

## Protective Effect of Korean Red Ginseng against Aflatoxin B<sub>1</sub>-Induced Hepatotoxicity in Rat

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Korean red ginseng (KRG), the steamed root of *Panax ginseng* Meyer, has a variety of biological properties, including anti-inflammatory, antioxidant and anticancer effects. Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) produced by the *Aspergillus* spp. causes acute hepatotoxicity by lipid peroxidation and oxidative DNA damage, and induces liver carcinoma in humans and laboratory animals. This study was performed to examine the protective effects of KRG against hepatotoxicity induced by AFB<sub>1</sub> using liver-specific serum marker analysis, histopathology, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling. In addition, to elucidate the possible mechanism of hepatoprotective effects, superoxide dismutase, catalase, glutathione peroxidase, and malondialdehyde were analyzed. Rats were treated with 250 mg/kg of KRG (KRG group) or saline (AFB<sub>1</sub> group) for 4 weeks and then received 150 µg/kg of AFB<sub>1</sub> intraperitoneally for 3 days. Rats were sacrificed at 12 h, 24 h, 48 h, 72 h, or 1 wk after AFB<sub>1</sub> treatment. In the KRG pre-treatment group, serum alanine aminotransferase, aspartate aminotransferase, and malondialdehyde levels were low, but superoxide dismutase, catalase, and glutathione peroxidase activities were high as compared to the AFB<sub>1</sub> alone group. Histopathologically, AFB<sub>1</sub> treatment induced necrosis and apoptosis in hepatocytes, and led to inflammatory cells infiltration in the liver. KRG pre-treatment ameliorated these changes. These results indicate that KRG may have protective effects against hepatotoxicity induced by AFB<sub>1</sub> that involve the antioxidant properties of KRG.

**Keywords:** *Panax ginseng*, Korean red ginseng, Aflatoxin B<sub>1</sub>, Antioxidant enzymes

### INTRODUCTION

Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is a metabolite produced by fungi, *Aspergillus flavus* and *Aspergillus parasiticus*, which contaminate grains. AFB<sub>1</sub> causes hepatotoxicity and liver carcinoma in humans and laboratory animals [1]. It is metabolized by cytochrome P-450 monooxygenases into reactive aflatoxin B<sub>1</sub>-8,9-epoxide, which binds to cellular macromolecules and causes injury to

the periportal regions of the liver. This damage appears as hemorrhage, parenchymal cell necrosis, and injury to intrahepatic bile ducts as well as elevated alanine and aspartate amino transferase levels [2-5]. In addition, AFB<sub>1</sub>-induced toxicity is due to lipid peroxidation and oxidative DNA damage [6,7]. The main active components of *P. ginseng* are ginsenosides which have been shown to have a variety of biological properties including anti-inflammatory, antioxidant, and anticancer

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effects. In addition, ginseng extract clearly reduce liver damage induced by certain chemicals including alcohol [8] or carbon tetrachloride [9,10]. Another study suggested that ginseng and/or ginsenoside can induce antioxidant enzymes essential for maintaining cell viability by lowering the level of oxygen radicals generated from intracellular metabolism [11].

This study was performed to examine the protective effects of Korean red ginseng (KRG) against hepatotoxicity induced by AFB<sub>1</sub>. We evaluated sequential pathological characteristics and liver-specific serum markers. In addition, antioxidant enzyme activities were analyzed in the liver to elucidate the mechanisms underlying the hepatoprotective effects of KRG.

## MATERIALS AND METHODS

### Animals and chemicals

Thirty-three 4-week-old male Sprague-Dawley rats weighing 85-100 g were obtained from Nara Biotech (Seoul, Korea). The rats were housed in polycarbonate cages at 23±1°C with 55±5% humidity and were maintained on a 12 h light/dark cycle. The animals had access to rodent chow (Jeil Feed Co., Daejeon, Korea) and water *ad libitum*. KRG extract was donated from KT&G (Seoul, Korea). The extracts were freshly dissolved in distilled water immediately before use. AFB<sub>1</sub> was purchased from Sigma-Aldrich (St. Louis, MO, USA). All animal studies were approved by the Institutional Animal Ethical Committee of Chungnam National University (approval no. 2009-1-26).

### Experimental procedures

After 1 wk of acclimation, the rats were randomly divided into three groups: control group ( $n=3$ ), KRG group ( $n=15$ ), and AFB<sub>1</sub> group ( $n=15$ ). The KRG group was given oral doses of KRG (250 mg/kg/d) or saline for 4 wk orally, and then received 150 µg/kg/d of AFB<sub>1</sub> intraperitoneally for 3 d. Control rats ( $n=3$ ) received an equivalent volume of corn oil. The rats were sacrificed while under ether anesthesia at 12 h, 24 h, 48 h, 72 h, or 1 wk after receiving the last dose of AFB<sub>1</sub> ( $n=3$  at each time point).

Immediately after sacrificing, the liver from each rat was removed and the left lobe was placed in 10% neutral buffered formalin for histopathological examination and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). The others lobes were used for antioxidant enzyme activity and thiobarbituric acid reactive substances (TBARS) assays.

### Blood chemistry

Blood was collected from the inferior vena cava while the rats were under ether anesthesia. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a Hitachi 7150 Chemistry analyzer (Hitachi Ltd., Tokyo, Japan).

### TUNEL

Apoptotic cells were detected by TUNEL using an ApopTag-peroxidase Kit (Intergen Co., Purchase, NY, USA). The procedure was performed according to the manufacturer's instructions.

### Hepatic antioxidant enzyme activity assay

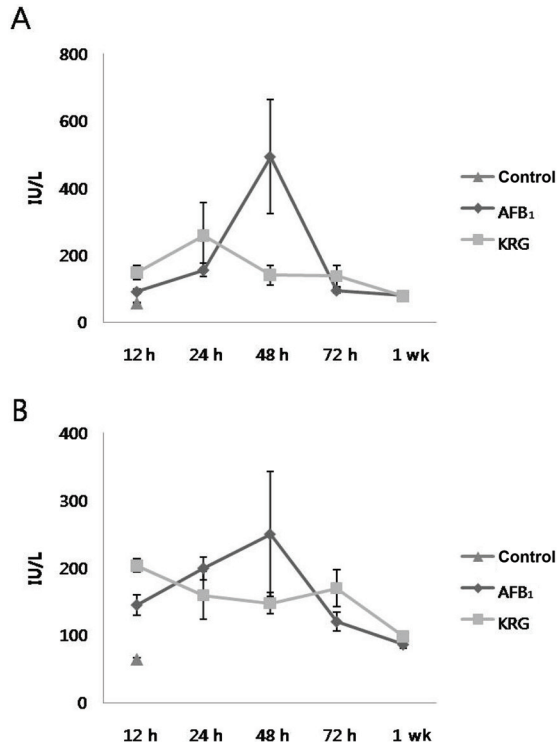
To elucidate the possible mechanism underlying the hepatoprotective effects of KRG, the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) were analyzed. The enzyme source fraction from liver tissue was prepared as follows. Liver tissue (1 g) was homogenized in a five-fold volume of the 0.25 mol/L sucrose buffer, centrifuged at 600 ×g for 20 min to remove any cell debris, and then the supernatant was centrifuged at 10,000 ×g for 10 min to remove the mitochondria. Finally, the supernatant was removed and further centrifuged at 105,000 ×g for 60 min to obtain the cytosolic fraction. The amount of protein in the mitochondrial and cytosolic fractions was measured using Bradford method [12] with bovine serum albumin as the standard.

### Superoxide dismutase activities assay

SOD activity was measured as described by Marklund and Marklund [13] with a slight modification. The cytosol supernatant (100 L) was mixed with 1.5 mL of Tris-EDTA-HCl buffer (pH 8.5), then 100 L of 7.2 mmol/L pyrogallol was added and the mixture was incubated at 25°C for 10 min. The reaction was terminated by the addition of 50 L of 1.0 M/L HCl and the absorbance was measured at 420 nm. One unit was defined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. The activity was expressed as U/mg protein.

### Catalase activities assay

CAT activity was measured using the method by Aebi [14] with a slight modification. The mitochondria pellet was dissolved in 1.0 mL of a 0.25 M/L sucrose buffer. The mitochondria solution (10 L) was then added to a cuvette containing 2.89 mL of a 50 mmol/L potassium phosphate buffer (pH 7.4), and the reaction initiated by

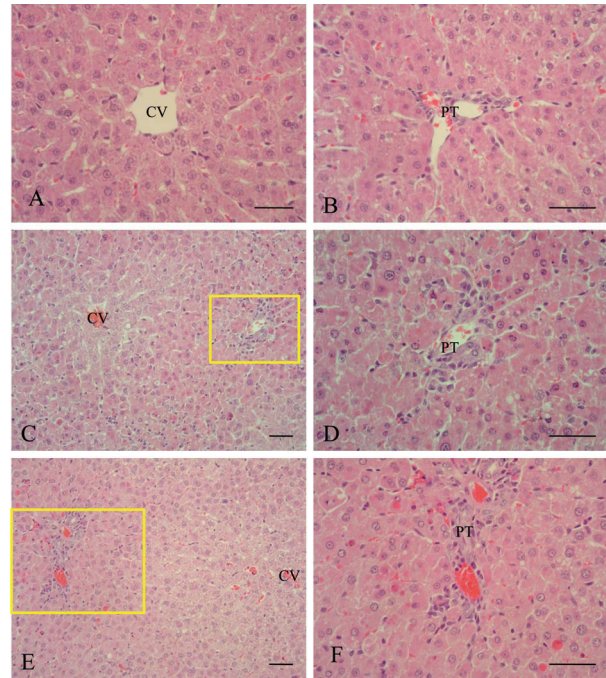


**Fig. 1.** Liver specific serum biomarker analysis in rats treated with Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) with or without Korean red ginseng (KRG). Serum alanine aminotransferase (A) and aspartate aminotransferase (B) were relatively low in group pretreated with KRG compared to the group treated only with AFB<sub>1</sub>.

the addition of 0.1 mL of 300 M/L H<sub>2</sub>O<sub>2</sub> in a final volume of 3.0 mL at 25°C. The decomposition of H<sub>2</sub>O<sub>2</sub> was measured at 240 nm for 5 min using a spectrophotometer. A molar extinction coefficient of 0.041 (mmol/L)<sup>-1</sup>·cm<sup>-1</sup> was used to determine the CAT activity. One unit of CAT activity was defined as the amount of enzyme which oxidized 1.0 μmol H<sub>2</sub>O<sub>2</sub> per min per mg protein.

### Glutathione peroxide activities assay

GPX activity was measured using the Paglia and Valentine method [15] with a slight modification. The reaction mixture contained 2.6 mL of a 0.1 M/L of Tris-HCl (pH 7.2) buffer, 100 μL of 30 mmol/L glutathione, and 100 μL of 6.0 mmol/L NADPH. The cytosolic supernatant (100 μL) was added to 2.9 mL of the reaction mixture and incubated at 25°C for 5 min. The reaction was initiated by the addition of 100 μL of 7.5 mmol/L H<sub>2</sub>O<sub>2</sub> and the absorbance was measured at 340 nm for 5 min. A molar extinction coefficient of 6.22×10<sup>3</sup> (mmol/L)<sup>-1</sup>·cm<sup>-1</sup> was used to determine the activity. One unit of GPX was defined as the amount of enzyme which oxidized 1.0 μmol NADPH per min per mg protein.



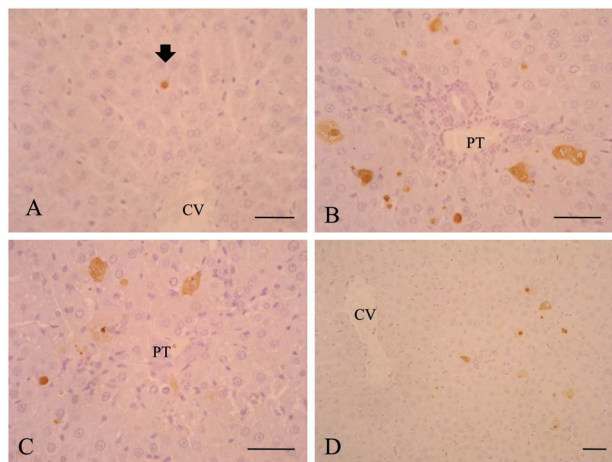
**Fig. 2.** Representative photomicrograph of liver lesions in rats treated with Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) with or without Korean red ginseng (KRG) pretreatment. H&E stain. (A,B) Centrilobular (CV) and periportal (PT) zone of the control group. Bar=50 μm. (C) Hepatic lesion found in the AFB<sub>1</sub>-treated group after 12 h; severe necrosis of hepatocytes and loss of hepatic cords were observed around the portal region. Bar=100 μm. (D) Higher magnification of (C). Severe hepatocyte necrosis was noted around the portal triad. Bar=50 μm. (E) Hepatic lesions of the KRG pretreated group after 12 h; mild necrosis in hepatocytes and loss of hepatic cords were observed around the portal region. Bar=100 μm. (F) Higher magnification of (E). Mild hepatocyte necrosis was noted around the portal triad. Bar=50 μm.

### ThioBarbituric acid reactive substances assay

Malondialdehyde (MDA) quantitation was performed using an OxiSelect™ TBARS assay kit (Cell Biolabs Inc., San Diego, CA, USA). Samples containing unknown quantities of MDA or MDA standards were first incubated with thiobarbituric acid at 95°C. After the brief incubation, the samples and standards can be read spectrophotometrically or fluorometrically. The unknown MDA content in the samples was determined by comparison with the MDA standard curve.

### Statistical analysis

The results are expressed as the mean±SD. Comparison between groups were carried out using a two-tailed Student's *t*-test. The threshold of significance was set a *p*<0.05.



**Fig. 3.** Apoptosis in liver of rats treated with Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) with or without Korean red ginseng (KRG) pretreatment. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) stain. Bar=50 μm. (A) Control group; few TUNEL-labeled apoptotic cells (arrow) were noted. (B) AFB<sub>1</sub> group; at 12 h many apoptotic cells were observed. (C) KRG pretreated group; at 12 h the number of apoptotic cells decreased compared to the AFB<sub>1</sub> group. (D) AFB<sub>1</sub> group; at 48 h the number of apoptotic cells decreased. CV, central vein; PT, portal triad.

## RESULTS

### Blood chemistry

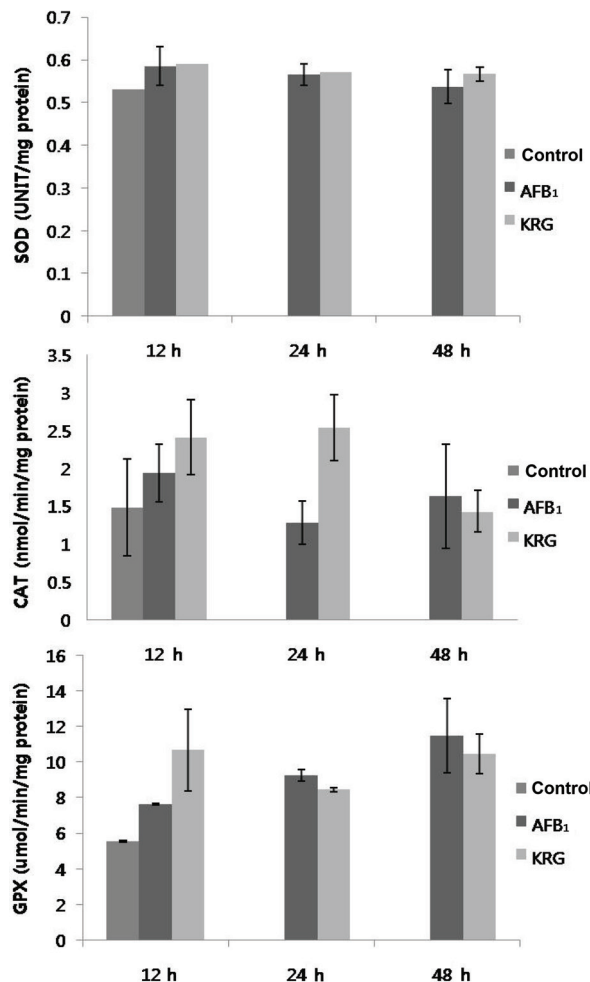
In the AFB<sub>1</sub> and KRG groups, the AST and ALT levels were increased starting at 12 h compared to the control group. In AFB<sub>1</sub> group, AST and ALT levels peaked at 48 h and then decreased. KRG pre-treatment ameliorated these changes (Fig. 1).

### Histopathological evaluation

Microscopically, significant pathologic changes in the liver were detected in all AFB<sub>1</sub>-treated rats. Histopathological changes were characterized by hepatic parenchymal cell necrosis, loss of hepatic cords, fatty changes, congestion, and hemorrhage in the centrilobular and periportal regions. In addition, inflammatory cells infiltration in the periportal region was observed. In the AFB<sub>1</sub> group, the appearance of severe liver lesions were observed after 12 h and 24 h of AFB<sub>1</sub> treatment. Liver lesions were still present at 48 h, but were less extensive and almost completely disappeared 1 wk after AFB<sub>1</sub> treatment. KRG pre-treatment significantly ameliorated these changes (Fig. 2).

### TUNEL assay

Positive TUNEL reactions were detected in hepatic parenchymal cells scattered throughout the liver in the AFB<sub>1</sub> rats. Apoptosis rates peaked at 12 h and remained

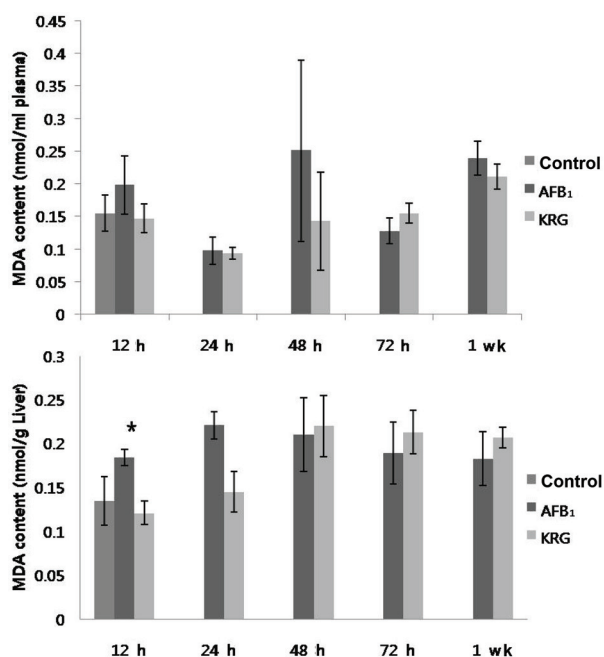


**Fig. 4.** Level of hepatic antioxidant enzymes in rats treated with Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) with or without Korean red ginseng (KRG) pretreatment. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) enzyme activity was increased after 12 h in the KRG and AFB<sub>1</sub> groups compared to the control group. These enzyme activities were relatively higher in the KRG group compared to the AFB<sub>1</sub> group.

elevated until 48 h after the AFB<sub>1</sub> treatment, but then gradually decreased until the end of the follow-up period. The number of apoptotic cells was decreased by KRG pre-treatment (Fig. 3).

### Hepatic antioxidant enzymes activities

AFB<sub>1</sub> treatment slightly increased antioxidant enzyme activity compared to the control group. SOD, CAT, and GPX activity levels were higher in the KRG group than in the AFB<sub>1</sub> group (Fig. 4). However, the hepatic MDA level was increased by treatment with AFB<sub>1</sub> compared to the control, and was significantly lower in KRG group than in AFB<sub>1</sub> group (Fig. 5).



**Fig. 5.** The levels of malondialdehyde (MDA), a natural byproduct of lipid peroxidation, in the plasma and liver of rats treated with Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) with or without Korean red ginseng (KRG) pretreatment. Plasma MDA levels were higher in the AFB<sub>1</sub> group compared to the control and KRG group at each time point except at 72 h. In addition, MDA levels in the liver were higher in the AFB<sub>1</sub> group than the control and KRG groups at 12 and 24 h. \*Significantly different from the control and KRG group.

## DISCUSSION

AFB<sub>1</sub>, a mycotoxin produced by the fungi *Aspergillus flavus* and *Aspergillus parviticus*, is known to be a potent hepatotoxin and hepatocellular carcinogen in experimental animals [16-18]. In the present study, AFB<sub>1</sub> treatment increased serum ALT and AST levels; they both increased until 48 h after AFB<sub>1</sub> treatment and then decreased in a time-dependent manner. These results concurred with our histopathological findings. After 12 h and 24 h of AFB<sub>1</sub> treatment, severe histopathological changes in the liver were noted. In addition, AFB<sub>1</sub> treatment resulted in significant increases in apoptosis after 12 h after which the number of apoptotic cells decreased in a time-dependent manner. The present results clearly indicate that AFB<sub>1</sub> induces damage in the liver during an early stage of exposure in which apoptosis of hepatocytes is involved. KRG pre-treatment clearly ameliorated these changes. Apoptosis is a form of cell death that permits the elimination of damaged or unwanted cells in multicellular organisms. The protective effect of *Panax ginseng* extract against the apoptotic cell death induced by PCB52 in human neuronal SK-N-MC cells has been

previously reported [19]. Likewise, ginsenoside Rh<sub>2</sub> induced apoptotic cell death in human breast cancer cell lines [20]. Further studies are now necessary to elucidate the effects of KRG on apoptotic cell death and the mechanisms underlying the protective effects of KRG against AFB<sub>1</sub>-induced hepatocyte apoptosis.

Lipid peroxidation is a well-defined mechanism of cellular damage in animals and plants. Oxidative modification of lipids can be induced *in vitro* by a wide array of pro-oxidant agents and occurs *in vivo* during aging and under certain pathologic conditions [21,22]. It was hypothesized that AFB<sub>1</sub>-induced hepatotoxicity is due to lipid peroxidation and oxidative DNA damage [6,7]. Lipid peroxides are indicators of cellular oxidative stress that decompose to form more complex and reactive compounds such as MDA and 4-hydroxynonenal. These aldehydic secondary byproducts of lipid peroxidation are generally accepted markers of oxidative stress. The enzymatic antioxidant system is one of the protective mechanisms including SOD which can be found in various cellular compartments and catalyze the disproportion of superoxide anions to hydrogen peroxide and oxygen [21,23]. H<sub>2</sub>O<sub>2</sub> is eliminated by various antioxidant enzymes such as CAT [21,24,25] and GPX [22,26] which convert H<sub>2</sub>O<sub>2</sub> into water. Toxic O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, and OH radicals are efficiently eliminated by non-enzymatic ( $\alpha$ -tocopherol,  $\beta$ -carotene, phenolic compounds, ascorbate, glutathione) and enzymatic antioxidants [27,28]. Red ginseng extracts are potent antioxidants that exert protective effects against the progression of oxidative stress-induced DNA damage [11,23,29]. In the present study, CAT, GPX, and SOD enzyme activities were higher in the KRG pretreatment group compared to the AFB<sub>1</sub> group. However, the level of MDA was lower in the KRG pretreated group. These results indicate that KRG prevented AFB<sub>1</sub>-induced hepatotoxicity through its antioxidant effects by increasing SOD, CAT, and GPX activity and reducing lipid peroxidation. In conclusion, KRG may be used to protect hepatocyte from oxidative injury caused by AFB<sub>1</sub>.

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