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원 저

백서 모델에서 알파 아마니틴에 의한 간독성에 대한 갯방풍의 보호 효과

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The Effect of Glehnia Littoralis on Alpha-amanitin Induced Hepatotoxicity in a Murine Model

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Purpose: *Glehnia littoralis* has been reported to have several pharmacological properties but no in vivo reports describing the protective effects of this plant on α-amanitin-induced hepatotoxicity have been published. α-Amanitin is a peptide found in several mushroom species that accounts for the majority of severe mushroom poisonings leading to severe hepatonecrosis. In our previous *in vitro* study, we found that α-amanitin induced oxidative stress, which may contribute to its severe hepatotoxicity. The aim of this study was to investigate whether *Glehnia littoralis* acetate extract (GLEA) has protective antioxidant effects on α-amanitin-induced hepatotoxicity in a murine model.

Methods: Swiss mice (n=40 in all groups) were divided into four groups (n=10/group). Three hours after giving α-amanitin (0.6 mg/kg, i.p.) to the mice, they were administered silibinin (50 mg/kg/d, i.p.) or *Glehnia littoralis* ethyl acetate extract (100 mg/kg/d, oral) therapies once a day for 3 days. After 72 hours of treatment, each subject was killed, cardiac blood was aspirated for hepatic aminotransferase measurement, and liver specimens were harvested to evaluate the extent of hepatonecrosis. The degree of hepatonecrosis was assessed by a pathologist blinded to the treatment group and divided into 4 categories according to the grade of hepatonecrosis.

Results: GLEA significantly improved the beneficial functional parameters in α-amanitin-induced hepatotoxicity. In the histopathological evaluation, the toxicity that was generated with α-amanitin was significantly reduced by GLEA, showing a possible hepatoprotective effect.

Conclusion: In this murine model, *Glehnia littoralis* was effective in limiting hepatic injury after α-amanitin poisoning. Increases of aminotransferases and degrees of hepatonecrosis were attenuated by this antidotal therapy.

Key Words: Alpha-amanitin, Glehnia littoralis, Antioxidant, Antidotes, Animal model

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INTRODUCTION

As outdoor activities have increased in recent years, wild poisonous mushroom taken from nature is misdiagnosed as edible mushroom, and the poisoning accident happens very often after ingesting it. According to the Korean Forest Service data from 2004 to 2013, a total of 53 poisoning accidents occurred, totaling 204

poisoning cases, of which 23 died[1]. Since not all poisoning accidents are reported, it is estimated that there are more poisoning accidents and deaths. In more than 90% of cases of ingestion, the type of mushroom is unknown because of difficulties in the exact identification of mushroom species. Most of the ingested mushrooms are either nontoxic or only gastrointestinal irritants, resulting in mild to moderate toxic effects[2]. Among these toxins, amatoxin have their most serious effect on the liver and account for 90% of fatal mushroom poisonings[3]. Alpha-amanitin, the main amatoxin, is readily absorbed from the gastrointestinal tract and carried to the liver via portal vein. Alpha-amanitin (α-amanitin) poisoning is characterized by liver necrosis, in many cases with acute hepatic insufficiency with subsequent complications including hepatic coma, coagulation disorders and renal failure[4-6]. Many other experiments revealed that α-amanitin may influence the activities of superoxide dismutase and catalase, enzymes crucial for the prevention of oxidative stress-related injury[7-9].

More and more attentions have been paid to the protective effects of natural antioxidants against drug-induced toxicities especially when free radical generations are involved. Glehnia littoralis is a perennial herb that grows on the sandy beaches of eastern China, Korea, Japan, and North-west America. The aqueous extract of Glehnia littoralis has been reported to have several pharmacological properties including antioxidant, anticancer, antiinflammatory, and some immunomodulatory properties[10-14]. In vitro previous study showed that Glehnia littoralis offered significant hepatoprotection against the oxidative damage induced by α-amanitin[15].

To date, no in vivo reports describing the hepatoprotective effect of this plant have been published. In this study, we investigated the effect of Glehnia littoralis on α-amanitin induced hepatotoxicity in a murine model used both histologic analysis and serum aminotransferase.

METHODS

1. Standard solutions and chemicals

Alpha amanitin standard and silibinin, ethanol, acetonitrile, ammonium acetate, dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Glehnia littoralis was collected by Professor Sung Dong Cho of Chosun University in South Jeolla Province (Republic of Korea) in October 2014. For the preparation of Glehnia littoralis ethyl acetate extract (GLEA), roots and rhizomes of Glehnia littoralis were washed with distilled water, air-dried at 60° C, and ground into fine powder by a grinder. The powder was refluxed with 10 vol (v/w) of 70% ethanol at 70° C for 24 hours, and the extraction procedure was repeated three times. The extract was filtered through filter paper, concentrated with a vacuum evaporator, and it was completely dried with a freeze drier. Silibinin was dissolved in DMSO and diluted in distilled water (in a way to have a final DMSO concentration of 1%). GLEA was prepared as suspension in distilled water. Drug solutions were prepared freshly just before the application. Silibinin was administered in doses of 50 mg/kg intraperitoneally (i.p.) and GLEA in doses of 100 mg/kg by oral gavage.

2. Animals and laboratory

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication number 85-23, revised 1996, latest revision in 2011), and was approved by Animal Care and Use ethical Committee of Chosun University (CIACUC2016-A0038). All efforts were made to minimize animal suffering and to reduce the number of animals used. White male laboratory mice with weight 20-22 g were used. Animals were housed 5 per cage with a 12-hour light/dark cycle and received temperature/humidity-controlled conditions. Food and water were provided ad libitum throughout the study. Following a seven-day acclimation period, animals were entered into the study.

3. Experimental procedures

Mice were randomly assigned to the individual four groups (n=10 for the sham group; sham, n=10 for the α-amanitin group; AMA, n=10 for the AMA and silibinin treated group; AMA+SIL, and n=10 for the AMA and GLEA treated group; AMA+GLEA). After dissolving it in

distilled water, α-amanitin was administered to all animals except those in the sham group in doses of 0.6 mg/kg intraperitoneal (i.p.) (in 0.2 mL distilled water). This amount was chosen because it represents 50% lethal dose value of α-amanitin in mice according to several publications and on the material safety data sheets provided by the commercial supplier of our extracted α-amanitin[16]. The sham group was given saline i.p. in the same volume. The silibinin (50 mg/kg, i.p.) and GLEA (100 mg/kg, oral) therapies were started at hour 3 following the administration of α-amanitin considering the frequent delays in presenting to a hospital after poisoning. Silibinin and GLEA were given once a day for 3 days. The AMA and sham groups were given the same volume of saline i.p. and orally. Twenty-four hours after the last treatment, blood was drawn from the hearts of animals under 10% diluted urethane anesthesia (1.5 g/kg, i.p.) to obtain serums. Liver tissues were removed for histopathological examination and kept in 10% formaldehyde. All chemistry tests for liver: aspartate aminotransferase (AST), alanine aminotransferase (ALT) were run on the Modular Analytics (Rosche, Indianapolis, Indiana, USA) reagents.

4. Histopathological study

The liver tissues were dehydrated in graded ethanol, embedded in paraffin, cut into 5 mm thick sections, and processed for routine hematoxylin and eosin (H & E) staining. The sections were examined under a light microscope. The independent board-certified pathologist performed the analysis of tissues in a blind fashion. The following parameters were investigated and graded in the liver: (a) degeneration and vacuolation of hepatic parenchyma, (b) congestion and hemorrhage, (c) portal mononuclear cell infiltration, (d) basophilic stippling, (e) granular cytoplasm, (f) eosinophilic debris, and (g) hyper activation of Kupffer cells. A grading score between 0 and 3 was given for each parameter[17].

5. Statistical analysis

The biochemical analysis results and histopathological grading scores were given in the form of mean (±SEM) and analyzed by SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA). The differences between the biochemical parameters were assessed with a one-way analysis of variance test. The differences between the groups in terms of histopathological parameters were examined with the Kruskal-Wallis test. Results where $p<0.05$ were considered statistically significant.

RESULTS

When the levels of AST and ALT were assessed, there was a significant increase in the aminotransferase levels in the mice that were given α-amanitin as compared with the sham group ($p<0.002$ for AST and ALT). However, the AST and ALT values in the AMA+SIL group were lower than those of the AMA group ($p<0.002$ for AST; $p<0.009$ for ALT). GLEA therapy of the α-amanitin administered mice resulted in a marked decrease in the serum AST and ALT levels as compared with the AMA ($p<0.002$ for AST and ALT) and AMA+SIL groups ($p<0.002$ for AST and ALT). However, they were still higher than those of the sham group ($p<0.009$ for AST; $p<0.002$ for ALT). The biochemical analysis results of the liver function tests of all groups were given in Table 1.

In the histopathological evaluation, which involved parameters such as degeneration and vacuolation of hepatic parenchyma, congestion and hemorrhage, portal mononuclear cell infiltration, granular cytoplasm, and eosinophilic debris, the toxicity that was generated with α-amanitin was significantly reduced by GLEA, result-

Table 1. Comparison of biochemical liver function test results by ANOVA test

Biochemical Parameters	AST	ALT
Sham group	67±5.8	22.4±1.3
AMA group	3316±311*	4598±572*
AMA+SIL group	1789±169*,†	2348±250*,†
AMA+GLEA group	1072.1±64*,‡	1169±33*,‡

AST, ALT: IU/L. Values are expressed as means±SEM.; n=10 all groups.
* $p<0.01$ difference from sham;
† $p<0.01$ difference from the AMA group;
‡ $p<0.01$ difference from AMA and AMA+SIL groups.
AMA: α-amanitin treated; AMA+SIL: α-amanitin and silibinin treated; AMA+GLEA: α-amanitin and Glehnia littoralis acetate extract treated.

ing in a hepatoprotective effect ($p<0.05$). However, Silibinin therapy did not show a significant decrease compared to GLEA treated group. The histopathological examination results pertaining to all groups and photos of sample cross-sections are given in Figures 1 and 2, respectively.

DISCUSSION

Amatoxin is thermally stable and is not destroyed by cooking, freezing or drying mushrooms. Amotoxin is known to be associated with phosphorylation of α-amanitin. Amatoxin is rapidly absorbed into the gastrointestinal tract and transported to the liver, inhibiting transcription by binding to RNA polymerase II and inhibiting mRNA synthesis[18]. Amatoxin is excreted in the bile and then enters the liver via enterohepatic circulation[19]. Alpha amanitin accounts for 60% of the amatoxins in A. phalloides, but this percentage may vary among various amanitin species[20].

The free radical intermediates that are generated in hepatocytes due to α-amanitin are associated with the production of increased reactive oxygen species. Subsequent intense oxidative stress would then lead to hepatocyte peroxidation, liver glutathione depletion, and death, contributing to severe hepatotoxicity. Damage to the liver is characterized by massive centrilobular necrosis, vacuolar degeneration, and a positive acid-phosphatase reaction[21,22]. In this experiment, we validated the use of a murine model to study hepatic injury from α-amanitin (measured objectively by increases in aminotransferases and histologic examinations of hepatocytic necrosis). Serum aminotransferase activity has been used for a long time as an index of liver damage[23]. Hepatocyte damage results in a change in transport membrane permeability and ultimately releases enzymes from the cells into the fluid. Thus, many releases of AST and ALT into the circulatory system imply serious damage to the liver tissue during α-amanitin-induced toxicity. In this study, the administration of α-amanitin to mice resulted in a rapid increase in the amount of AST and ALT, indicating that hepatic toxicity was observed. And we showed that histopathological changes such as degeneration and vacuolation of hepatic parenchyma, congestion and hemorrhage, portal mononuclear cell infiltration, granular cytoplasm, and eosinophilic debris in the liver tissue in the mice that received α-amanitin. All these changes show that α-amanitin administration leads to cellular disruption and hepatocellular necrosis in the liver.

Treatment of poisonings caused by amatoxin-containing mushrooms involves gastrointestinal decontamination, supportive measures, antidotes and, if liver failure

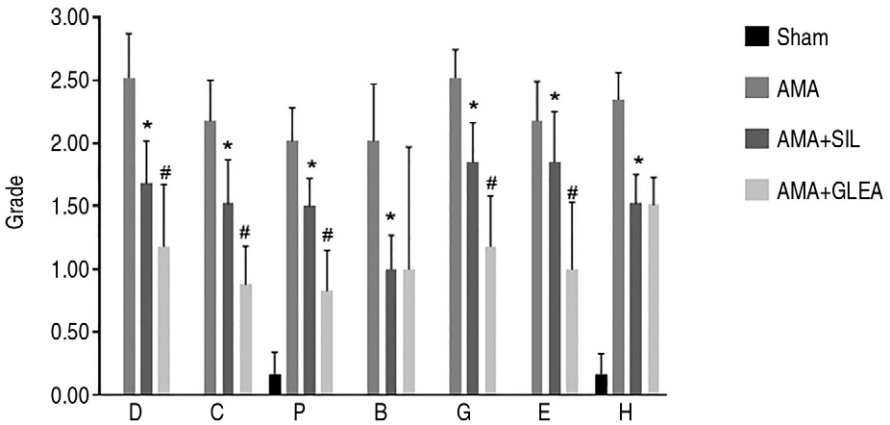

Fig. 1. Comparison of effects of GLEA and silibinin on the histopathological parameters of the liver in alpha amanitin-induced toxicity by Kruskal-Wallis test. (n=10 for each group; bars indicate means±SEM). * $p<0.05$ difference from the AMA group; # $p<0.05$ difference from the AMA group

D: degeneration and vacuolation of hepatic parenchyma, C: congestion and hemorrhage, P: portal mononuclear cell infiltration, B: basophilic stippling, G: granular cytoplasm, E: eosinophilic debris, H: hyperactivation of Kupffer cells, AMA: α-amanitin treated group; AMA+SIL: α-amanitin and silibinin treated group; AMA+GLEA: α-amanitin and Glehnia littoralis acetate extract treated group.

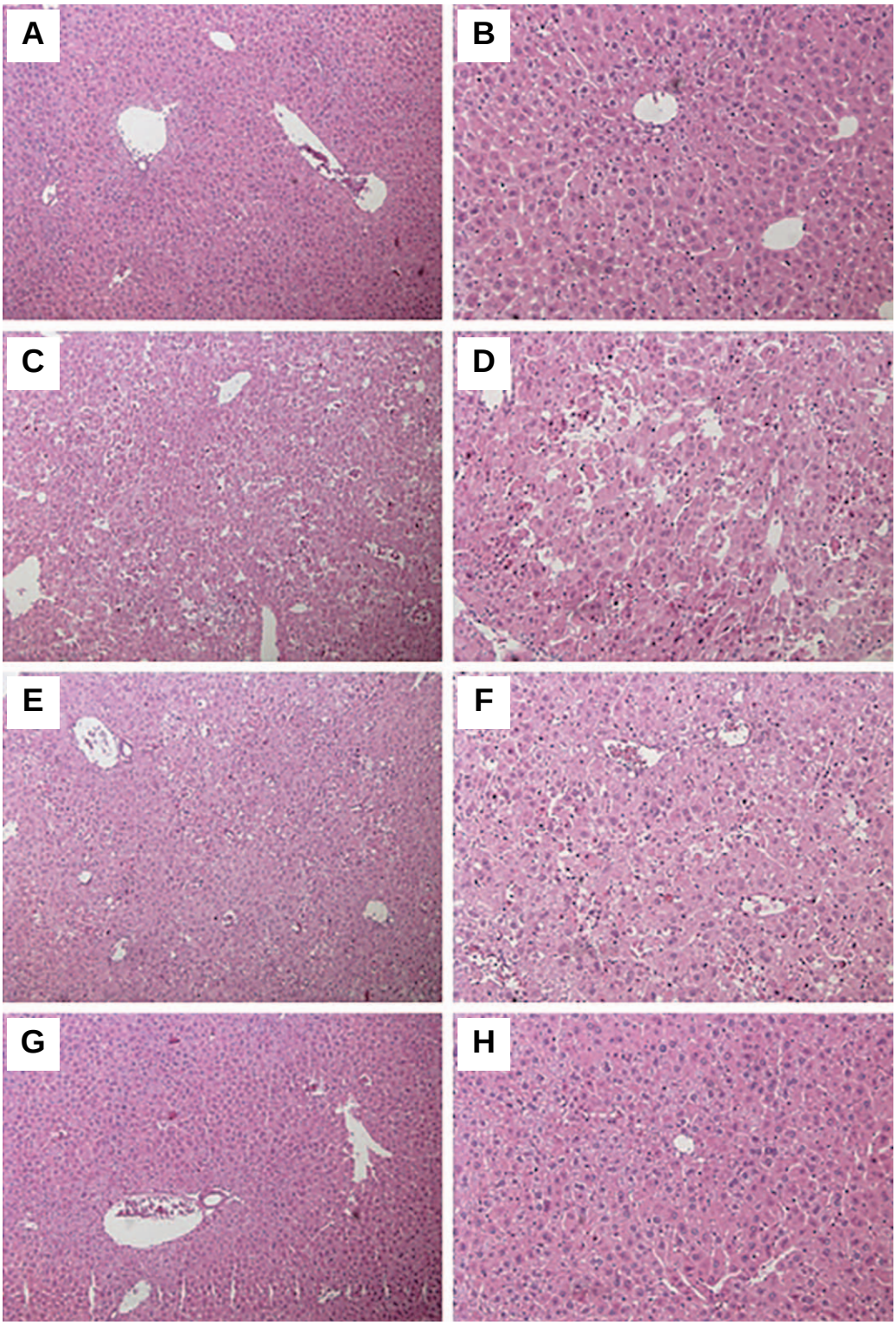

Fig. 2. Representative histological sections (×100 and ×200 enlargement after hematoxylin-eosin staining) of mice livers from Sham, AMA, AMA+SIL and AMA+GLEA groups. (**A**, **B**) Sham group. Normal liver tissue sample. Ordinary hepatocytes, portal area, and central vein are displayed. (**C**, **D**) AMA group. Widespread congestion, hepatic vacuolization, eosinophilic debris, vacuolar degeneration can be seen in hepatocytes. (**E**, **F**) AMA+SIL group. Focal pericentral congestion, eosinophilic debris, and vacuolar degeneration can be seen in hepatocytes. (**G**, **H**) AMA+GLEA group. Mild vacuolar degeneration can be seen in hepatocytes. AMA: α-amanitin treated group; AMA+SIL: α-amanitin and silibinin treated group; AMA+GLEA: α-amanitin and Glehnia littoralis acetate extract treated group.

occurs, liver transplantation. In antidotal therapy, various substances (silibinin, steroids, cimetidine, thioctic acid; benzylpenicillin; acetylcysteine) have been widely used in the past to treat α-amanitin poisonings[24]. In experimental and clinical studies today, all these abovenamed drugs are said to remain inadequate in treating alpha amanitin poisoning, they do not even have any noticeable advantage over one another[16,22,25]. A systematic review of the treatment of Amanita poisoning shows that there is no treatment policy backed by a high level of evidence. Therefore, it is necessary to wait for further study results. Until then, it is necessary to consider treatments such as silymarin, penicillin, and N-acetyl cysteine in addition to conservative treatment. Future clinical research should focus on confirming the efficacy of various antioxidant and antiinflammatory agents. For this reason, the efficacy of GLEA in poisoning was compared with the silibinin therapy in our study.

Our study demonstrated that GLEA had a protective effect in α-amanitin induced hepatotoxicity by significantly improving the functional and histological parameters. GLEA treatment reduced the activities of hepatic enzymes to control levels. Moreover, where silibinin failed to relieve toxicity in α-amanitin induced hepatotoxicity, GLEA brings hope for mushroom poisonings with its positive effects. The protective effect of GLEA may be associated with its property of reducing the oxidative stress that was elevated with α-amanitin due to its potent antioxidant and radical scavenging effects. Animal-based experimental evidence supports the protective effect of GLEA in various types of tissue injuries mediated by products of oxidative stress[13,26].

GLEA improved increased nitric oxide (NO) level in liver[27,28]. NO is one of the reactive mediators released in the liver through endothelial cells, macrophages, hepatocytes, and Kupffer cells in response to different stimuli and thus induces hepatic injury. It is likely that cytokines and the NO pathways are interconnected and that both are involved in the systems responsible for inflammatory reaction of the organ. GLEA caused decreased inflammatory cell accumulation and prevented inflammation-induced tissue injury. Alpha amanitin does not only cause hepatocyte necrosis but also may lead to apoptotic cell death and it is also a strong apoptosis inductor. Apoptosis might contribute to pathogenesis of the severe liver injury in the course of amanitin intoxication, particularly during the early phase of poisoning[29]. All these suggest that GLEA' s effects on NO balance, its antiinflammatory properties, and its effects on apoptosis may have contributed to its hepatoprotective action in α-amanitin toxicity.

This study is an experimental mouse model of α-amanitin poisoning. An animal model may not represent the same antioxidant response seen in human clinical model, it is unfortunate that this study did not show the change of hepatic enzyme and histological examination by time. Quercetin, isoquercetin, rutin, chlorogenic acid, and caffeic acid have been isolated as the major antioxidative constituents in GLEA. In addition, imperatorin, isoimperatorin, ferulate and polyine compounds have been reported as the compounds in GLEA. However, it was not possible to measure the protective effect by extracting each component in this study. The pharmacokinetics and pharmacodynamics of orally ingested α-amanitin may differ from α-amanitin injected intraperitoneally. Also, intravenous administration of silibinin is ideal, but it was administered intraperitoneally in this experiment.

CONCLUSION

In this murine model, green tea extract was effective in limiting hepatic injury after α-amanitin poisoning. Increases of aminotransferases and degrees of hepatonecrosis were attenuated by this antidotal therapy. The mechanisms involved in this action may be the prevention of free radical damaging cascades, oxidant radical release, and its prevention from proinflammatory processes. But further experimental and clinical studies are required to confirm these findings and to reveal its mechanisms of action more clearly.

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