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Original Article

Ganoderic acid A attenuated hepatic impairment by down-regulating the intracellular JAK2-STAT3 signaling pathway in induced mushroom poisoning

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Abstract: Background: Mushroom poisoning is one of the most prominent public health problems. However, there is no special antidote so far. In the present study, we verified that Ganoderma lucidum may be an effective approach for treatment of acute mushroom poisoning. Methods: A retrospective study was performed within the past 20 years, we compiled information on the treatment of α-Amatoxin mushroom poisoning with Ganoderma lucidum by evaluating the mortality rate and liver function before and after treatment. Moreover, we explore the potential underlying mechanism of Ganoderma lucidum in the treatment of α-amanita poisoning in both in vivo animal experiments and in vitro cell experiments. Results: In our study, a total of 556 cases of mushroom poisoning were integrated over the past 20 years, the primary outcome was in-hospital mortality. Specificity, descriptive data of ALT, AST, BA and STB were evaluated for the effectiveness of protection to acute liver damage. From 1994 to 2002, there were 55 cases of mushroom poisoning in which 372 individuals were poisoned, 129 individuals died, with a mortality of 35%. Since 2002, after being treated with Ganoderma lucidum, surprisingly, the mortality decreased to 0%, and all the 184 patients were cured, the hepatic impairment improved significantly within 10 days. Based on a multivariate logistic regression analyses, after adjusting for age, gender and baseline clinical indicators, it was found that Ganoderma lucidum treatment was effective in reducing the morbidity (OR = 0.58), and Ganoderma lucidum treatment also showed an improvement in liver enzymes and in shortening the length of hospitalization significantly. Meanwhile, the main components of Ganoderma lucidum, Ganoderic acid A could significantly improve the survival rate and liver function in α-Amatoxin poisoned mice and may effectively inhibit the JAK2-STAT3 pathway, which could contribute to the detoxification in poisoned patients. Conclusion: Ganoderma lucidum is very effective in treating mushroom poisoning by α-amanita and is worth promoting.

Keywords: Ganoderma lucidum, Ganoderma lucidum A, mushroom poisoning, α-amatoxin, liver toxicity

Introduction

Mushroom poisoning is a prominent public health problem affecting public health [1], the number of deaths from mushroom poisoning with gooseberry-peptide containing mushrooms exceeds 90% of the total number of deaths from mushroom poisoning in China [2]. Gooseberry-peptide associated mushroom poisoning is a disease characterized by acute liver damage caused by the consumption of mushrooms containing α-Amatoxin [3] (**Figure 1A**). Dozens of mushrooms containing amatoxin have been found in China, and amatoxin originated mainly from Helicoverpa armigera and Ringstalk mushrooms [4]. Even though the awareness of being wary of poisonous mushrooms has increased, lots of people are still poisoned every year [5].

α-Amanitin is mainly responsible for the severe liver and kidney injury observed [6]. It is well established that α-Amanitin inhibits RNA polymerase II, thereby interfering with the transcription process [7]. RNA polymerase II transcribes all protein-coding genes and many noncoding RNAs in the eukaryotic genome. It lacks the ability to initiate transcription and cannot sus-

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Figure 1. Pictures of Amanita (A) and Ganoderma lucidum (B).

tain transcription through long DNA sequences. As a result, a series of proteins and protein complexes are required to interact with Pol II to regulate its activity and perform these essential functions [8]. However, research shows that even avoiding RNA polymerase II inactivation with structural inhibitors of α-Amatoxin did not alleviate late mortality in surviving animals [7, 9, 10], suggesting that occupancy inhibition of RNA polymerase II was not the only pathway of liver injury attributed to α-Amatoxin.

The current study confirmed that α-Amatoxin causes damage by potentially inducing an acute inflammatory response [7], which is due to continuous release of such things as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and interleukin IL23A (IL-23A). JAK2 is a member of the Janus family of tyrosine kinases, the JAK2/STAT3 signaling pathway is a signal transduction pathway stimulated by cytokines, which was discovered in recent years, and it participates in many important organisms such as Inflammatory diseases, apoptosis and immune regulation. What's more, the JAK2/STAT3 signal pathway is involved in the expression of cytokines, growth factors and hormones [11]. Many inflammatory factors, cytokines and growth factors signal, including NF-κB, interleukin 27 and epidermal growth factor, control many important cellular processes, including inflammatory and immune responses, cell proliferation and development [12-17].

Ganoderma lucidum is known as the "King of Mushrooms" with non-toxicity [18] (**Figure 1B**). It is a precious herbal medicine of medicinal and food origin. With a history of over 2,000 years, Ganoderma lucidum is an oriental mushroom that has been used for thousands of years in East Asia to improve health and longevity [18-23]. However, the underlying pathogenesis by which Ganoderma lucidum treatment exerts protective effect remains not fully understood. Ganoderic acid A (GAA) is the main active ingredient of Ganoderma lucidum [24, 25]. Which has high medicinal value and a wide range of pharmacological effects, and exhibits significant anticancer activities treat various human diseases including bronchitis, allergies, hepatitis, hypertension and immunological disorder [26-28].

In this report, we show that Ganoderma lucidum inhibited the effect of α-amanitin poisoning, which significantly improved survival and liver damage, while its main component, GAA, may downregulate the JAK2-STAT3 pathway leading to detoxification.

Materials and methods

Retrospective study

A retrospective study was performed in Xiangya Hospital, during the period from January 1, 1994 to December 31, 2022, Diagnosis of acute mushroom poisoning was confirmed based on clinical manifestations, mushroom ingestion history, laboratory test results, epidemiological data and consultation with a mycologist. The exclusion criteria were as follows: patients with hepatic disease or the other diseases that caused elevated liver enzymes, poisonous mushrooms co-ingested with other poisonous substances, patients <14 years old and cases not followed to a known outcome [29].

Patients who were diagnosed, one group was treated with Ganoderma lucidum, and both groups received conventional treatments as follows: adequate gastric lavage, blood purification, correction of electrolyte disorders and acidosis, symptomatic treatments, several antidotes such as N-acetylcysteine and silibinin.

This study was approved by the ethics committee of Xiangya Hospital (Ethical Review Number 2023030724). Data were collected from the electronic medical record system of the hospital patient records database using a designed medical chart. Parameters extracted (if available) from the included cases were as follows: gender, age, liver function. The primary outcome was in-hospital mortality and length of hospitalization, the secondary outcome included the laboratory indicators of liver function and clinical symptoms, laboratory indicators were determined upon patient arrival at the hospital.

α-Amanita toxin peptide determination

The analyzer used a Waters 600 high-performance liquid chromatograph, and toxin detection was performed at the Toadstool Institute, College of Life Sciences, Hunan Normal University. Briefly, 3 ml of blood was obtained and incubated at room temperature for 1 h, and serum (400 μl) was obtained and used for analysis (20 μl).

Animal and α-amatoxin poisoning model

Female BALB/c mice weighing 20 to 25 g were purchased from Shanghai SLAC and bred at the experimental animal center, Central South University. The mice used were specific-pathogen-free (SPF) grade and intraperitoneally injected with the indicated drugs. The animal protocol was approved by the Ethics Committee of Xiangya Hospital. All experiments strictly followed the guidelines for the investigation of experimental pain in conscious animals to minimize animal suffering and improve animal welfare.

Twenty-four mice were randomly divided into three groups: the control group, α-Amatoxin poisoning model group (0.6 mg/ml) and GAA treatment group (20 mg/kg). After 7 days of adaptive feeding, the control and model groups were injected intraperitoneally with normal saline or α-Amatoxin, and GAA was administered within 30 minutes after the administration of α-Amatoxin. Mice were anesthetized with 1% sodium pentobarbital [30, 31].

Drugs and reagents

Ganoderma lucidum and GAA (purity ≥97%) were purchased from Weikeqi Biotechnology Co., Ltd. (Chengdu, Sichuan Province, China), and α-amatoxin was purchased from Med Chem Express LLC. Antibodies were purchased from Abcam and Cell Signaling Technology.

Blood collection for detecting renal and liver function

Blood was centrifuged at 900 G for 10 min at room temperature, blood was taken from the inferior vena cava into EDTA-containing tubes. The blood was immediately centrifuged at 920 G for 10 min at 4°C. The plasma supernatant was collected into tubes and stored at -80°C until determination of aspartate aminotransferase, alanine aminotransferase, creatinine, urea and total bilirubin. Plasma biochemical parameters were measured on an Auto Analyzer (PRESTIGE 24i, PZ Cormay S.A.) and renal function and liver function were examined on 7600 automatic biochemical analyzers.

Cell culture

The normal mouse liver cell line AML12 cells (Also known as Alpha Mouse Liver 12, and we purchased from Procell Life Science & Technology Co., Ltd.) were cultured in AML-12 specialized Medium (DMEM/F12 + 10% FBS + 10 μg/ml Insulin + 5.5 μg/ml Transferrin + 5 ng/ml Selenium + 40 ng/ml Dexamethasone + 1% P/S). Purchase from cultured in Expansion Media (BI), all cell lines were routinely tested for mycoplasma contamination by using a MycoAlert mycoplasma detection kit (Lonza, Rockland) and found to be negative.

Western blot analyses

Cells or tissues were lysed in RIPA Lysis Buffer (DingGuo, China) supplemented with protease inhibitors and phosphatase inhibitors (Selleck, USA). Protein concentrations were measured with BCA reagent (Beyotime, China) on a Beckman Coulter DU-800 spectrophotometer. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with different antibodies as described in the Key Resources Table. The immunoblots were imaged using a gel image analysis system (LI-COR, USA).

Real-time RT PCR analyses

Total RNA was extracted using Magzol reagent (Magen, China), and reverse transcription reactions were performed using HiScript II Q RT

SuperMix for qPCR (Vazyme, China) according to the manufacturer's instructions. Then, 40 cycles of quantitative reverse-transcription PCR (qRT-PCR) were conducted in 96-well plates using Ultra SYBR Mixture (CWBIO, China) on the QuantStudio3 Real-Time PCR System. The fold change in gene expression was calculated by the 2-(DCtexperimental group-DCtcontrol group) method. The sequences of the primers were as follows: 5' to 3', GAPDH AGGTCGGTGTGAACGGATTTG, 3' to 5' GGGGTCGTTGATGGCAACA, 5' to 3', JAK2 TTGTGGTATTACGCCTGTGTATC, 3' to 5' ATGCCTGGTTGACTCGTCTAT, STAT3, 5' to 3'-GAGAGCAGAAGGGAGCAA, and 3' to 5' CTCACAGAGTGGGGCAA.

Histological analysis of liver and kidney

Routine histological procedures for qualitative structural analysis of the liver and kidney were performed in four mice from each group, the 4% paraformaldehyde-fixed transverse section of the liver and kidney was processed for the routine hematoxylin-eosin staining.

Statistical analysis

Continuous variables with normal distribution were presented as the mean ± standard deviation (SD), and those without normal distribution were presented as the median with interquartile range. The correlation of risk or protective factors with in-hospital mortality was performed to confirm Ganoderma lucidum is a protective factor for liver function and patient survival with logistic regression, and the results were reported as odds ratios (ORs) with 95% confidence intervals (CIs). The Mann Whitney U test and Kruskal-Wallis H test were used for comparison of continuous variables without normal distribution between two groups and among three groups, for normal distribution data t-test and ANOVA were used, respectively. *P* values <0.05 was considered statistically significant. All statistical analyses were completed using SPSS software 24.0.

Results

Ganoderma lucidum significantly lowered the mortality rate and improved liver function significantly in patients with amanitin poisoning

From January 1, 1994 to December 31, 2002, there were 55 acute mushroom poisoning cases, 172 females and 200 males, with a mean age of 43.2±15.23 years and the mortality rate of 35% (129 deaths of 372 individuals). However, there have been no deaths in 184 cases from January 1, 2002 to December 31, 2022 since our department began to use Ganoderma to treat α-amatoxin poisoning, no adverse effects have been observed. There were 91 females and 93 males with mean age of 32.6±13.4 years, and deterioration of liver function led to an increase in mortality, which was significantly reduced after treatment with Ganoderma lucidum. Moreover, the liver function was almost restored to normal levels within 7 to 10 days in Ganoderma lucidum treatment group. Based on multivariate logistic regression analyses, after adjusting for age, gender and baseline clinical indicators, we found that Ganoderma lucidum was effective in reducing the morbidity (OR = 0.58), and showed an improvement in hepatic function and shorten hospitalization time (**Figure 2**, **Tables 1**, **2**).

GAA remarkably reduced mortality and improved liver function in α-Amatoxin poisoned mice

To investigate the role of GAA in the underlying mechanism of α-Amatoxin detoxification, we compared the normal control group (NC), α-Amanitin group and GAA-treated group. The survival analysis of NC, α-Amanitin and or GAA mice indicated subjected to α-Amanitin. In line with clinical observations, GAA rescued 62.5% of mice and attenuated the liver injury marked after α-Amanitin treatment (**Figure 3**).

GAA may inhibit the JAK2/STAT3 pathway

STAT3 is phosphorylated by the tyrosine kinase JAK2 in the JAK2-STAT3 signaling pathway, which binds to and transactivate its DNA response elements that are involved in acute injury and inflammatory responses. Phosphorylated p65 targets NF-κB to particular gene subsets by activating p65 and p-RNAP II promoter recruitment. We therefore investigated whether GAA may affect this pathway when treated with α-Amanitin. After administration of α-Amanitin, the AML12 cells were treated with different concentrations of GAA. Surprisingly, the expression of JAK2, STAT3 was significantly upregulated in the α-Amanitin group but significantly downregulated in the GAA-treated group. Moreover, this phenomenon was significantly and positively correlated with the intervention dose of GAA (**Figure 4**).

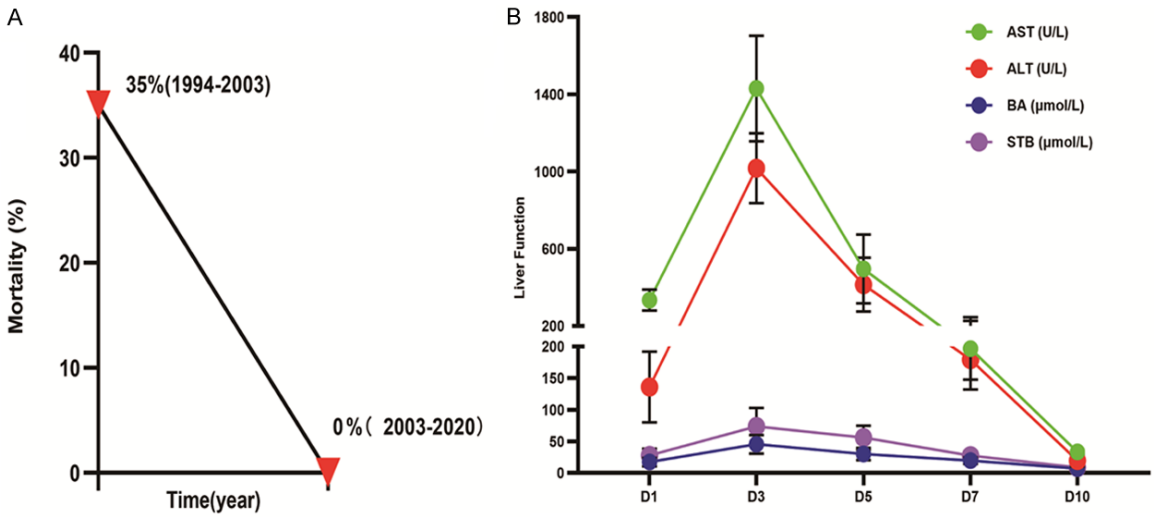

Figure 2. The change trend of the mortality and curves of liver function after treatment with Ganoderma lucidum.

Table 1. Demographic information, clinical characteristics and regression of liver function of the mortality rate of mushroom poisoning patients by α-amatoxin before and after treatment with Ganoderma lucidum for mushroom poisoning

Items	1994-2003	2003-2020	*P* value
Number of patients poisoned	372	184	
Males/females, n	200/172	93/91	0.41
Age (year)	43.2±15.23	32.6±13.4	0.18
Plasma concentration of α-Amanita toxin peptide (ng/ml)	36.00±19.07	35.10±22.61	0.718
D1-STB (μmol/L)	26.98±13.76	27.78±10.46	0.55
D3-STB (μmol/L)	120.47±23.16	73.84±28.92	<0.001
D5-STB (μmol/L)	193.19±22.47	55.86±18.86	<0.001
D7-STB (μmol/L)	261.23±49.75	27.47±6.10	<0.001
D1-BA (μmol/L)	17.82±3.26	17.34±6.58	0.376
D3-BA (μmol/L)	93.38±18.14	45.41±14.56	<0.001
D5-BA (μmol/L)	324.20±36.32	29.99±9.78	<0.001
D7-BA (μmol/L)	661.23±49.75	19.64±5.22	<0.001
D1-ALT (U/L)	129.38±41.29	136.02±55.76	0.681
D3-ALT (U/L)	1887.23±104.60	1017.96±180.63	<0.001
D5-ALT (U/L)	3793.02±136.21	414.21±139.12	<0.001
D7-ALT (U/L)	4661.21±105.00	179.10±47.05	<0.001
D1-AST (U/L)	373.12±66.84	333.61±54.34	0.192
D3-AST (U/L)	2791.71±143.12	1430.48±272.95	0.01
D5-AST (U/L)	7862.25±293.10	495.86±178.62	<0.001
D7-AST (U/L)	22754.10±106.36	196.69±49.26	<0.001
Hospital stay, d	16.30±5.50	8.16±4.32	0.036

Discussion

There is no specific antidote for mushroom poisoning due to α-Amanitin, and we have found that Ganoderma lucidum is effective in treating mushroom poisoning. The mortality rate reached 35% before 2002, which was partly attributed to most of the patients being in critical condition. However, there had been no deaths up until now since our department began to use Ganoderma lucidum, and no adverse effects observed, too.

Table 2. Multivariate Logistic regression analysis of α-amatoxin induced liver injury Association between the Ganoderma lucidum Treatment and mortality in patients with mushroom poisoning

	Estimate	SE	Wald X^2	*P* value	OR
Age	0.17	0.10	1.32	0.18	1.13
STB	0.64	0.12	0.82	0.008	1.04
BA	0.61	0.26	0.41	0.04	1.01
ALT	0.35	0.18	0.08	<0.001	1.16
AST	0.46	0.30	1.58	<0.001	1.18
Gender	-0.17	0.21	-1.63	0.41	0.84
Treatment of Ganoderma lucidum	-0.54	0.20	-2.66	0.01	0.58

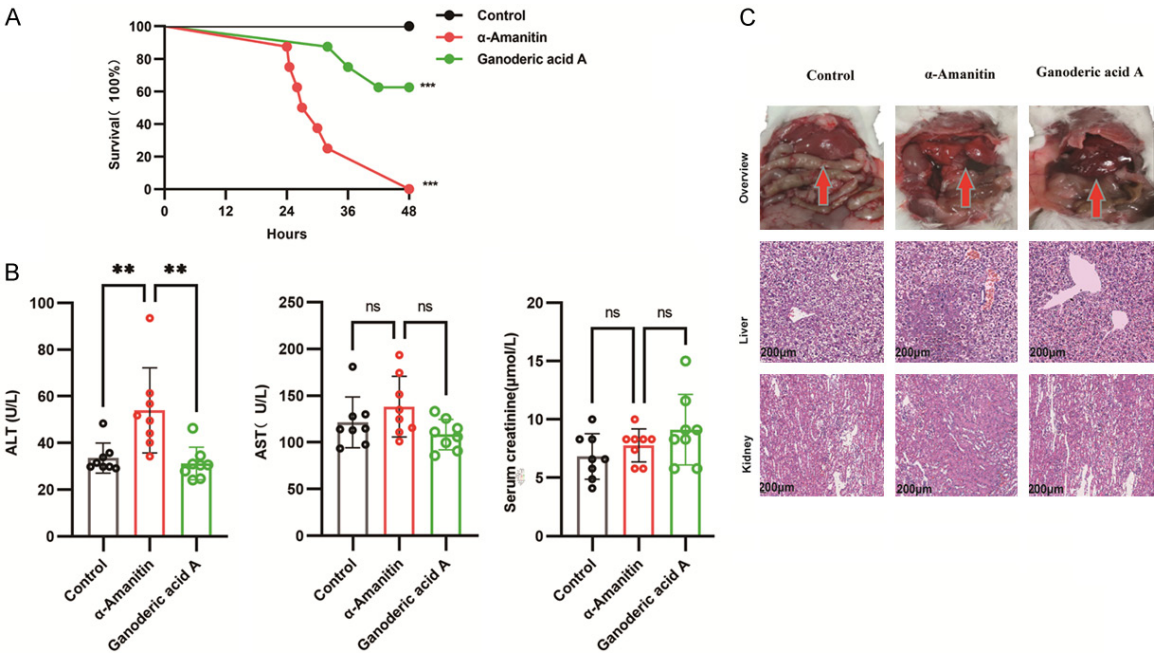

Figure 3. (A) a survival analysis of NC, α-Amanitin and or Ganoderic acid A mice indicated subjected to α-Amanitin (n = 10). (B) plasma concentrations of ALT, AST and SCr in mice 24 hours after α-Amanitin or Ganoderic acid A injection (n = 8), (C) representative images of liver tissue hematoxylin and eosin (H&E) staining of liver from three group mice. Data were pooled from at least two independent experiments. Circles represent individual mice. Error bars indicate ±SDs. ns, not significant, **P<0.01, ***P<0.001. Statistics are by one-way analysis of variance (ANOVA) or survival curve comparison [log-rank (Mantel-Cox) test].

Ganoderma lucidum contains a variety of carbohydrates [32]. GAA content accounts for more than half of Ganoderma lucidum, and the determination of GAA content has been used as the scientific basis for judging the quality of Ganoderma lucidum. Studies showed that the metabolic kinetics of the main GAA metabolites have been investigated, and GA-C2 is the most abundant reduction product of GAA, one study found the structure and activity analysis, the specific substituents of C-3 and C-15 of GAA seemed to provide the effects [27]. Furthermore, GAA can play an anti-oxidant and anti-inflammatory role and has an important regulatory effects on the human immune system [33]. Previous studies have confirmed that Ganoderma can effectively protect against liver damage caused by carbon tetrachloride and inhibit inflammation and tumorigenesis in the colon also [9, 34]. Our results showed that GAA reduced mortality ang had optimal inhibitory activity against liver injury, we identified the active GAA monomers with anti-liver toxicity activity in mushroom poisoning due to α-Amanitin.

Studies on RNAP II were shown to be of outmost importance in the development process [10]. After entering the cells, amanitin non-

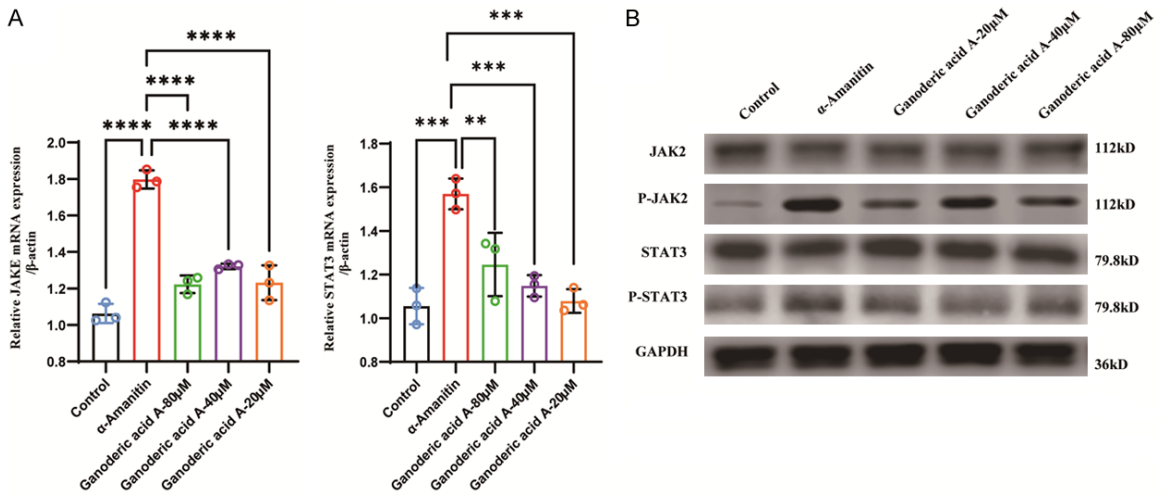

Figure 4. Ganoderic acid A return to RNA polymerase II activity via JAK2/STAT3 pathway. A. JAK2 and STAT3 were determined by chromatin immunoprecipitation-RT-PCR in melanoma cell lines (AML12). *P* values were calculated using one-way ANOVA and Dunnett's multiple comparison test. Results are presented as mean ± SD, n = 3, **P<0.01, ***P<0.001; B. Western-blot analysis of the quantity of phosphorylated (p) JAK2 and STAT3, total JAK2, total STAT3 in the livers of mice of indicated genotypes at the indicated time points after α-Amanitin and or Ganoderic acid A. n = 3 independent biological repeats.

covalently binds and inhibits the activity of RNA polymerase II in the nucleus, causing a decrease in mRNA levels and blocks protein synthesis, resulting in cell necrosis [35]. Hepatocellular damage in poisoned mice is mainly characterized by the rupture of the nucleolus and the continuous decrease in RNA polymerase II in the cell, the underlying mechanism of amanitin poisoning is inhibiting the activity of RNA polymerase II, and polymyxin B may help with this. However, suppressing or avoiding RNA polymerase II inactivation with structural inhibitors of α-Amatoxin did not alleviate late mortality simply, indicating that RNA polymerase II was not the unique target of inflammatory injuries. The JAK2-STAT3 signaling pathway is involved in acute inflammatory responses and tissue injury [36], we found that GAA alleviated the activation of the JAK2-STAT3 pathway caused by α-Amatoxin which was regulated in terms of transcription, and this mechanism will be further explored in our future studies.

In summary, our study showed Ganoderma lucidum is very effective in treating mushroom poisoning by α-amanita. GAA, as the most promising active monomer in α-Amatoxin, GAA attenuated live function development by down-regulating the intracellular JAK2-STAT3 signaling pathway, which suggesting that it might be a promising candidate drug.

This study had some limitations. Older paper medical records may be incomplete, thus, the corresponding indicators and original data were not saved, so parts of the data may not be displayed. Otherwise, the mechanisms can be further explored in terms of additional aspects, such as in vitro and in vivo experiments with activators and inhibitors of JAK/STAT3. We will take them into account in further research on this topic.

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Disclosure of conflict of interest

None.

Abbreviations

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ANOVA, one-way analysis of variance; GAA, Ganoderic acid A; RT, room temperature; qRT-PCR, Real-time quantitative polymerase chain reaction; NAC, N-acetylcysteine; SPF, specific-pathogen-free.

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