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Toxicity of red *Liriope platyphylla* manufactured by steaming process on liver and kidney organs of ICR mice

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Red *Liriope platyphylla* (RLP) produced by steaming process has been reported to enhance the secretion of insulin and nerve growth factor (NGF). However, there has been no report on the toxicity of RLP in the specific organs of mice. To investigate the toxic effect of RLP, we tried to observe a significant alteration on body weight, food/water intake, organ weight, liver pathology and kidney pathology in female ICR mice received 12.5, 25.0 and 50.0 mg/kg body weight/day of RLP via gavage for 10 days. Out of seven organs including brain, heart, lung, liver, kidney, spleen and ovary, two organs (heart and lung) showed significantly decreased weights in the medium dosage RLP-treated group, whereas weights of other organs were maintained at constant levels in all dosage groups. In the liver toxicity analysis, no significant increase of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate amino-transferase (AST) were detected in any RLP-treated group compared to vehicle-treated group. The specific pathological changes induced by most of toxic compounds were not observed in the liver in microscopic examination. Furthermore, in the kidney toxicological analysis, a significant enhancement of the blood urea nitrogen (BUN) concentration was detected in the high dosage RLP-treated group compared to the vehicle-treated group. However, the serum creatinine (CA) concentration on the serum biochemistry as well as the pathological changes in microscopic examination were not significantly different between the vehicle- and RLP-treated groups. Therefore, these results suggest that RLP does not induce any specific toxicity in liver or kidney tissues of mice, although the BUN level slightly increased in 50.0 mg/kg of RLP-treated group.

Key words: Red *Liriope platyphylla*, steaming process, toxic effect, liver, kidney

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Liriope platyphylla (LP) used as the base material of RLP have been widely used a herbal medicine for the treatment of human chronic diseases such as asthma, diabetes, dementia, and atopic dermatitis in Korea, Japan, and China [1,2]. Especially, it effectively stimulates insulin secretion as well as the insulin receptor signaling pathway in some cell lines and animal models [3-6]. Also, the differentiation of naive PC12 cells as well as improvement of learning and memory in mice were induced LP through upregulation of NGF secretion [7-10]. Furthermore, it relieved symptoms of atopic dermatitis in NC/Nga mice after phthalic anhydride (PA) treatment [11].

Moreover, several studies in recent year were attempted to enhance the therapeutic effects of LP on diabetes and neurodegenerative disorders, because LP have a therapeutic effects on the various chronic disease. In an effort to try, RLP was recently manufactured from LP by steaming

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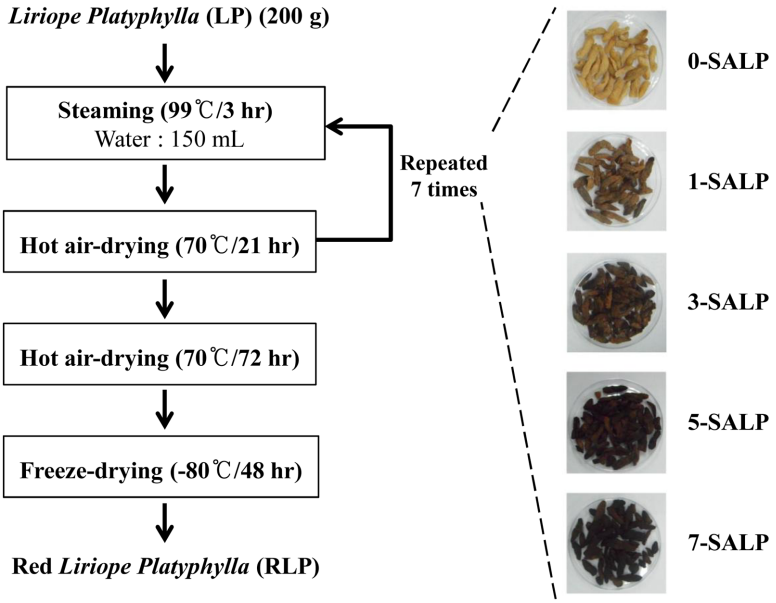

Figure 1. Manufacturing process of RLP. (A) Schematic process for RLP manufacture comprising two repeating steps (3 hr of steaming and 24 hr of air-drying) carried out a total of seven times. (B) Alteration of RLP color in each repeated step.

process [3], which is a traditional method for reducing toxicity and minimizing side effects associated with chemical, pharmacological or bioactive changes [12-15]. RLP greatly stimulated the insulin secretion from INS cells, although no significant alteration of cell viability was detected upon LP treatment. Additionally, the expression and phosphorylation levels of most components of the insulin receptor signaling pathway significantly increased upon RLP treatment, whereas a similar expression pattern of glucose transporter-2 was observed. Furthermore, RLP treatment induced the down-regulation of glucose concentration as well as the up-regulation of insulin concentration in a streptozotocin-induced diabetic model [3]. Despite the increase of attention and publication for therapeutic effects of RLP, its toxicity on the specific target organs of mice have not investigated until now.

Therefore, in this study, we investigated RLP toxicity in liver and kidney organs of ICR mice following short-term treatment. The results show that there is a scientific basis for determining the optimal concentration of RLP as a therapeutic drug for the treatment of human chronic diseases.

Materials and Methods

Preparation of sample

Roots of LP were collected from plantations in the Miryang area (Korea) and dried in a hot-air drying machine (JSR, Seoul, Korea) at 60ºC. To prepare extracts of RLP at seven different steaming frequencies (7-SALP), a specific process comprising two steps (200 g of dry roots was steamed at 99ºC for 3 hr followed by air-drying at 70ºC for 24 hr) was carried out for different numbers of repetitions a total of seven times (Figure 1). The steamed roots (RLP) were reduced to powder using an electric blender. Then, aqueous extracts of RLP were purified for 2 hr at 100ºC using circulating extraction equipment (IKA Labortechnik, Staufen, Germany) after adding 200 mL of distilled water. In addition, a solution of RLP extracts was concentrated to dry pellets in a rotary evaporator (EYELA, Tokyo, Japan) and stored at -80ºC until needed. Analyses for the general compounds in LP and RLP were performed as previously described [11,16].

HPLC analysis of LP and RLP

Composition of LP and RLP were analyzed using the iLC 3000 HPLC system (Interface Engineering, Seoul, Korea) equipped with a Corona® CAD® Detector (ESA Bioscinece, Inc., Chelmsford, MA, USA). Chromatographic separation was performed on a CAPCELL PAK MG C18 (4.6 mm×250 mm, particle size 5 μm, Shiseido Co. Ltd., Tokyo, Japan). The mobile phase consisted of solvent A (deionized water) and solvent B (acetonitrile) using the following gradient elution program: 0-25 min, 30-90% of solvent B and 25-40 min, 90% of solvent B.

A flow rate of 1.0 mL/min was applied for the sample analysis, and the nebulizer gas was compressed nitrogen. The gas flow rate and gas pressure were maintained at 1.53 L/min and 35±2 psi, respectively. The output signal of the detector was recorded using Clarity™ chromatography software (DataApex, Prague, The Czech Republic).

Care and use of animals and experimental design

The animal protocol used in this study was reviewed and approved based on the ethical and scientific care procedures of the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC; Approval Number PNU-2011-000220). Female ICR mice were purchased from Samtako (Osan, Korea) and handled at the Pusan National University Laboratory Animal Resources Center according to National Institutes of Health guidelines. All mice were given a standard irradiated chow diet (Purina Mills Inc., Seoungnam, Korea) *ad libitum* and maintained in a specific pathogen-free state under a strict light cycle (12 hr light-dark cycle) at a temperature of 22±2ºC and at 50±10% relative humidity.

Eight-week-old female ICR mice (n=24) were assigned to one of the following four groups: vehicle-treated group (n=6), low dosage RLP-treated group (n=6), medium dosage RLP-treated group (n=6), and high dosage RLP-treated group (n=6). As a control, one group of ICR mice received a comparable volume of daily water via gavage (vehicle-treated group), whereas the others received 12.5 mg/kg body weight/day of RLP (low dosage RLP-treated group), 25.0 mg/kg body weight/day of RLP (medium dosage RLP-treated group), and 50.0 mg/kg body weight/day of RLP (high dosage RLP-treated group) via gavage. After RLP treatment for 10 days, all animals were immediately sacrificed using CO_2 gas, followed by preparation of blood and tissue samples. The dose and duration of RLP administration were referred to the previous results that RLP was significantly stimulated the insulin secretion and glucose transporter expression in ICR mice treated with 50 mg/kg for 10 days [3].

Measurement of body weight, organ weights, food intake and water intake

Clinical signs as well as the number of animals that died were recorded more than twice a day for 10 days. Also, alterations of the body weight were observed with an electronic balance (Mettler Toledo, Greifensee, Switzerland) at every day according to KFDA guideline. And the weight of seven organs collected from the sacrificed mice were also measured the same method used to detect the body weight. Furthermore, the food intake and water consumption of mice were daily measured using electronic balance and a measuring cylinder.

Serum biochemical analysis

After final administration of RLP, all ICR mice were fasted for 8 hr, after which blood was collected from abdominal veins of mice and incubated for 30 min at room temperature. Serum was then obtained by centrifugation of blood. Serum biochemical components, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), and creatinine (CA), were assayed using an Automatic Serum Analyzer (HITACHI 747, Tokyo, Japan). All assays were measured using fresh serum and conducted in duplicate.

Histopathology

Liver and kidney tissues collected from ICR mice were fixed with 10% formalin for 12 hr, embedded in paraffin wax, and sectioned into approximately 4 μm slices. Liver and kidney sections were then stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, USA), after which pathological changes were examined using Leica Application Suite (Leica Microsystems, Switzerland).

Statistical analysis

Tests for significance between the RLP- and vehicle-treated ICR mice were performed using a one-way analysis of variance (ANOVA) test of variance (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL). All values are reported as the mean±standard deviation (SD). A P value<0.05 was considered to be significant.

Results

Composition of LP and RLP

In order to analysis the difference of composition between LP and RLP, the general compounds and total phenolic contents were measured in LP and RLP extracts. As shown Figure 2A, overall distributions of the main components of both LP and RLP were shown

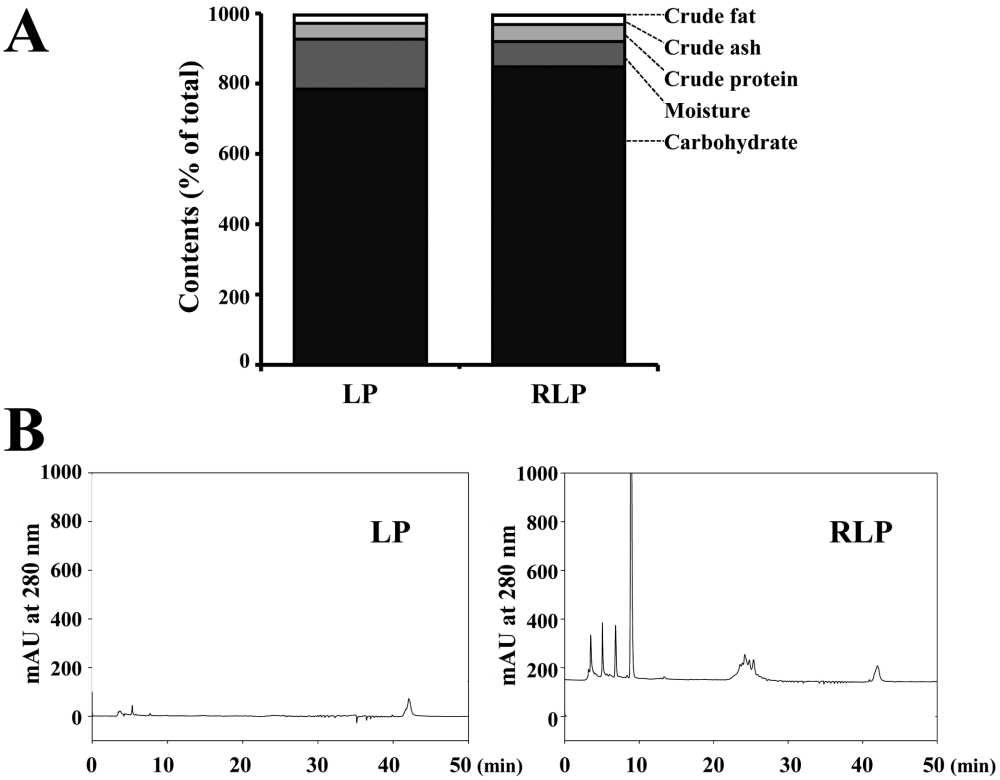

Figure 2. Composition of main components (A) and total phenolic compounds in LP and RLP (B).

to be similar despite some differences. In the case of LP, the most abundant component was carbohydrates, followed by moisture, crude protein, crude ash, and crude fat. However, RLP treatment increased the levels of carbohydrates, protein, crude ash, and crude fat were higher in RLP than LP, while moisture was lower in RLP than LP. Furthermore, total phenolic contents as shown by antioxidant activity significantly increased upon RLP (Figure 2B).

Effects of RLP on pathological symptoms in ICR mice

To evaluate the phenotypical toxicity of RLP, clinical signs on the mice phenotype and mortality were observed in ICR mice over 10 days. No significant pathological symptoms, including melancholy, hypokinesia, gait abnormality, and tremors, were detected in mice treated with RLP. Furthermore, dead mice were not observed in all RLP treatment group. These results show that RLP may not induce any pathological symptoms or mortality, and that the minimum lethal dose (MLD) is higher than 50 mg/kg of RLP.

Effects of RLP on body weight, food intake, and water intake of ICR mice

Changes in body and organ weights of mice were measured as an indicator of animal toxicity. Significant alteration of body weight for 10 days was not detected in any of RLP-treated groups compared to the vehicle-treated group (Figure 3A). Furthermore, food and water intake were maintained at the same levels as the vehicle-treated group (data not shown). Therefore, the above results suggest that RLP treatment dose not induced any significant increase on body weight, food intake, and water intake.

Effects of RLP on organ weights of ICR mice

To evaluate the effects of RLP on organ weights, the gross findings as well as the weights of seven organs (brain, heart, lung, liver, kidney, spleen, and ovary) were observed in vehicle-treated, low, medium, and high dosage RLP-treated groups (Data not shown). No pathological changes were observed in any of the organs in all treatment groups. For organ weights, only two

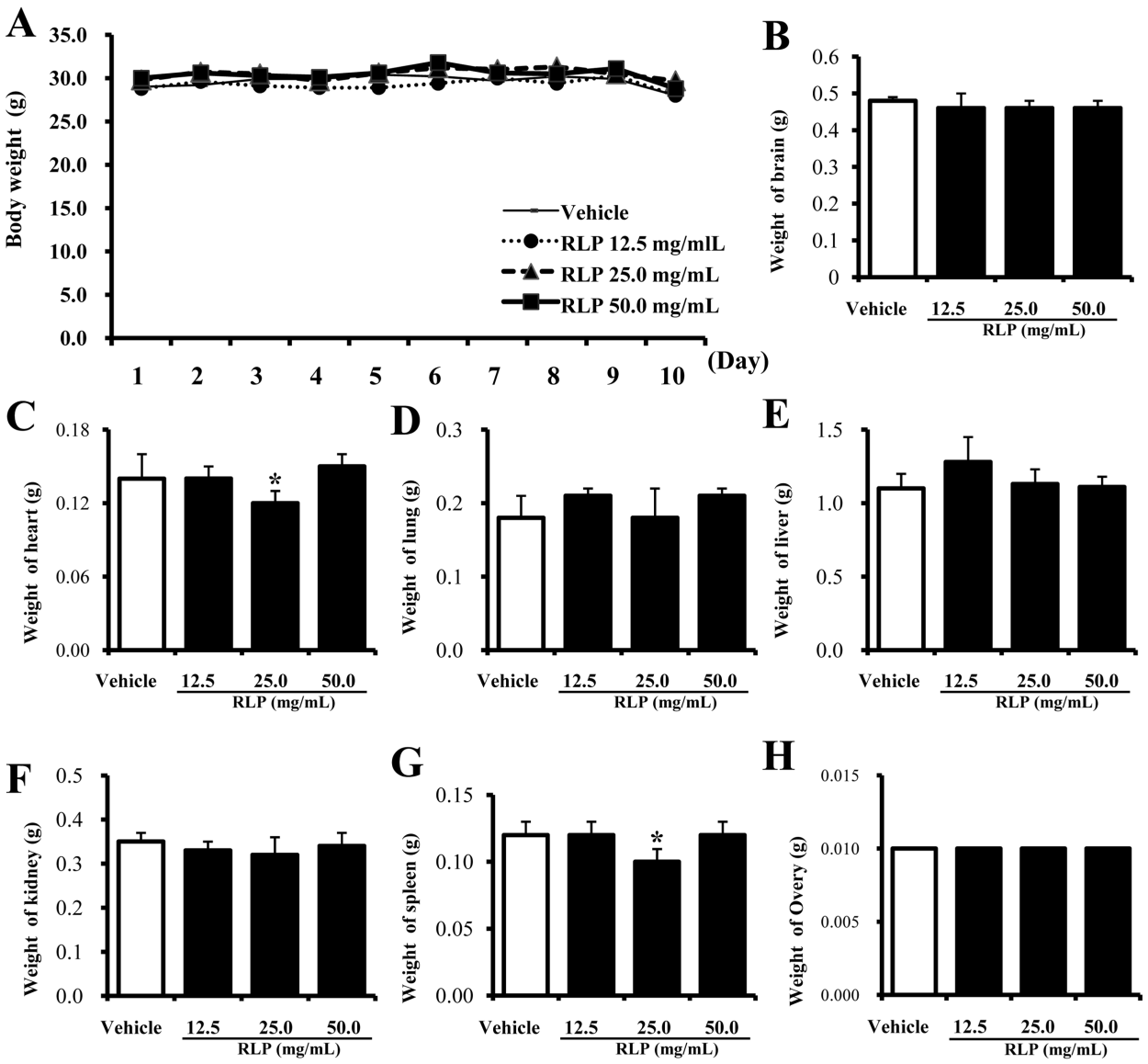

Figure 3. Effects of RLP on body and organ weights of ICR mice. At 24 hr after final RLP treatment, final body weight (A) and each organ weights (B-H) of ICR mice were measured daily using an electronic balance. Data represent the mean±SD from three replicates. **P*<0.05 is the significance level compared to vehicle-treated group.

organs of total weighted organs underwent specific alterations in RLP-treated groups. Specifically, heart and spleen weights significantly decreased in the medium dosage RLP-treated group compared with the vehicle-treated group, whereas they did not change in the other groups (Figure 3C and G). Furthermore, lung and liver weights slightly increased in some RLP-treated groups compared to the vehicle-treated group, although the changes were not significant (Figure 3D and E). However, brain, kidney, and ovary weights were maintained at constant levels as shown in Figure 3B, F, and H. These results suggest that RLP treatment does not induce any significant toxic effects on organ weights of ICR mice.

Effects of RLP on liver toxicity in ICR mice

To investigate RLP toxicity in liver organs of ICR mice, alteration of several enzymes related to liver metabolism was investigated in blood serum. There were no increase in the levels of four liver toxicity indicators, specifically ALP, AST, ALT, and LDH. The serum level of ALP did not undergo any significant alteration, although it slightly increased in the medium dosage RLP-treated group (Figure 4Aa). On the other hand, the levels of AST and ALT decreased in the medium dosage

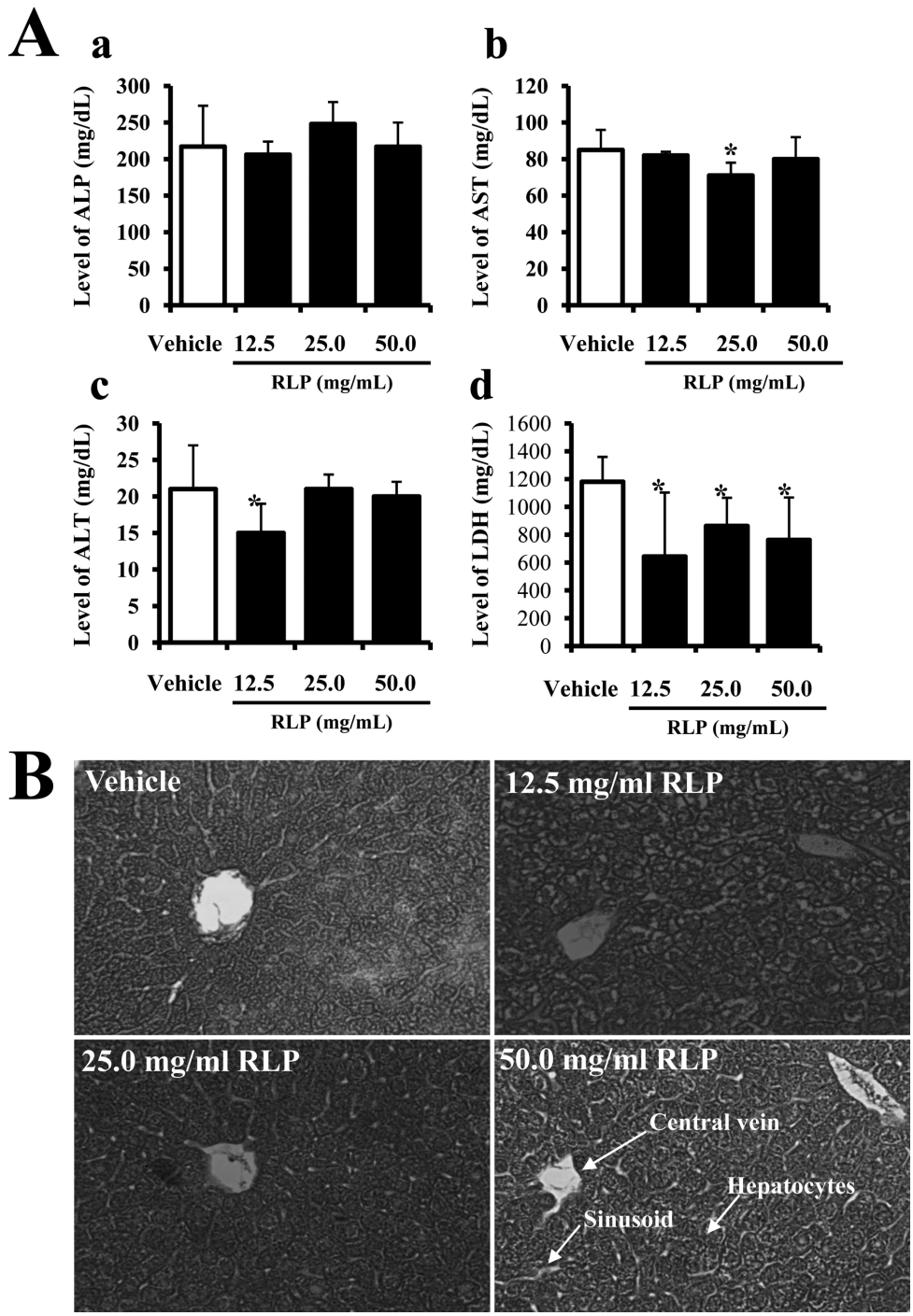

Figure 4. Effects of RLP on liver toxicity in ICR mice. Blood was collected from abdominal veins of vehicle- and RLP-treated mice. Serum concentrations of ALP (Aa), AST (Ab), ALT (Ac), and LDH (Ad) were analyzed in duplicate using a serum biochemical analyzer as described in Materials and Methods. (B) Liver tissue of ICR mice was prepared on a histological slide, and cellular morphology was viewed at 200x magnification. Data represent the mean±SD from three replicates. **P*<0.05 is the significance level compared to vehicle-treated group.

(Figure 4Ab) and low dosage RLP-treated groups (Figure 4Ac). Furthermore, the LDH level significantly decreased in mice of all treatment groups compared with the vehicle-treated group (Figure 4Ad). Meanwhile, to investigate histological alterations, the liver sections stained with H&E were observed with microscope. No significant pathological changes were detected in liver tissue of mice in all groups (Figure 4B). These results

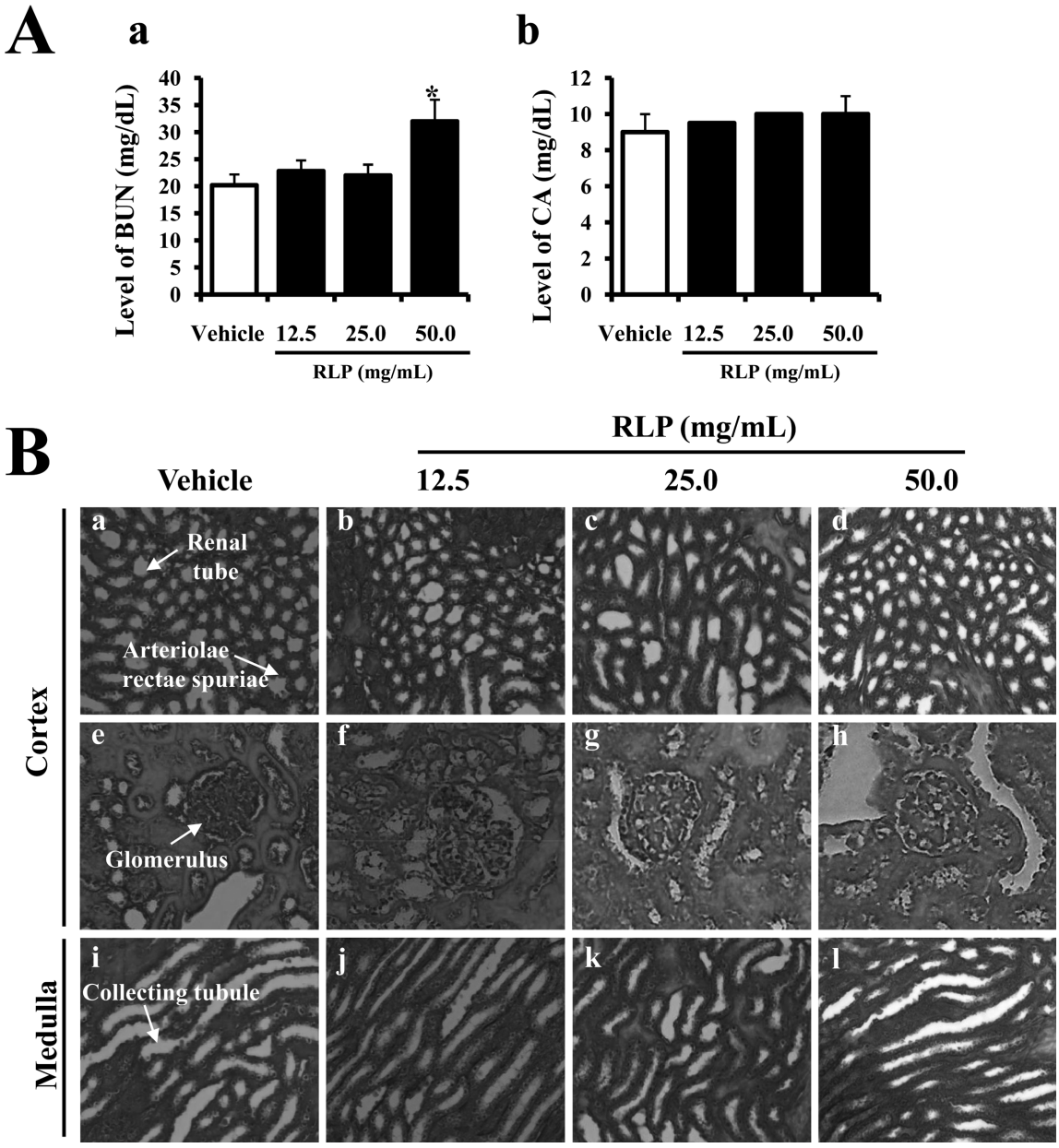

Figure 5. Effects of RLP on kidney toxicity in ICR mice. Blood was collected from abdominal veins of vehicle- and RLP-treated mice. Serum concentrations of BUN (Aa) and CA (Ab) were analyzed in duplicate using a serum biochemical analyzer as described in Materials and Methods. (B) Cortex (Ba-h) and medulla regions (Bi-l) of kidney tissue of ICR mice was prepared on a histological section, and the cellular morphology was viewed at 200x magnification. Data represent the mean±SD from three replicates. **P*<0.05 is the significance level compared to vehicle-treated group.

suggest that RLP treatment for a short time does not induce any toxicity in liver organs of ICR mice.

Effects of RLP on kidney toxicity in ICR mice

Finally, the kidney toxicity was investigated in ICR mice using serum biochemical and microscopic examination. In the serum biochemical analysis, a significant increase in the BUN level was observed only in the high dosage RLP-treated group compared to the vehicle-treated group, whereas the other two groups maintained their levels (Figure 5Aa). However, the level of CA did not significantly change in any group of the mice, although all RLP-treated groups exhibited slight increases of this level (Figure 5Ab). Furthermore, microscopic findings showed that no specific pathological symptoms were detected in any of the RLP-treated groups. Especially,

most of the kidney cells maintained their normal structures (Figure 5B). These results suggest that RLP treatment for a short time does not induce specific toxic effects in kidney organ of ICR mice, although the BUN level increased in the high dose RLP-treated group.

Discussion

The beneficial effects of medicinal plants widely used for the treatment of human chronic diseases can be increased by several manufacturing processes, including purification, extraction, steaming, heating, acidic treatment, enzyme treatment, and microorganism treatment [17,18]. Of these methods, steaming process has been applied to medicinal plants in order to increase therapeutic effects or improve novel functions. In the case of Korean Red Genseng (KRG), steaming process has been shown to induce increases in the levels of certain components, including ginsenosides F4, Rg3, Rg5, acidic polysaccharides, and phenolics, as well as transformation of new compounds such as nonsaponinpolyaceylene, maltol, and amino acids [17-22]. However, in the case of sosihotang, which is used to treat chronic liver disease, steaming process has been shown to induce an increase in only one component (gisenoside Rg3), whereas other components such as saikosaponin A, homogenetisic acid, baicalin, glycyrrhizin, and 6-gingerol are decreased. Although this process does not alter the levels of many compounds, treatment with steamed sosihotang has been shown to have strong neuroprotective effects in HT22 cells [12]. RLP used in this study was manufactured by carrying out specific steaming process comprising two steps (200 g of dry roots was steamed at 99ºC for 3 hr followed by air-drying at 70ºC for 24 hr) for different numbers of repetitions a total of seven times. Secretion of insulin from INS cells and NGF from B35 cells significantly increased upon RLP treatment compared with LP [3]. Although RLP has been reported to have stronger therapeutic effects than LP, it was not reported whether RLP could induce any toxicity on the specific organs of animals.

In the toxicological evaluation process, body and organ weights were used as primary indicators of toxicity for various substances [23]. Of these organs, the liver and kidney are considered as major target organs since they contain the most enzymes and metabolic pathways for xenobiotic excretion [22]. Especially, an increase in liver weight can further progress to abnormal fat accumulation, obesity, and necrosis of liver cells [24-29]. In this study, significant alteration of liver and kidney weights was not detected in any of the RLP-treated groups. Although a slight increase of liver weight was detected in the low dosage RLP-treated group, these increases were not statistically significant. This variation on the liver weight was thought to cause by several artificial factors except for toxicity. Therefore, our data indicate that RLP does not have any toxic effects on liver or kidney weight.

Meanwhile, liver toxicity is confirmed based on alterations in the levels of four enzymes related to liver metabolism of theses enzymes. ALT is found in the serum as well as various tissues [28], while AST is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells [27]. Especially, these two enzymes are released into the blood when liver cells are disrupted by various factors. Therefore, alteration of the level of either enzyme is commonly used as a marker of altered liver health. In addition, the concentration of ALP is used to diagnose liver disease or bone disorders. Especially, ALP activity increases under conditions of bile secretion disease, which include primary biliary cirrhosis as well as extra- and intra-hepatic cholestasis [30]. Furthermore, LDH is present in almost all animal tissues and is up-regulated in response to cell damage [30,31]. Therefore, when a liver toxicant is administered to animals, the damaged liver cells release a large amount of ALP into the blood. In this study, we used above factors related with toxicants in order to detect RLP toxicity on the liver and kidney of mice. Our results showed that RLP did not have any toxic effects on liver tissue of RLP-treated mice based on the serum levels of four enzyme indicators. Also, we believe that non-toxicity of RLP in the mice was correlated with the characteristics of RLP major component such as carbohydrate, protein and ash fat.

Generally, various pathological alterations can be observed histologically in liver tissue under toxic conditions. For example, CCl_4, a popular liver toxicant, can induce massive coagulative necrosis in the central vein as well as degenerative changes with fatty alteration of the necrotic border region [30]. However, in our study, such pathological changes were not detected in liver sections of ICR mice after RLP treatment.

Kidney toxicity was also evaluated based on alteration of the serum levels of two factors, specifically BUN and CA. BUN analysis can be used as a measurement of the

amount of BUN and is widely used an indicator of renal health [31]. CA is naturally primarily produced from amino acids in the kidney and liver, after which it is transported to muscles [32]. Upon kidney trauma, the levels of these two factors dramatically increase in the blood serum. Furthermore, an increased BUN/CA ratio can be observed upon upper gastrointestinal tract bleeding, hemocytotripsis, inflammation, drugs administration and fever [32]. In this study, we measured the levels of BUN and CA after RLP treatment in order to evaluate RLP toxicity in the kidney. In our results, the BUN level was higher only upon treatment with 50.0 mg/mL of RLP compared to vehicle, whereas the CA level was maintained at a constant level. Therefore, our results suggest that a high concentration of RLP induces an increase in the serum level of BUN. However, we couldn't put our finger one the reason for the BUN increase at 50 mg/kg RLP treated group. Therefore, further studies were required to identify the correlation between the BUN increase and RLP components. Meanwhile, the pathological alterations in kidney tissue can be well induced by paraquat (1'1'-dimetythyl-4'4'-bipyridyliumion), which is a non-selective contact and acute herbicide. Specifically, necrosis of the proximal tube has been observed in paraquat-treated mice [33]. However, in this study, RLP-treated kidney was not shown any pathological alteration which had been observed in the medulla or cortex region of paraquat-treated kidney.

Taken together, these results show that three different concentrations of RLP did not have any specific toxic effects on liver and kidney tissues of ICR mice. Further, the toxicity data on RLP provide vital information on the development of RLP as a therapeutic drug for the treatment of various chronic diseases, including diabetes, dementia, asthma, and atopic dermatitis.

Acknowledgments

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