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Hepatoprotective Effects of Aqueous Extract from Lingzhi or Reishi Medicinal Mushroom *Ganoderma lucidum* (Higher Basidiomycetes) on α-Amanitin–Induced Liver Injury in Mice

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ABSTRACT: The Lingzhi or Reishi mushroom *Ganoderma lucidum* is a well-known traditional medicinal mushroom that has been shown to have obvious hepatoprotective effects. The aim of this study was to evaluate the hepatoprotective effects of *G. lucidum* aqueous extracts (GLEs) on liver injury induced by α-amanitin (α-AMA) in mice and to analyze the possible hepatoprotective mechanisms related to radical scavenging activity. Mice were treated with α-AMA prepared from *Amanita exitialis* and then administrated with GLE after the α-AMA injection. The hepatoprotective activity of the GLE was compared with the reference drug silibinin (SIL). α-AMA induced a significant elevation in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and provoked a significant reduction of superoxide dismutase (SOD) and catalase (CAT) activities and a significant increment of malondialdehyde (MDA) content in liver homogenate. Treatment with GLE or SIL significantly decreased serum ALT and AST levels, significantly increased SOD and CAT activities, and decreased MDA content in liver compared with the α-AMA control group. The histopathological examination of liver sections was consistent with that of biochemical parameters. The results demonstrated that GLE induces hepatoprotective effects on acute liver injury induced by α-AMA; these protective effects may be related in part to the antioxidant properties of GLE.

KEY WORDS: medicinal mushrooms, α-amanitin, *Ganoderma lucidum*, hepatoprotective effect, antioxidant

ABBREVIATIONS: α-AMA, α-amanitin; **ALT,** alanine aminotransferase; **AST,** aspartate aminotransferase; **CAT,** catalase; **GLE,** *Ganoderma lucidum* aqueous extract; **HPLC,** high-performance liquid chromatography; **LD_{50},** median lethal dose; **MDA,** malondialdehyde; **SIL,** silibinin; **SOD,** superoxide dismutase

I. INTRODUCTION

Most fatal mushroom poisonings are caused by some species of the genus *Amanita*. In Europe, Asia, and North America, the lethal *Amanita* species includes *A. phalloides*, *A. virosa*, *A. verna*, *A. ocreata*, and *A. bisporigera*.[1] In China, the main fatal *Amanita* species are *A. fuliginea* and *A. exitialis*.[2] It has been demonstrated that α-amanitin (α-AMA) seems to be the primary lethal constituent that inhibits RNA polymerase II and therefore blocks the synthesis of proteins, leading to cell death.[3–5] Amanitin uptake in hepatocytes is high and the liver is the main target, so acute liver failure is the characteristic symptom of amatoxin poisoning.[6] Treatment of amatoxin poisoning includes toxin elimination, optimum symptomatic and supportive care, antidotes, and liver transplantation.[6–8] A wide variety of antidotes for amatoxin poisoning have been reported, but no efficient and specific antidotes are available. Enjalbert et al.[1] summarized the clinical data related to amatoxin poisoning in the literature over the past 20 years, and statistical analysis indicated that the most commonly used benzylpenicillin showed little efficacy; silybin and *N*-acetylcysteine seemed to be the most effective therapeutic antidotes. In recent years, the experimental data for the efficacy of the antidotes are still incongruous or conflicting. The

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experiments in human hepatocyte cultures intoxicated with α-AMA demonstrated that benzylpenicillin, silibinin (SIL), and acetylcysteine had better efficacy,[9–11] whereas mouse model studies with α-AMA showed that *N*-acetylcysteine, benzylpenicillin, cimetidine, thioctic acid, and silybin were ineffective antidotes.[12]

The Lingzhi or Reishi medicinal mushroom *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Ganadermataceae, Polyporales, higher Basidiomyetes) is a well-known traditional medical species, which has been used for hundreds of years as a clinical remedy for the treatment of a variety of ailments. More recent research has verified its multiple pharmacological effects including antitumor, immunomodulation, anti-inflammation, antibacteria, hepatoprotection, antioxidation, and radical scavenging; its bioactive components include triterpenoids, polysaccharides, sterols, steroids, peptides, and other bioactive ingredients.[13–15] Previous studies indicated that *G. lucidum* extracts and its main bioactive constituents (polysaccharide, triterpenoid, proteoglycan, and peptides) had obvious protective effects against liver injury induced by carbon tetrachloride and D-galactosamine and that the possible hepatoprotective mechanisms might be ascribed to its antioxidation and free radical scavenging activity.[16–19] Clinical utility of *G. lucidum* as a remedy for *Amanita* poisoning has been reported in China, and the results showed that the clinical efficacy was obvious, with a significant decrease in mortality.[20,21] However, experimental data about the protective effects of *G. lucidum* on liver damage induced by amanitin is still lacking.

The purpose of this study was to evaluate the hepatoprotective availability of *G. lucidum* aqueous extract (GLE) on liver injury induced by α-AMA in mice and analyze the possible hepatoprotective mechanisms related to radical scavenging activity.

II. MATERIALS AND METHODS

A. Samples and Chemicals

Ganoderma lucidum was purchased from Yongchun Medical Mushroom Institute (Changde, China) and the voucher specimen was stored in College of Life Sciences, Hunan Normal University. Alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) determination kits were purchased from Shanghai Rongsheng Biotech Co. Ltd.; superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), and Coomassie Brilliant Blue protein determination kits were from Jianchen Bio-Engineering Institute (Nanjin, China). The standard sample of α-AMA was purchased from Merck-Calbiochem, SIL was from Sigma (St. Louis, MO), and 0.9% saline solution was from Hubei Pharmaceutical Co. Ltd. All other chemicals used were of the highest commercial grade.

B. Preparation of *Ganoderma lucidum* Aqueous Extract

The fruiting bodies of *G. lucidum* were cut into small pieces and boiled in distilled water at a ratio of 1: 5 (w/v) at 100°C for 30 minutes then filtrated with Whatman No. 1 filter paper. The residue was extracted and filtrated again in the same way. The two collections of the filtrate were put together and freeze-dried. The extracts obtained were stored in a refrigerator at 4°C till use.

C. Preparation and Identification of α-AMA

The peptide toxins were extracted from the lethal *A. exitialis* according to methods described in our previous reports.[22,23] The extracts of the peptide toxins were separated and purified using a reversed-phase high-performance liquid chromatography (HPLC) method on a semipreparation Waters Delta 600 HPLC system (Waters Inc., Milford, MA) with a Waters 2487 variable ultraviolet detector (Waters Inc.). Separations were carried out at 40°C on a Sinochrom ODS-BP reverse phase HPLC column

(250 × 10 mm internal diameter, particle size, 10 μm; Dalian Elite Analytical Instruments Co. Ltd., China). The mobile phases and elution profile were performed according to the conditions described in our previous report.[22] The mobile phase flow rate was 2 mL/min and the injection amount was 2 mL. The compound with a retention time close to that of the α-AMA standard sample was collected and purified repeatedly by HPLC under the same conditions until the purity reached 95%. The compound was further identified as α-AMA based on the spectral methods including liquid chromatography-mass spectrometry and nuclear magnetic resonance; the data obtained were consistent with the data reported by Yang et al.[24] and Deng et al.[25]

D. Animals and Treatments

Kunming mice (SPF class, 160 male and 160 female, 20 ± 2 g weight) were purchased from Hunan SLAC-Jingda Laboratory Animal Co. LTD (no. eligible: SCXK [Xiang] 2009–0004). The mice were kept in cages under a constant temperature (20 ± 4°C) and light cycle (12 hours of dark, 12 hours of light) and were provided with standard mouse feed and water. All procedures involving the use of laboratory animals were in accordance with Chinese Animal Care and Use Guidelines.

1. Determination of the LD_{50} of α-AMA in Mice

Kunming mice (n = 60) were divided randomly into 6 groups (10 mice each) for administration by intraperitoneal injection of 0.4 mL α-AMA at different concentrations (0.10, 0.15, 0.23, 0.35, 0.53, and 0.80 mg/kg body weight). The numbers of deaths in each group were recorded 96 hours after injection. The median lethal dose (LD_{50}) was calculated using the modified Karber's method.[26]

2. The Time-Dose Effect of α-AMA on Biochemical Activities in Mice

Mice (n = 150) were divided randomly into 3 groups (50 mice each) for intraperitoneal injection of 0.4 mL α-AMA at a dose of 0.2, 0.4 and 0.6 mg/kg body weight, respectively. Another 10 mice constituted the control group and were administered with the same volume of 0.9% saline solution. Ten mice from every α-AMA group were killed to determine biochemical activities, including ALT, AST, SOD, CAT, and MDA, at 0, 24, 48, 72, and 96 hours after injection, respectively.

3. The Dose Effect of GLE on the Biochemical Activities in α-AMA-Injured Mice

Seventy mice were divided into 7 groups, namely 5 groups treated with GLE and 2 control groups, including a normal control group injected intraperitoneally with 0.4 mL of 0.9% saline solution and a control group injected with 0.4 mL of α-AMA at a dose of 0.6 mg/kg body weight. Different doses of GLE (10, 50, 100, 500, and 1000 mg/kg/day) dissolved in 0.9% saline solution were administrated intragastrically (via gavage tube) 4 hours after α-AMA injection in mice in the 5 groups treated with GLE. Each mouse received 0.4 mL, and GLE gavage was performed every 6 hours. In the meantime, the 2 control groups were given an equal volume of saline solution every 6 hours. All mice were killed to determine biochemical activities (ALT, AST, SOD, CAT, and MDA) 48 hours after injection with α-AMA.

4. The Effect of GLE and SIL on the Biochemical Activity in α-AMA-Injured Mice

Forty mice were divided into 4 groups, namely a group treated with GLE, a group treated with SIL, a normal control group, and an α-AMA control group. GLE (a dose of 500 mg/kg/day) was administered intragastrically in the mice in the GLE-treated group , and the mice in the SIL-treated group were injected intraperitoneally with SIL (a dose of 20 mg/kg/day) in 0.9% saline solution. All of the procedures were the same as those described in the previous sections. All mice were killed for biochemical tests and histopathological analysis

TABLE 1: Lethal Dose (LD_{50}) of α-Amanitin (α-AMA) in Mice (n = 10)

α-AMA Dose (mg/kg body weight)	Dead mice (n)	Mortality (P)
0.10	0	0.0
0.15	0	0.0
0.23	0	0.0
0.35	2	0.2
0.53	8	0.8
0.80	10	1.0

48 hours after injection with α-AMA.

E. Biochemical Determinations

Blood samples were collected from mice and serum were isolated by centrifugation. The liver tissues used for biochemical tests were washed with cold 0.9% saline solution. Liver homogenate was prepared with saline solution at a ratio of 1:9 (w/v). The serum ALT and AST activity, the liver SOD and CAT activity, and the content of MDA in liver homogenate were determined by the detection kits according to the manufacturers' manuals.

F. Histological Examination

Liver tissue was fixed in 10% buffered formalin, subsequently dehydrated, and embedded in wax. The tissue wax was cut into 3-μm sections. The sections were stained with both hematoxylin and eosin for histological examination under a light microscope.

G. Statistical Analysis

Data are expressed as means ± standard deviations. Differences between values were analyzed by 1-way analysis of variance with a Tukey test. $P < 0.05$ was considered statistically significant; $P < 0.01$ was considered extremely significant.

III. RESULTS

A. LD_{50} of α-AMA in Mice

The number and mortality of mice at different α-AMA doses are recorded in Table 1. The LD_{50} of α-AMA was determined to be 0.41 mg/kg body weight according to the modified Karber's method, and the 95% confidence limits were 0.36–0.48 mg/kg body weight. The LD_{50} of α-AMA was consistent with the data reported by Wieland.[3]

B. Time-Dose Effect of α-AMA on Biochemical Activities in Mice

The results (Fig. 1A, B) showed that the activities of ALT and AST were time- and dose-dependent. During 48 hours after α-AMA injection, the ALT and AST activities rose and reached a climax, then showed a downward trend from 48 to 96 hours. Meanwhile, the higher the dose, the higher the activities of ALT and AST. ALT activity is more sensitive to α-AMA than AST activity. ALT activity in mice in groups treated with any dose of α-AMA was significantly higher than in the control group. SOD and CAT activities showed a downward trend generally within 96 hours, and the values in mice receiving any dose of α-AMA were significantly lower than in the control group, except SOD activity at a dose of 0.2 mg/kg (Fig. 1C, D). The accumulation of MDA in liver homogenate significantly increased as time and dose increased (Fig. 1E).

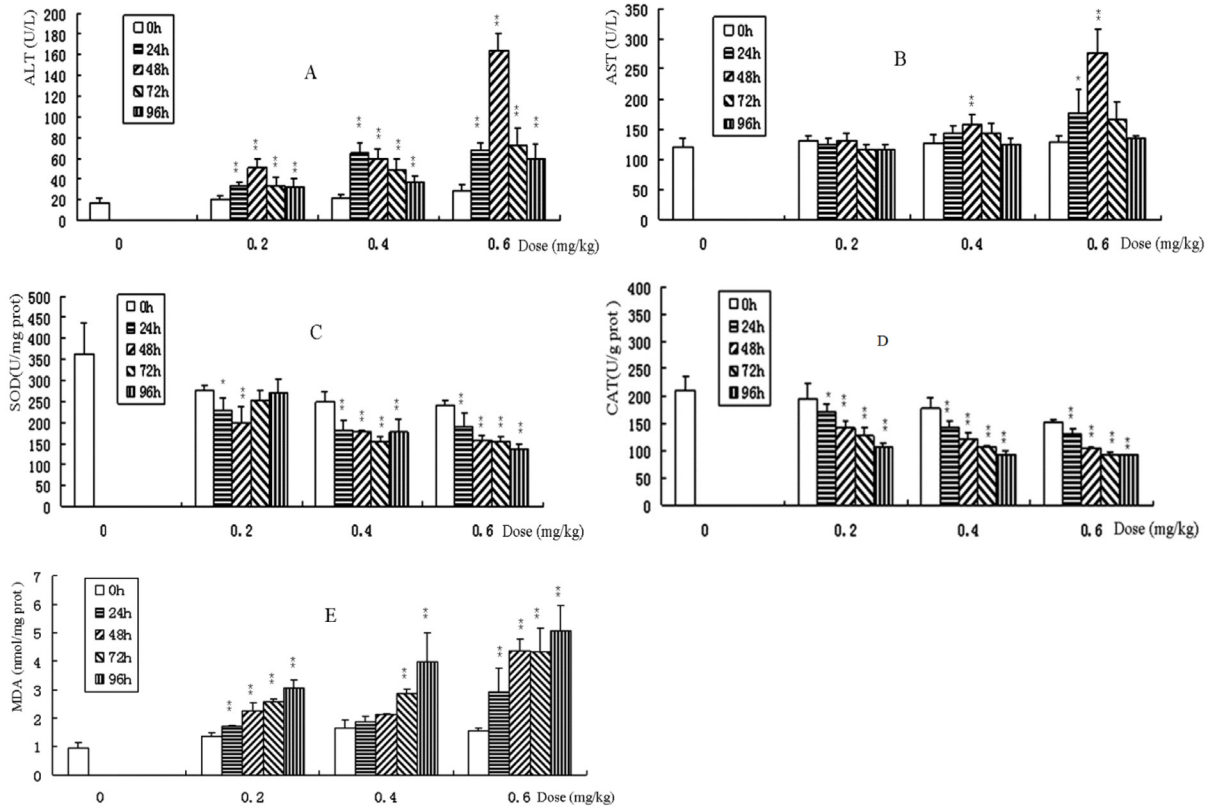

FIG. 1: The time-dose effect of α-amanitin (α-AMA) on biochemical activity in mice: alanine aminotransferase (ALT) (**A**), aspartate aminotransferase (AST) (**B**), superoxide dismutase (SOD) (**C**), catalase (CAT) (**D**), and malondialdehyde content (**E**). Values are expressed as mean ± standard deviation (n = 10). **P* < 0.05, ***P* < 0.01 compared with the control group.

C. The Dose Effect of GLE on Biochemical Activities in α-AMA-injured Mice

The results (Fig. 2) showed that α-AMA induced severe hepatocellular injuries, with a significant elevation in serum ALT and AST activities, and provoked a significant reduction of SOD and CAT activities and a significant increment of MDA content in the liver homogenate when compared with the normal control group ($P < 0.01$). Treatment with GLE at all doses tested decreased serum ALT and AST levels significantly compared with the α-AMA control group. Higher doses led to lower ALT and AST activity. When the dose of GLE exceeded 100 mg/kg/day, the levels of ALT and AST were stable, but the liver SOD and CAT activities increased and MDA content decreased when the treatment dose of GLE rose. A dose of 500 mg/kg/day resulted in a significant increase of SOD and CAT activities and a significant decrease of MDA content compared with the α-AMA control group ($P < 0.01$). Because of this we chose a GLE dose of 500 mg/kg/day for further research.

D. The Effect of GLE and SIL on Biochemical Activities in α-AMA-injured Mice

Treatment with GLE or SIL could decreased serum ALT and AST levels compared with the α-AMA control group, but only GLE significantly ($P < 0.01$) decreased the ALT and AST. Treatment with GLE or SIL significantly prevented the decrease of SOD and CAT activities and the elevation of

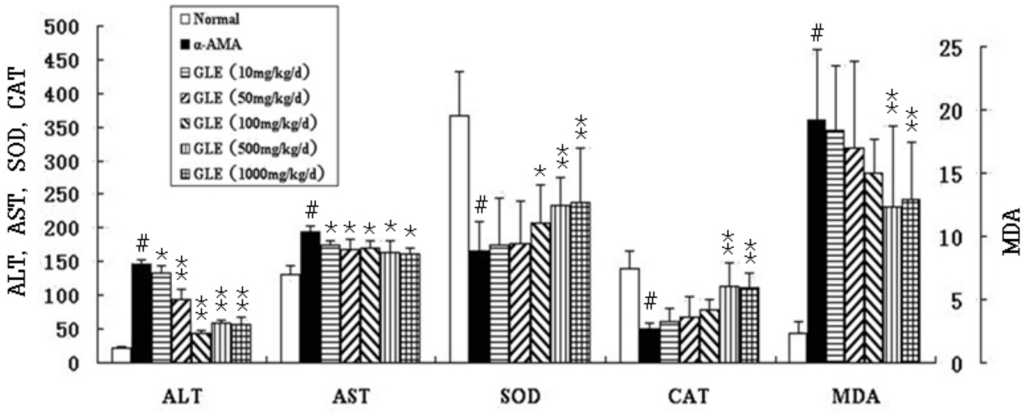

FIG. 2: Dose effect of *G. lucidum* aqueous extract (GLE) on biochemical activities in α-amanitin (AMA)-injured mice. Values are expressed as mean ± standard deviation (n = 10). #*P* < 0.01 compared with normal control group; **P* < 0.05, ***P* < 0.01 compared with α-AMA control group. ALT, alanine aminotransferase (U/L); AST, aspartate aminotransferase (U/L); CAT, catalase (U/mg protein); MDA, malondialdehyde (mmol/mg protein); SOD, superoxide dismutase (U/mg protein).

MDA in the liver compared with the α-AMA control group ($P < 0.01$). The results showed that GLE had a better hepatoprotective effect on α-AMA-induced liver injury in mice than SIL. Results are shown in Fig. 3.

E. Histopathological Observation

Histological observation of the liver provided further evidence to support the hepatoprotective role of GLE. Photomicrographs of liver sections from normal mice showed that a limpid central vein and hepatic cords that radiated out in all the directions and hepatocytes with normal hepatic architecture (Fig. 4A). Photomicrographs of liver sections from mice intoxicated with α-AMA showed a central vein with congestion and random hepatic cords and necrosis of hepatocytes with collapse of hepatic architecture (Fig. 4B). Figure 4C and D indicates that treatment with GLE and SIL were able

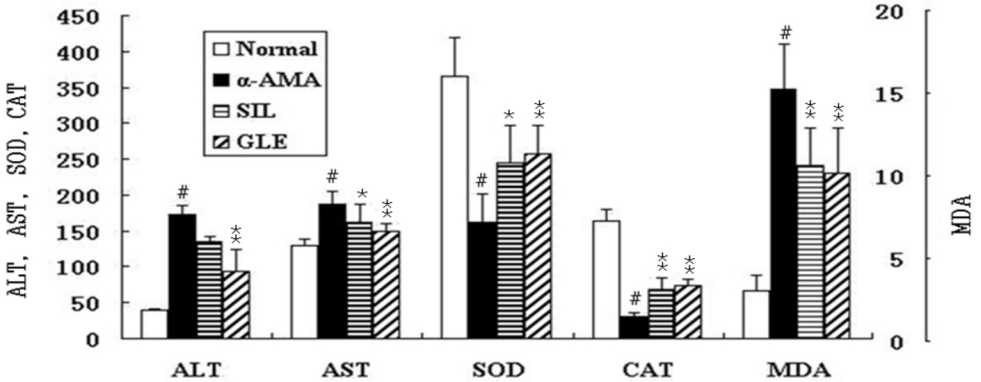

FIG. 3: Effect of *G. lucidum* aqueous extract (GLE) and silibinin (SIL) on biochemical activities in α-amanitin (AMA)-injured mice. Values are expressed as mean ± standard deviation (n = 10). #*P* < 0.01 compared with normal control group; **P* < 0.05, ***P* < 0.01 compared with α-AMA control group. ALT, alanine aminotransferase (U/L); AST, aspartate aminotransferase (U/L); CAT, catalase (U/mg protein); MDA, malondialdehyde (mmol/mg protein); SOD, superoxide dismutase (U/mg protein).

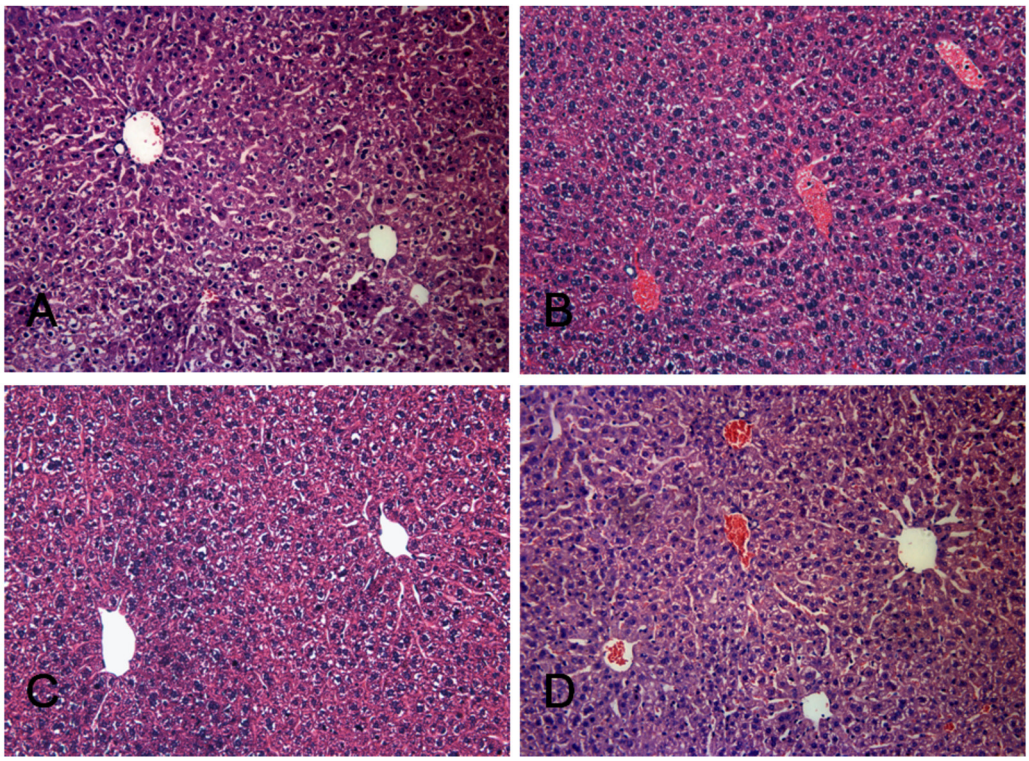

FIG. 4: Histopathological changes in liver sections after α-amanitin (α-AMA)intoxication and treatment with *G. lucidum* aqueous extract (GLE) and silibinin (SIL)(magnification ×100). **A:** Normal control. **B:** α-AMA control. **C:** GLE treatment group. **D:** SIL treatment group.

to relieve the histopathological changes of hepatic cells injured by α-AMA. The hepatic lesions reversed by GLE were almost comparable to those in the normal control group. These histopathological observations further confirmed the hepatoprotective potential of GLE.

IV. DISCUSSION

According to the clinical data from a 20-year retrospective analysis reviewed by Enjalbert et al.,[1] SIL and *N*-acetylcysteine seem to be the most effective antidote to poisoning by mushrooms containing amatoxin. In more than a dozen European countries, SIL is currently registered and licensed as a antidote specifically for the treatment of *A. phalloides* intoxication.[27] SIL is the major bioactive extract from the seeds and fruits of the milk thistle *Silybum marianum* Gaertn. The hepatoprotective mechanisms of SIL involve different biochemical events, such as the stimulation of the synthetic rate of ribosomal RNA species through stimulation of polymerase I and ribosomal RNA transcription,[28] blockage of the uptake of toxins such as α-AMA,[29] and its antioxidation and free radical scavenging properties.[28,30] On the basis of the antioxidation and free radical scavenging characteristics of SIL and the experimental data showing that amanitin generates free radicals, Zheleva et al.[31] proposed a hypothesis that free radical reactions might contribute to severe α-AMA hepatotoxicity. Magdalan et al.[32] assessed the effect of α-AMA and α-AMA with some antidotes on lipid peroxidation and the activities of SOD and CAT in human hepatocyte cultures. The results indicated that α-AMA could probably enhance lipid peroxidation and the addition of antidotes (especially *N*-acetyl-L-cysteine and SIL) to the culture medium provided effective protection against lipid peroxidation in human hepatocytes intoxicated with α-AMA.

G. lucidum as a well-known traditional medical mushroom that has obvious hepatoprotective effects on liver injury induced by some toxins such as carbon tetrachloride and D-galactosamine; the possible mechanisms may be ascribed to its antioxidation and free radical scavenging activities.[16–19] In addition, it has been used as an antidote for treatment of AMA poisoning in China, and the clinical efficacy is obvious.[20,21] Therefore we speculated that GLE might have protective effects against liver injury induced by α-AMA and that the possible hepatoprotective mechanisms might relate to radical scavenging activity. This study indicated that α-AMA-induced hepatic damage was manifested by a significant increase in the activities of marker enzymes (AST, ALT) in serum. Administration of GLE could significantly ($P < 0.01$) decrease serum ALT and AST levels. The biochemical results were consistent with histopathological examination of liver sections. In addition, we showed that the GLE has a better hepatoprotective effect than SIL, which may be due in part to the difference of injection method and concentrations; because GLE is a crude extract and usually administrated orally or intragastrically in clinical trials and experiments, the dose reached 500 mg/kg/day. However, SIL has low water solubility and a dose of 20–48 mg/kg/day has shown promise as a clinical antidote to acute *Amanita* poisoning.[28]

Furthermore, our experimental data indicated that α-AMA could provoke a significant reduction of SOD and CAT activities and a significant increment of MDA content in liver homogenate. We also showed that administration of GLE or SIL could significantly increase SOD and CAT activity and reduce the content of MDA in the liver. These data demonstrated that α-AMA could generate free radicals and lipid peroxidation, which may contribute to its hepatotoxicity, and administration with GLE or SIL could provide effective protection against lipid peroxidation. These results were consistent with the report by Magdalan et al.[32] In the reports by Zheleva et al.[31] and Magdalan et al., α-AMA could increase SOD activity and decrease CAT activity, and the authors suggested that the increased lipid peroxidation was caused by the pro-oxidant properties of the toxin that prevailed over its antioxidant properties with accumulation of α-AMA in the liver. In our present study, although α-AMA decreased both SOD and CAT activity, the decrease of CAT was more obvious than the decrease of SOD. We suggest that the decrease of SOD was caused by the accumulation of highly reactive free radicals at a higher α-AMA concentration. The decrease of SOD and CAT encouraged lipid peroxidation, which led to deleterious effects such as loss of integrity and function of cell membranes. GLE and SIL act as antioxidants and free radical scavengers and could decrease lipid peroxidation and then stabilize cell membranes and protect the liver from injury induced by α-AMA.

Previous studies indicated that polysaccharides, triterpenoids, and peptide components in *G. lucidum* were the bioactive constituents responsible for its hepatoprotective effects. Further studies are ongoing to assess the hepatoprotective effects of these constituents on α-AMA-induced liver injury.

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