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Research article

Protective effect of ultrasonication-processed ginseng berry extract on the D-galactosamine/lipopolysaccharide-induced liver injury model in rats

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ABSTRACT

Background: Acute hepatic failure is a life-threatening critical condition associated with rapid deterioration of liver function and liver transplantation. Several studies have shown that *Panax ginseng* Meyer has antidiabetic and hepatoprotective effects. However, the hepatoprotective effect of ginseng berry is still unveiled. In this study, we evaluated the hepatoprotective effects of ultrasonication-processed ginseng berry extract (UGBE) on acute hepatic failure model in rats.

Methods: Ginseng berry extract (GBE) was ultrasonically processed. The GBE, silymarin, and UGBE were orally administered to male Sprague-Dawley rats for 4 wk. Twenty-four h after the last administration, rats were challenged with D-galactosamine (D-GalN)/lipopolysaccharide (LPS).

Results: After ultrasonication, the component ratio of ginsenosides Rg2, Rg3, Rh1, Rh4, Rk1, Rk3, and F4 in GBE had been elevated. Administration of UGBE significantly increased the survival rate of D-GalN/LPS-challenged rats. Pretreatment with UGBE significantly decreased serum alanine aminotransferase, aspartate aminotransferase, and total bilirubin levels in D-GalN/LPS-challenged rats in a dose-dependent manner. The levels of enzymatic markers for oxidative stress (superoxide dismutase, glutathione peroxidase, catalase, and glutathione) were increased by UGBE treatment in a dose-dependent manner. Tumor necrosis factor alpha level, inducible nitric oxide synthase activities, and nitric oxide productions were reduced by UGBE treatment. In addition, hemeoxygenase-1 levels in liver were also significantly increased in the UGBE-treated group. The protein expression of toll-like receptor 4 was decreased by UGBE administration. Hematoxylin and eosin staining results also supported the results of this study showing normal appearance of liver histopathology in the UGBE-treated group.

Conclusion: UGBE showed a great hepatoprotective effect on D-GalN/LPS-challenged rats via the toll-like receptor 4 signaling pathway.

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1. Introduction

Acute liver failure (ALF) is an uncommon but life-threatening critical condition in which rapid degeneration of liver function results from catastrophic injury to the liver [1]. ALF usually occurs with no pre-existing liver disease, within a relatively short period of time [2]. There are many causes of ALF, such as viruses, drugs, toxins, alcohol, metabolic disease, or chronic autoimmune hepatitis [3,4]. Liver transplant is known to be the only effective therapy; however, there are critical drawbacks such as a shortage of liver

donors or the rapidity of the progression of ALF [5]. Therefore, prevention of ALF may prove to be more important than curative therapy.

D-galactosamine (D-GalN) is a hexosamine derived from galactose that causes necrosis of liver cells [6]. D-GalN induces liver damage through a decrease in the cellular uridine-5'-triphosphate (UTP) concentration and a reduction in the synthesis of hepatocyte RNA [7]. Metabolism of D-GalN by D-glucosamine pathways results in transcriptional blockade in the liver [8]. Lipopolysaccharide (LPS) is a representative endotoxic compound from the outer leaflet of

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Gram-negative bacteria, which can induce an inflammatory reaction [9]. Treatment of D-GalN in combination with LPS makes the liver more sensitive to LPS [10]. LPS induces intrahepatic inflammation by the activation of Kupffer cells and chemical mediators such as superoxide, nitric oxide (NO), and tumor necrosis factor alpha (TNF- α) [11]. Throughout oxidative stress, treatment of D-GalN/LPS (G/L) induces severe hepatic damage [12]. Considering these facts, the G/L-induced ALF experimental model is useful for ALF studies [13].

Panax ginseng Meyer is one of the most widely used medicinal herb, which has a long medicinal history in East Asia [14]. It is famous for having beneficial effects on diseases associated with the livers, brain, and heart [15]. Ginsenosides, which are representative compounds of *P. ginseng*, have been proved to contribute to these diverse biological effects [16]. Ginsenosides are triterpene saponins that belong to the steroidal family, which are dispersed in the roots, leaves, and berries of *P. ginseng* [17,18]. Among the ginsenosides identified (approximately 40), ginsenosides Rg1, Rb1, Re, and Rd are the most common ones [19].

P. ginseng berry extract (GBE) is known to have antiobesity and antidiabetic effects, which may be due to ginsenoside Re, which is the major compound of *P. ginseng* [20]. In our experiments, however, ultrasonication-processed *P. ginseng* berry extracts (UGBEs) were used. After the ultrasonication process, the composition of GBE was changed. Ginsenosides Rh1, Rh4, Rg2, Rg3, Rk1, Rk3, and F4 have rapidly been increased, while ginsenoside Re has been decreased. Several studies have examined the physiological effects of these compounds on the liver [21].

In the present study, we investigate the hepatoprotective effect of UGBE on the G/L-induced ALF model in rats, from an anti-oxidative perspective. Silymarin and GBE were used as positive controls. To identify the hepatoprotective effect of UGBE, the survival rate, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (T. bilirubin) levels were measured. Furthermore, the activity and expression of several enzymes related to oxidative stress and cytokines were evaluated.

2. Materials and methods

2.1. Materials

D-GalN, LPS (*Escherichia coli* O111:B4), and silymarin were purchased from Sigma-Aldrich Co., LLC (St. Louis, MO, USA). The glutathione peroxidase (GPx), catalase (CAT) activity, and NO assay kits were purchased from Biovision, Inc. (San Francisco, CA, USA). The TNF- α enzyme-linked immunosorbent assay (ELISA), glutathione (GSH), bilirubin, and albumin assay kits were also obtained from Biovision, Inc. The hemeoxygenase-1 (HO-1) ELISA kit was purchased from Enzo Life Sciences, Inc. (New York, NY, USA). The inducible nitric oxide synthase (iNOS) ELISA kit was purchased from CUSABIO (Wuhan, China). The toll-like receptor 4 (TLR4) ELISA kit was purchased from MyBioSource (San Diego, CA, USA). The anti-TLR4 antibody was purchased from Abcam (Cambridge, UK). The secondary antibody (Rabbit/Mouse) was purchased from Dako Real Envision Detection System (Agilent, Santa Clara, CA, USA) GBE and UGBE were kindly supplied by Professor Sung Kwon Ko, Semyung University (Jecheon, Korea). Ginsenoside standards were purchased from Chromadex (Irvine, CA, USA). Other essential materials and reagents were purchased from Sigma-Aldrich Co., Limited Liability Company.

2.2. Preparation and analysis of UGBE

UGBE and GBE water solvent extracts were kindly provided by Professor Sung Kwon Ko of Semyung University. We analyzed the

constituents of UGBE and GBE by the Waters 1525 binary HPLC system (Waters, Milford, MA, USA). Separation of UGBE was performed on an analytical column (Eurosphere, 100-5 C18, 250 mm \times 3.0 mm, 5 μ m; Knauer, Berlin, Germany) by gradient elution at room temperature. The eluent was a mixture of acetonitrile for HPLC (A) and distilled water for HPLC (B). The elution process was performed according to the following conditions: 0 min, 17% of A; 25 min, 25% of A; 50 min, 40% of A; 105 min, 60% of A; 110 min, 100% of A. The flow rate was 0.8 mL/min, injection volume was 20 μ L, and chromatograms were acquired by a UV/VIS Waters 2478 Dual λ Absorbance Detector (Waters) at 203 nm. After 10 h of ultrasonication, ginsenosides Rb1, Rb2, Rd, Re, Rf, Rg1, Rg2, Rg3, Rg6, Rh1, Rh4, Rk1, Rk3, F1, and F4 were identified. UGBE solution was orally administered to the rats once/d at doses of 100 mg/kg body weight (b.w.), 250 mg/kg b.w., and 500mg/kg b.w.

2.3. Animal models

Specific pathogen-free male Sprague-Dawley rats (200–250g) were purchased from Samtako Bio (Osan, Korea). The rats were group housed in pathogen-free cages in a room controlled for temperature (24–25°C) and humidity (70–75%), on a 12-h dark/light cycle, and were provided with filtered tap water and fed a normal laboratory diet from Samtako Bio. Animals were fasted for 24 h prior to being sacrificed, but were allowed free access to tap water throughout. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chung-Ang University, in accordance with the guide for the Korean Association for Laboratory Animals, Korea (CAUIACUC-20140031).

The 54 specific pathogen-free male Sprague-Dawley rats were randomly divided into six groups—a control group, an ALF group, a silymarin group, and three experimental groups. The rats in the control and ALF groups were given 2mL/kg b.w. distilled water once/d. The silymarin group was given 150 mg/kg b.w. silymarin once/d. The experimental groups were given 100 mg/kg b.w., 250 mg/kg b.w., and 500 mg/kg b.w. UGBE once/d. Each solution was administered for 28 d. During the administration period, food and water consumption and body weight were checked daily. One h following the last administration, D-GalN (300mg/kg, intraperitoneal injection; i.p.) and LPS (30 μ g/kg, i.p.intraperitoneal injection; i.p.) were injected simultaneously to all rats except those in the control group (normal saline, 5 mL/kg, i.p.intraperitoneal injection; i.p.). Twenty-four h after G/L injection, all rats were sacrificed by cervical dislocation. Blood and liver samples were removed immediately after sacrificing. This procedure is summarized in [Supplementary Fig. 1](#).

Another experiment was carried out in the same manner for lethality analysis (previously described). Following G/L injection, the survival rate was assessed over 10 d.

2.4. Measurements of serum biochemical parameters

Serum samples were diluted for optimal reactions before being measured. Serum ALT and AST activities were measured by the International Federation of Clinical Chemistry methods using Beckman-Coulter reagents (Beckman-Coulter, Brea, CA, USA) [22]. The T. bilirubin level was measured by a bilirubin kit. Serum TNF- α protein expression was measured by ELISA. All assay procedures were progressed according to the manufacturer's instructions.

2.5. Measurements of hepatic biochemical parameters

Activities of superoxide dismutase (SOD), GPx, CAT and GSH levels were measured by colorimetric assays. The activity of hepatic iNOS was measured by an ELISA kit (CUSABIOS, Wuhan, China). NO

production was measured by an NO colorimetric assay kit. Hepatic HO-1 protein expression was measured by ELISA. All assay procedures were progressed according to the manufacturer's instructions.

2.6. Measurement of TLR4 protein expression

The hepatic protein expression of TLR4 was measured by ELISA. In brief, liver tissues were stored overnight at -20°C , followed by washing with phosphate-buffered saline (PBS) and homogenization. Homogenates were centrifuged for 5 min at $5,000g$ at $2-8^{\circ}\text{C}$. The supernatant was collected and assayed immediately. All other assay procedures were processed in accordance with the manufacturer's instructions.

For immunohistochemistry assay, liver samples were perfused with 10% formaldehyde solution. After perfusion, liver samples were collected and immersed for 2 wk at room temperature. After immersion 2 wk, liver samples were embedded in liquid paraffin and cut into $5\text{-}\mu\text{m}$ -thick sections. The TLR4 antibody (1:100) was used for staining of the expression of TLR4 with the Dako Real EnVision Detection System Rabbit/Mouse (1:200).

Stained tissues were observed with a Leica DMR 6000 microscope and images were photographed with a Leica DM 480 camera (Wetzlar, Hesse, Germany) at 40×10 magnifications.

2.7. Hematoxylin and eosin staining

Liver samples for hematoxylin and eosin (H&E) staining were perfused with 10% formalin solution and harvested in same solution. Two wk later, the liver tissues were embedded in liquid paraffin, cut into $4\text{-}\mu\text{m}$ -thick sections, and stained with H&E. For pathological observation under a light microscope, 20–30 views were randomly selected from each side at a magnification of $200\times$ and different optical fields were measured.

2.8. Statistical analysis

Data are expressed as the mean \pm standard error of the mean. Significant differences between means were analyzed by a Student *t* test or Tukey's multiple comparisons after analysis of variance using SPSS Statistics 24 (IBM, Armonk, NY, USA). Differences were considered to be significant at $p < 0.05$.

3. Results

3.1. Phytochemistry of UGBE

Table 1 shows the composition ratio of GBE and UGBE by the HPLC analysis [23]. Ginsenosides Rh1, Rh4, Rg2, Rg3, Rk1, Rk3, and F4 were rapidly increased after ultrasonication process. Among these ginsenosides, the composition ratio of ginsenosides Rg2, Rh1, and F4 was largely increased, which are produced by the process of manufacture of red ginseng [24]. Furthermore, ginsenosides Rg3, Rh4, and Rk1 were newly produced by ultrasonication process, which were not identified in GBE. HPLC chromatogram data also proved these changes (Figs. 1A and 1B). While other ginsenosides were increased by ultrasonication process, ginsenoside Re was significantly decreased in UGBE.

3.2. Body weight gain, food intake, water consumption, and survival rate

There was no significant difference in body weight gain, food intake, or water consumption between the different treatment groups before G/L challenge (data not shown). However, as shown

Table 1
Composition ration of GBE and UGBE

Ginsenoside	GBE	UGBE
Rb1	0.758 \pm 0.179	0.072 \pm 0.052 [#]
Rb2	0.594 \pm 0.114	0 [#]
Rd	1.534 \pm 0.182	0.025 \pm 0.007 [#]
Re	11.169 \pm 0.158	0.288 \pm 0.037 [#]
Rf	0.330 \pm 0.115	0 [#]
Rg1	0.567 \pm 0.013	0.033 \pm 0.004 [#]
Rg2	0.801 \pm 0.215	2.278 \pm 0.368 [*]
20S-Rg3	0	0.432 \pm 0.063 [*]
20R-Rg3	0	0.400 \pm 0.059 [*]
Rg6	0.044 \pm 0.026	0.445 \pm 0.063 [*]
Rh1	0.629 \pm 0.095	1.350 \pm 0.208 [*]
Rh4	0	0.083 \pm 0.011 [*]
Rk1	0	0.2071 \pm 0.030 [*]
Rk3	0	0.039 \pm 0.005 [*]
F1	0.193 \pm 0.149	0.035 \pm 0.017 [#]
F4	0.191 \pm 0.026	1.210 \pm 0.137 [#]

Data represent mean \pm SEM

GBE, *P. ginseng* berry extract; SEM, standard error of the mean; UGBE, ultrasonication-processed *P. ginseng* berry extract

* $p < 0.05$ increased composition ratio of UGBE compared with the same ginsenoside in GBE

$p < 0.05$ decreased composition ratio of UGBE compared with the same ginsenosides in GBE [23]

in Fig. 2, the survival rate of the liver-damaged group was significantly decreased. Ten d following G/L treatment, only 22.2% of the damaged rats had survived. On the contrary, the UGBE-treated (250 mg/kg and 500 mg/kg) group showed a huge increase in survival rate (77.8% and 66.7%, respectively). This increase was higher than that in the silymarin-treated (150 mg/kg) group (55.6%). With respect to the GBE- (250 mg/kg) and UGBE-treated (100 mg/kg) groups, only 33.3% and 44.4% of the nine rats, respectively, survived.

3.3. Serum ALT, AST, and T. bilirubin levels

Serum ALT, AST, and T. bilirubin levels are shown in Table 2. In the G/L group, serum ALT, AST, and T. bilirubin levels were significantly increased compared with those in the control group, and were reduced by the treatment with 250 mg/kg and 500 mg/kg UGBE and 150 mg/kg silymarin. The value for 250 mg/kg UGBE was similar to that for 150 mg/kg silymarin. Therefore, it can be concluded that UGBE ameliorated liver function and attenuated the liver damage induced by G/L treatment.

3.4. Hepatic SOD, GPx, and CAT activities, and GSH level

Hepatic SOD, GPx, and CAT activities, and GSH level were measured in order to evaluate the antioxidant capacity of the liver (Table 3). Treatment with UGBE (100 mg/kg, 250 mg/kg, and 500 mg/kg) or silymarin (150 mg/kg) significantly recovered the antioxidant capacity of the liver damaged by G/L. However, GBE (250 mg/kg) did not recover the decreased antioxidant capacity of the liver.

3.5. Hepatic iNOS activity and NO levels

Figs. 3A and 3B show the hepatic iNOS activity and NO levels in the different treatment groups. The iNOS activity and NO levels in the liver were significantly increased after G/L treatment. Treatment with silymarin or UGBE significantly decreased iNOS activity and NO levels to such an extent that UGBE protected oxidative stress from NO production. However, treatment with GBE did not affect the iNOS activity or NO level in the liver.

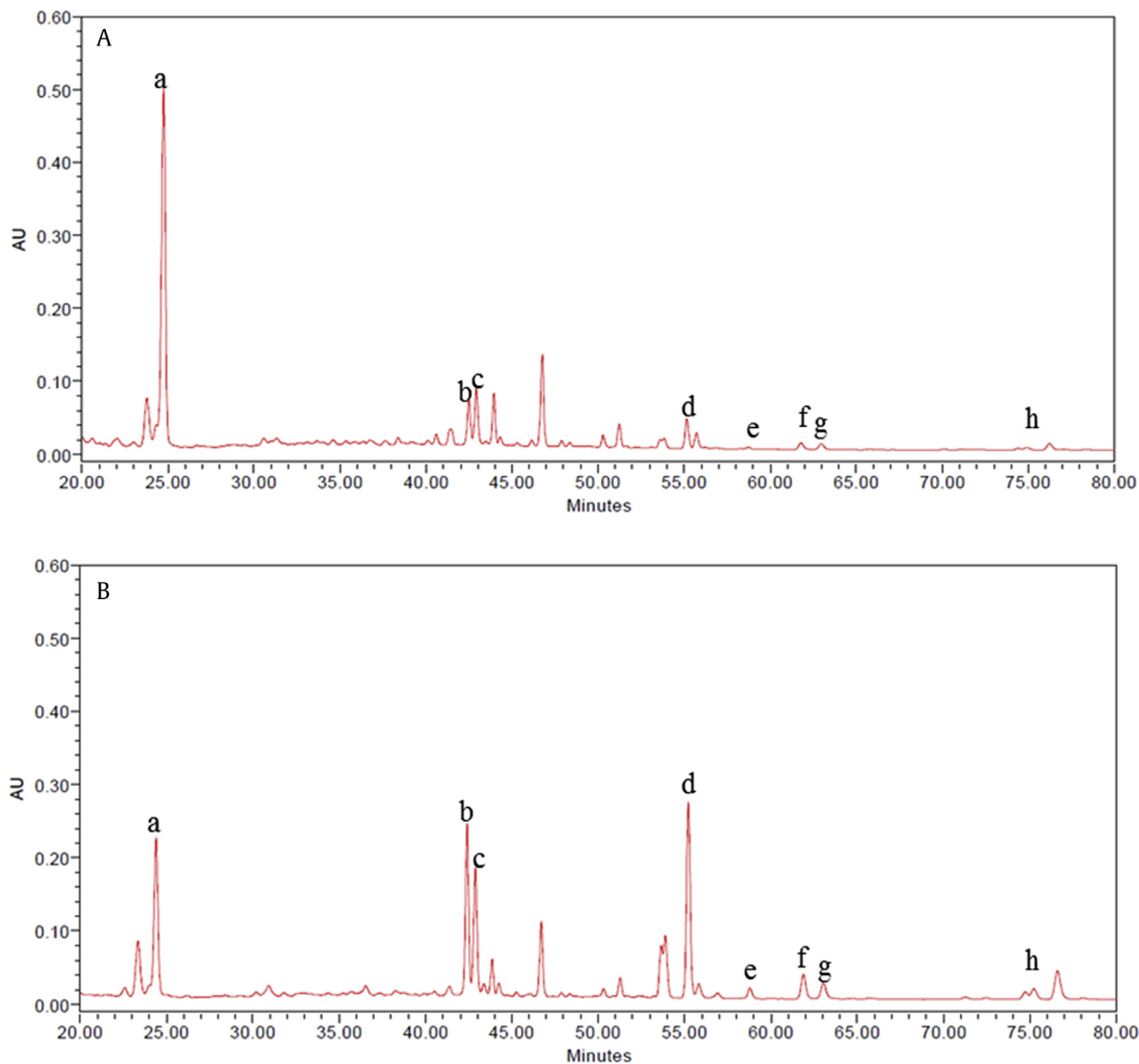


Fig. 1. HPLC chromatogram of ginsenosides detected from the ginseng berry extracts processed with ultrasonication. Separation of sample was performed on an analytical column by gradient elution at room temperature. Chromatograms of (A) GBE and (B) UGBE were obtained using a UV/VIS Waters 2478 Dual λ Absorbance Detector at 203 nm. The lowercase letters represent the following: a, ginsenoside Re; b, ginsenoside Rg2; c, ginsenoside Rh1; d, ginsenoside F4; e, ginsenoside Rk3; f, ginsenoside Rh4; g, ginsenoside Rg; and h, ginsenoside Rk1. AU, absorbance units; HPLC, high-performance liquid chromatography; GBE, *P. ginseng* berry extract; UGBE, ultrasonication-processed *P. ginseng* berry extract.

3.6. Protein expression of serum TNF- α and hepatic HO-1

As shown in Fig. 3C, G/L challenge caused a great increase in serum TNF- α protein expression compared with that of the control group, which was reduced with silymarin or UGBE treatment. The suppression of TNF- α caused by the treatment with 250 mg/kg UGBE was similar to that of 500 mg/kg UGBE, which were more potent than 150 mg/kg silymarin. UGBE also increased hepatic HO-1 protein expression in a dose-dependent manner (Fig. 3D). However, there was no significant increase in HO-1 protein expression, with only slight upregulation in the silymarin-treated group. These results suggest that UGBE effectively prevents the inflammatory response to G/L treatment and enhances HO-1 protein expression, contributing to the antioxidant capacity of the liver.

3.7. Protein expression of TLR4

A G/L challenge caused a great increase in TLR4 protein expression compared with that of the control group, which was reduced with silymarin or UGBE treatment (Fig. 4A). Suppression of TLR4 caused by the treatment with 250 mg/kg UGBE was similar to that of 500 mg/kg UGBE, which were more potent than 150 mg/kg silymarin. Fig. 4B shows the representative immunohistochemistry result of TLR4 in each rat.

3.8. Histopathological study

Fig. 5 shows the H&E staining result in the different treatment groups. Compared with the control group, G/L challenge group

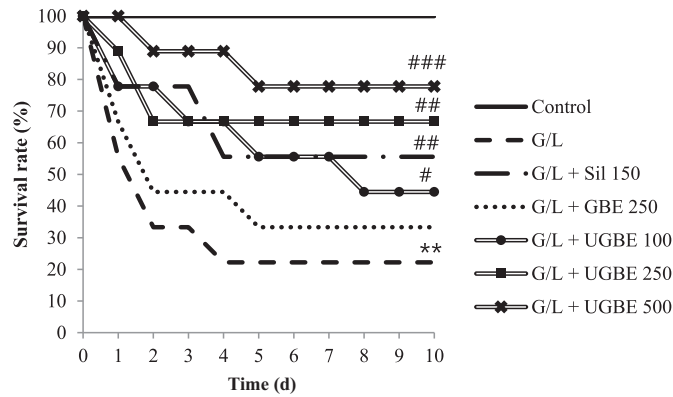


Fig. 2. Survival rate of rats in different treatment groups. Serum samples were collected from different groups. The sample number in each group is 0 ($n = 9$). ** $p < 0.01$ compared with control. # $p < 0.05$ versus G/L. ## $p < 0.01$ versus G/L. ### $p < 0.005$ versus G/L. GBE, *P. ginseng* berry extract; G/L, D-galactosamine/lipopolysaccharide; Sil, silymarin; UGBE, ultrasonication-processed *P. ginseng* berry extract.

Table 2
TaSerum AST, ALT, and T. bilirubin levels in different treatment groups

	AST (IU/L)	ALT (IU/L)	T. Bilirubin (mg/dL)
Control	92.43 ± 10.94	39.29 ± 7.11	0.14 ± 0.04
G/L	3425.917 ± 766.71***	2590.3 ± 591.63***	1.08 ± 0.3*
G/L + Sil 150	1452.1 ± 236.56##	697.6 ± 196.94###	0.47 ± 0.1##
G/L + GBE 250	2478 ± 508.65	1872.6 ± 205.36	0.99 ± 0.1
G/L + UGBE 100	2468.08 ± 912.39#	1907.33 ± 170.45	0.74 ± 0.14#
G/L + UGBE 250	1706.5 ± 306.76##	616.17 ± 237.13##	0.54 ± 0.12###
G/L + UGBE 500	303.57 ± 178.15###	203.14 ± 122.79###	0.30 ± 0.09###

Data represent mean ± SEM

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GBE, *P. ginseng* berry extract; G/L, D-galactosamine/lipopolysaccharide; SEM, standard error of the mean; T. bilirubin, total bilirubin; UGBE, ultrasonication-processed *P. ginseng* berry extract

* $p < 0.05$, compared with control

*** $p < 0.005$ compared with control

$p < 0.05$ compared with G/L

$p < 0.01$ compared with G/L

$p < 0.001$ compared with G/L

showed severe abnormal changes in the hepatic lobules. In addition, inflammatory cell infiltration, centritubular necrosis, and inflammatory foci were frequently detected (black arrows in Fig. 5B). These histological changes reduced in the silymarin- or UGBE-treated groups. Especially, UGBE 500 mg/kg-treated group showed normal appearance in liver morphology so that the

Table 3
Hepatic SOD, GPx, and CAT activities and GSH levels in different treatment groups

	SOD (U/mg)	GPx (U/mg)	CAT (U/mg)	GSH (ng/mg)
Control	83.20 ± 5.99	25.59 ± 5.38	24.81 ± 9.48	10.41 ± 2.84
G/L	15.26 ± 1.92***	10.19 ± 2.28**	7.85 ± 3.37***	3.65 ± 1.54***
G/L + Sil 150	52.72 ± 11.55##	17.73 ± 0.7###	17.02 ± 3.89#	6.44 ± 1.74#
G/L + GBE 250	26.62 ± 9.11	10.94 ± 3.77	6.78 ± 1.27	3.45 ± 1.23
G/L + UGBE 100	37.87 ± 3.63##	15.87 ± 0.45##	15.77 ± 3.92#	6.51 ± 2.33#
G/L + UGBE 250	54.62 ± 3.51##	19.66 ± 0.4###	18.70 ± 3.83##	9.41 ± 5.86#
G/L + UGBE 500	71.70 ± 2.08###	22.05 ± 1.92###	23.45 ± 3.55###	9.47 ± 2###

Data represent mean ± SEM

CAT, catalase; GBE, *P. ginseng* berry extract; G/L, D-galactosamine/lipopolysaccharide; GPx, glutathione peroxidase; GSH, glutathione; Sil, silymarin; SEM, standard error of the mean; SOD, superoxide dismutase; UGBE, ultrasonication-processed *P. ginseng* berry extract

* $p < 0.05$ compared with control

*** $p < 0.005$ compared with control

$p < 0.05$ compared with G/L

$p < 0.01$ compared with G/L

$p < 0.001$ compared with G/L

appearance of histopathology is similar to that of the control group. This grading of histological lesions in the liver confirmed that UGBE significantly reduced hepatic injury induced by G/L challenge.

4. Discussion

Owing to its various physiological effects, *P. ginseng* gained significant interest for use in a variety of medicinal applications [25]. Several studies have shown the effect of ginseng berry on diabetes and obesity, which is attributed to ginsenoside Re [20]. However, other protective effects of ginsenosides on liver failure have yet to be unveiled. In the present study, we aimed to produce a novel GBE containing a high concentration of active ginsenosides and to investigate the effect of UGBE on ALF.

We examined the composition ratio of UGBE by HPLC. Among several trials with time variations, we selected 10 h ultrasonication processing for the highest content of ginsenosides Rg2, Rh1, and F4, and total saponin (data not shown). After 10 h of ultrasonication processing, the composition ratio of ginsenosides Rg2, Rg3, Rh1, Rh4, Rk1, Rk3, and F4 was markedly increased, while ginsenoside Re was decreased (Table 1). Formation of ginsenosides in UGBE has been reported to be related to enzymatic production, fermentation, or bacterial metabolisms. In the present study, ultrasonication processing in a specific condition facilitated this kind of reactions instead of physiological function. However, the other transformation mechanisms involved in ginsenoside Rg3, Rk1, and F4 still remain to be unveiled and should be researched further. Ginsenosides Rg2, Rg3, Rh1, Rk1, and F4 are most common ginsenosides in red ginseng, which are known to have a protective effect against liver injuries [24,26,27]. With the newly developed ultrasonication processing, we may have produced more effective ginsenosides than with typical red ginseng manufacturing. Ginsenoside Rk1 has been reported to have an anticancer effect against hepatocellular carcinoma cells [28,29]. Ginsenoside Rg3 is known to have antioxidative and hepatoprotective effects by the HO-1 enzyme activity [30]. G Rh1 has shown an anti-inflammatory function via inhibition of iNOS and cyclooxygenase-2 function [31]. Ginsenoside F4 has an apoptotic effect, which contributes to the protection of damaged organs [32].

In the present study, we significantly increased the levels of the representative indices of liver injury (ALT, AST, and T. bilirubin), oxidative stress (SOD, GPx, GSH, CAT, iNOS, and HO-1), and inflammation (TNF- α) by G/L injection, for building an ALF rat model. However, administration of UGBE recovered the liver function and increased survival time, dose dependently. H&E staining results also supports these results (Fig. 5). The combined administration of D-GalN and LPS caused liver failure and rapid

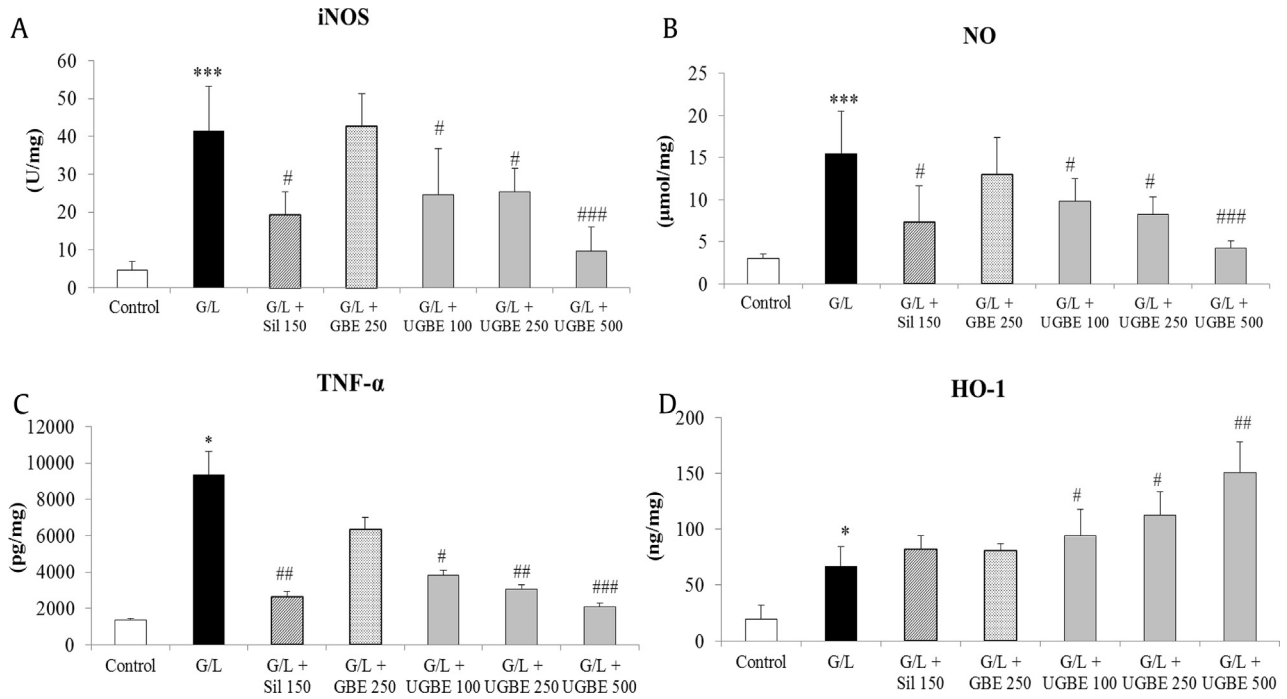


Fig. 3. Activities of iNOS, NO levels, TNF- α levels, and HO-1 expression. (A) Activity of iNOS in the livers. (B) NO levels in the livers. (C) Serum TNF- α protein expression. (D) Hepatic HO-1 protein expression. Liver samples were collected from different groups. The sample number in each group is 9 ($n = 9$). Data represent mean \pm SEM. * $p < 0.05$ compared with control. *** $p < 0.005$ compared with control. # $p < 0.05$ compared with G/L. ## $p < 0.01$ compared with G/L. ### $p < 0.001$ compared with G/L. GBE, *P. ginseng* berry extract; G/L, D-galactosamine/lipopolysaccharide; HO-1, hemoxygenase-1; iNOS, inducible nitric oxide synthase; NO, nitric oxide; SEM, standard error of the mean; Sil, silymarin; TNF- α , tumor necrosis factor alpha; UGBE, ultrasonication-processed *P. ginseng* berry extract.

death within 1–2 d [5]. In the G/L-treated group, more than 60% of rats died within 2 d. Oral administration of UGBE significantly increased the survival rate. These survival effects at a higher concentration of UGBE are greater than that in the silymarin-treated group. Meanwhile, GBE did not affect the survival rate of the G/L-treated group. The D-GalN-induced ALF model is characterized by increased serum levels of ALT and AST [33]. In our experiment, serum ALT, AST, and T. bilirubin levels were reduced in the high-concentration-UGBE-treated group (Table 2). The differences between GBE and UGBE on liver protection may be due to the

difference in composition ratio of ginsenosides, especially ginsenosides Rk1, Rg2, Rg3, Rh1, and F4.

The enzyme-dependent antioxidant system plays a key role in the ALF condition [34–37]. The treatment of G/L caused an imbalance of reactive oxygen species (ROS) production and a reduction of the antioxidant capacity of the liver [38]. Overproduction of ROS impaired antioxidant enzymes such as SOD, CAT, and GPx in the ALF condition in rats [39–42]. TNF- α is an important proinflammatory cytokine that triggers liver damage in the G/L-induced ALF mode [43,44]. The findings of the present

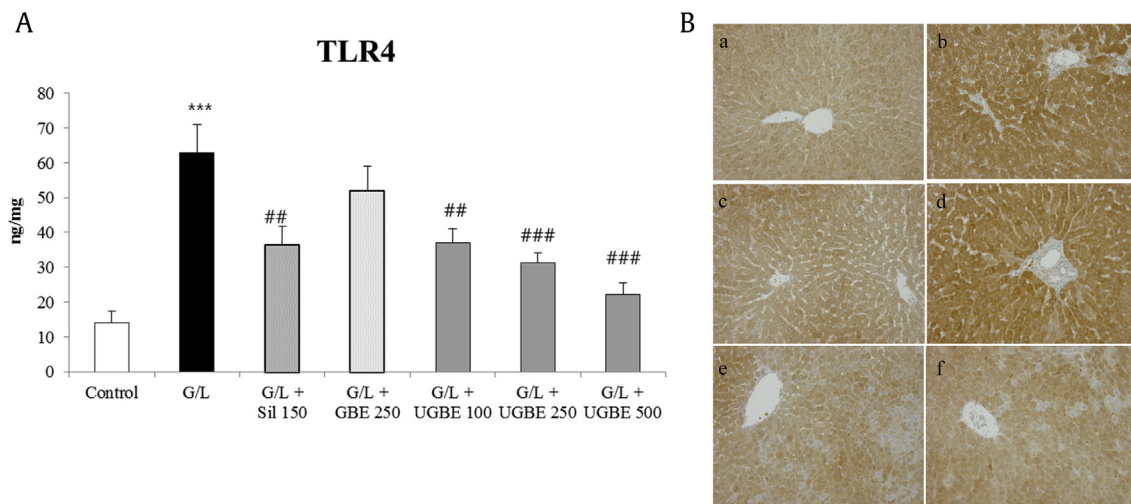


Fig. 4. Effect of UGBE on TLR4 expression in G/L-challenged liver injury model. (A) Protein expression of TLR4 in the liver. (B) Photomicrographs of TLR4 immunohistochemistry assay: a, control group; b, G/L group; c, G/L + silymarin (150 mg/kg) group; d, G/L + GBE (200 mg/kg) group; e, G/L + UGBE (250 mg/kg) group; and f, G/L + UGBE (500 mg/kg) group. The sample number in each group is 9 ($n = 9$). Data represent mean \pm SEM. *** $p < 0.005$ compared with control. ## $p < 0.01$ compared with G/L. ### $p < 0.005$ compared with G/L. GBE, *P. ginseng* berry extract; G/L, D-galactosamine/lipopolysaccharide; SEM, standard error of the mean; Sil, silymarin; TLR4, toll-like receptor 4; UGBE, ultrasonication-processed *P. ginseng* berry extract.

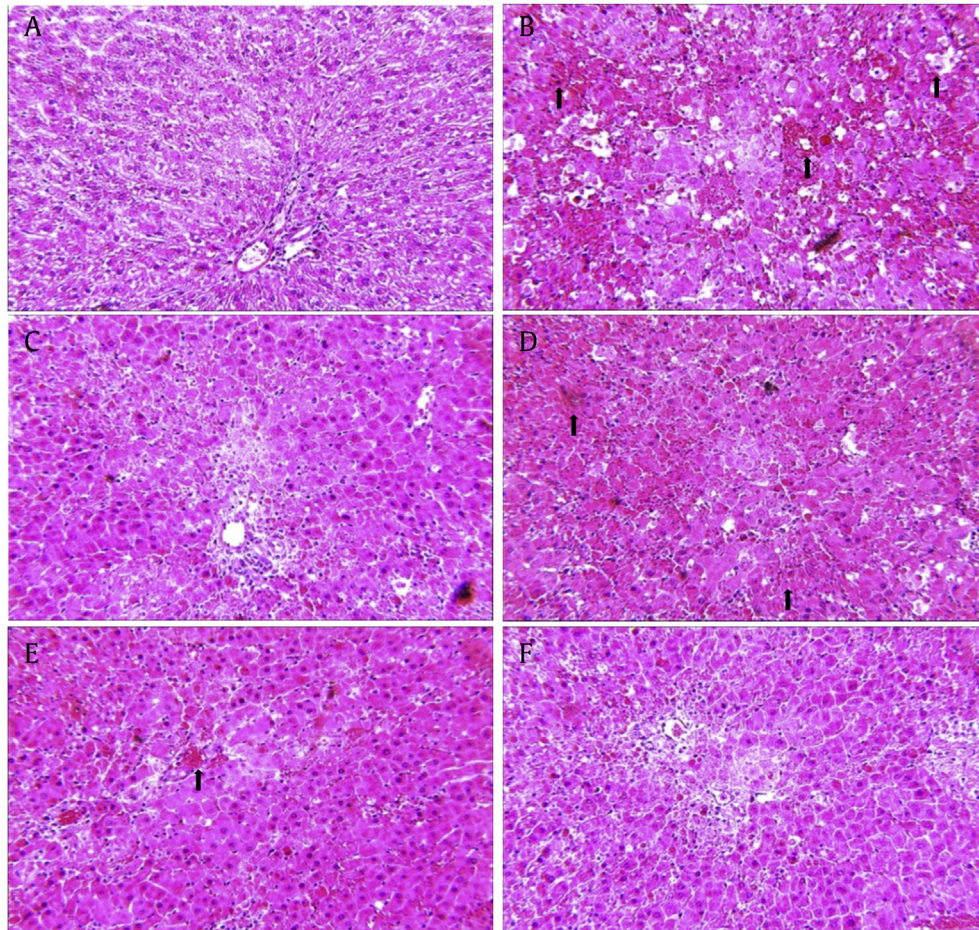


Fig. 5. Effect of UGBE on liver histopathological changes. Representative hematoxylin and eosin-stained high magnification (original magnification 200 \times) of liver tissue from different groups. (A) Control group. (B) G/L group. (C) G/L + silymarin (150 mg/kg) group. (D) G/L + GBE (200 mg/kg) group. (E) G/L + UGBE (250 mg/kg) group. (F) G/L + UGBE (500 mg/kg) group. GBE, *P. ginseng* berry extract; G/L, D-galactosamine/lipopolysaccharide; UGBE, ultrasonication-processed *P. ginseng* berry extract.

study revealed that administration of UGBE protects antioxidative enzymes from oxidative damage (Table 3). Hepatic TNF- α levels were also decreased by UGBE treatment in a dose-dependent manner. Although it has not been investigated that the oral administration of UGBE directly scavenges ROS, there is a strong likelihood that UGBE increased the capacity of antioxidant, and ameliorated hepatotoxicity and oxidative stress induced by G/L challenge.

HO-1 plays a key role in cytoprotection by inhibiting the leukocytic response and ameliorating hepatic microvascular perfusion from LPS-induced oxidative damage [45]. Bilirubin is one of the key mediators of the HO-1-mediated cytoprotection process for intracellular homeostatic balance and inflammation against oxidative stress [46]. In accordance with many other findings, an increased HO-1 expression in the G/L-treated group was significantly increased. Furthermore, the protein expression of HO-1 increased dose dependently according to UGBE treatment (Fig. 3D). This result indicates that UGBE enhanced the hepatoprotective effect by upregulating the protein expression of HO-1 and is likely mediated by ginsenoside Rg3 [30]. However, the role of iNOS in liver injury still remains controversial [47]. NO, which is produced by iNOS, rapidly reacts with ROS to form a peroxynitrite (ONOOK) that is very harmful to the liver [48]. However, during this process, NO acts as a scavenger of ROS [49]. In this study, UGBE ameliorated G/L-induced hepatotoxicity in view of the

former action. It is assumed that this action was due to ginsenoside Rh1 [31].

LPS, a main ligand of TLR4 which is related in innate immune reaction, is thought to play a key role in the progression and pathogenesis of ALF [50,51]. Impaired gut barrier integrity, by ethanol and its metabolite, induces TLR4-mediated hepatic inflammation and damage by an increase of blood LPS concentration and arrival of LPS to the liver by portal blood [52]. In our findings, the protein expression of TLR4 was upregulated by G/L treatment. However, the treatment of UGBE attenuated TLR4 protein expression in a dose-dependent manner (Fig. 4). Although the exact correlation between effective ginsenosides and the expression of TLR4 is still not clear, UGBE is supposed to have a hepatoprotective effect through TLR4.

Based on these results, it can be summarized that G/L-induced ALF condition was alleviated by oral administration of UGBE. Oral administration of UGBE lengthened the survival time of G/L-treated rats and reduced serum ALT, AST, and T. bilirubin levels. The antioxidant capacity also increased in the UGBE-treated group compared with the damaged liver group. This hepatoprotective effect may be mediated by an increase in HO-1 protein expression and suppression of iNOS levels. These protective effects are related to the inhibition of TLR4. Consequently, these findings strongly support the possibility of *P. ginseng* berry being developed as a preventative remedy for liver injury or as a functional food.

Conflicts of interest

All authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jgr.2017.07.007>.

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