of alcohol.

Acknowledgments

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