# Protective effects of compound ammonium glycyrrhizin, L-arginine, silymarin and glucurolactone against liver damage induced by ochratoxin A in primary chicken hepatocytes

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**Abstract.** Ochratoxin A (OTA) is a mycotoxin that is produced by fungi in improperly stored food and animal feed. It exhibits nephrotoxic, hepatotoxic, embryotoxic, teratogenic, neurotoxic, immunotoxic and carcinogenic effects in laboratory and farm animals. In the present study, the hepatotoxicity of OPA was investigated in chicken primary hepatocytes. On this basis, the cytoprotective effects of compound ammonium glycyrrhizin (CAG), L-arginine (L-Arg), silymarin (Sil) and glucurolactone (GA) were investigated *in vitro*. Hepatocytes were treated with OTA, which resulted in a significant decrease in cell viability and increases in serum aspartate transaminase and alanine transaminase activities, as determined by an MTT assay and commercial kits, respectively. Furthermore, following OTA treatment, the levels of hepatic antioxidants, such as superoxide dismutase and glutathione, were decreased, and the lipid peroxidation product malondialdehyde was increased, compared with the control group. However, pretreatment with CAG, L-Arg, Sil and GA significantly ameliorated these alterations and Sil exerted the optimum hepatoprotective effect. The apoptotic rates were measured by flow cytometry and the results revealed that OTA increased cell apoptosis. The four types of hepatoprotective compounds employed in the present study decreased the apoptosis rate and significantly reversed OTA-induced increases in the mRNA expression levels of caspase-3, which was determined by reverse transcription-quantitative polymerase chain reaction. Furthermore, B-cell lymphoma-2 (Bcl-2) mRNA expression was increased in OTA-treated cells when pretreated with CAG, L-Arg, Sil and GA. However, no alterations in the mRNA expression of Bcl-2-associated X were observed in the L-Arg and GA groups, compared with the OTA-only group. These results indicate that OTA may exhibit hepatotoxicity in chickens and that CAG, L-Arg, Sil and GA may protect the liver against this via anti-oxidative and antiapoptosis mechanisms. In addition, CAG and GA are likely to mediate their effects through the mitochondrion-dependent apoptosis pathway; however, the exact hepatoprotective mechanism of L-Arg and GA require further investigation. Therefore, CAG, L-Arg, Sil and GA are potential candidates for the prevention and treatment of chicken liver injury.

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*Key words:* ochratoxin A, hepatotoxicity, hepatoprotective drugs, oxidative stress, cell apoptosis

## Introduction

Ochratoxin A (OTA) is produced by the *Penicillium verrucosum* fungus and various species of aspergillus, and among the mycotoxins with great public health and agroeconomic importance (1). It is a widespread contaminant of a variety of animal and human food and is not easy decomposed, and dietary intake of OTA may be unavoidable (2,3). As the elimination of OTA from the body is slow, it accumulates in the tissues and fluids of humans and animals that consume food contaminated with this toxin (4). Notably, reports have demonstrated that even exposure to low concentrations of OTA in domestic and experimental animals leads to morphological and functional alterations in renal and hepatic tissues. In addition, it has been classified as potentially carcinogenic, genotoxic and teratogenic to humans (group 2B according to the International Agency for Research on Cancer classification) (5,6).

It has been indicated that the mechanisms underlying OTA toxicity may include the inhibition of protein synthesis, reactive oxygen species (ROS) formation, lipid peroxidation, altered calcium homeostasis and impaired mitochondrial oxidation reactions (7-9). Studies have demonstrated that OTA exhibits a dose-dependent inhibition of HepG2 human hepatoma cell viability, presenting typical sigmoid curves (10,11). Furthermore, OTA has been demonstrated to enhance ROS levels and oxidative damage in certain immortalized renal and cancer cell lines, including HK-2 human renal proximal tubular epithelial cells, primary rat proximal tubular cells, LLC-PK1 proximal tubular cells, HepG2 and CaCo-2 human

colonic adenocarcinoma cells (9,12-14). In addition, it established that oxidative stress leads to the induction of numerous cellular processes, which include apoptosis and the arrest of growth, as well as the stimulation of certain transcription factors. It is thought that apoptosis activation may be among the primary cellular mechanisms underlying OTA-induced toxicity, particularly renal toxicity (15). However, whether OTA induces oxidative stress and apoptosis, and its potential role in chicken primary hepatocytes, remains unknown.

Glycyrrhizin is the most important and recognized bioactive component of licorice root. This compound has been employed for >20 years in patients suffering from chronic hepatitis in China and Japan (16-19). The major components of compound ammonium glycyrrhizin (CAG) are glycyrrhizin, glycine and methionine, and this compound is reported to be an effective anti-inflammatory, anticancer, antihepatotoxic and antioxidant agent (20-22). L-arginine (L-Arg) is a semi-essential amino acid that has roles in the synthesis of protein and creatine, and is also involved in nitrogen balance (23). It also functions as a scavenger of free radicals and is a substrate for nitric oxide synthase, which means it has protective effects in endothelial damage. Certain previous studies have demonstrated that L-Arg exerts protective effects in certain chronic diseases (24,25). Silymarin (Sil), a hepatoprotective agent, is a flavonolignan that is extracted from milk thistle (*Silybum marianum*) that has been employed as a natural treatment for various liver diseases for a number of decades (26). It has been reported that Sil may exhibit anti-inflammatory, antioxidant and anticancer properties, which have been associated with its potential therapeutic effects (27). Glucurolactone (GA) is a conventional hepatoprotective drug that is used in epidemical hepatitis, cirrhosis of the liver and poisoning due to food and drugs. It acts as a hepatic antidote and immune regulator. Thus, it is reasonable to investigate the protective effects of CAG, L-Arg, Sil and GA on OTA-induced injury in chicken primary hepatocytes.

China faces a food shortage and available food is frequently contaminated with mycotoxins (28), particularly OTA. As a site of metabolism, the liver is an important target for the majority of xenobiotics, and the effect of OTA on this organ remains uncertain. To assess the potential for hepatoxicity following OTA exposure, the present study performed experiments in chicken primary hepatocytes to evaluate the potential protective effect of four hepatoprotective agents against liver disease in chickens.

## Materials and methods

*Materials*. OTA was purchased from Fermentek, Ltd. (Jerusalem). Dulbecco's modified Eagle's medium (DMEM) was obtained from Hyclone; GE Healthcare Life Sciences (Logan, UT, USA). Collagenase (type IV), HEPES and MTT reagent were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). CAG was produced in-house (per 100 g containing 2.8 g ammonium glycyrrhizin, 2 g glycine and 2 g methionine).

*Cell culture*. Hepatocytes were isolated from a male Hy-line variety brown chicken by an improved two-step collagenase perfusion method (29). A total of ~20 chickens were obtained from the Nanjing Tangquan Chicken Farm (Nanjing, China) and treated at a controlled temperature (24˚C) under a 12-h light-dark cycle and fed standard laboratory chow and water *ad libitum*. Chickens were housed in accordance with the National Institutes of Nanjing Agriculture University for the Care and Use of Laboratory Animals. The chickens were raised until 5 months old, weighing 1-1.5 kg, before experiments. The current study was approved by the Animal Care and Use Committee of Nanjing Agricultural University, (Nanjing, China; license number: SYXK2017-0007). The liver was separated from the chicken after ligating the blood vessels such as the pancreaticoduodenal veins, mesenteric vein and inferior caval vein, which are located across the liver. The liver was subsequently perfused with saline solution A (33 mM/l HEPES, 127.8 mM/l NaCl, 3.15 mM/l KCl, 0.7 mM/l $Na_2HPO_4$•$12H_2O$, 0.6 mM/l EGTA, pH 7.4) for 30 min and saline solution B (33 mM/l HEPES, 127.8 mM/l NaCl, 3.15 mM/l KCl, 0.7 mM/l $Na_2HPO_4$•$12H_2O$, 3 mM/l $CaCl_2$, pH 7.4) for 15 min at 37˚C. Subsequently, 0.5% collagenase IV was used to digest the liver at a flow of 20 ml/min for 20-25 min at 37˚C. Hepatocytes were separated under aseptic conditions and cultured in DMEM containing 10% fetal bovine serum (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 0.5 mg/l bovine insulin (cat. no. I8040; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The hepatocytes were seeded in plates, diluted to a final concentration of $5x10^5$ cells/ml and incubated at 37˚C in a humidified incubator with an atmosphere of 5% $CO_2$.

*OTA cytotoxicity detection by MTT assays and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurement*. Hepatocytes were seeded in 96-well plates at a density of $5x10^5$ cells per well in 0.1 ml DMEM and were exposed to increasing concentrations of OTA (0.25, 0.5, 1, 2 and 4 $\mu$g/ml) for 24 h. Cell viability was assayed by the MTT assay [1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan Thiazolyl blue formazan; cat. no. 57360-69-7; Sigma-Aldrich; Merck KGaA] (30). MTT stock solution (5 mg/ml) was then applied to each of the wells, and the cells were incubated in a humidified atmosphere for 4 h. The absorbance of the samples was measured using a microtiter plate reader at a dual wavelength mode of 490 and 655 nm. DMSO was used to dissolve the formazan. Cell culture supernatants were collected and assayed for ALT (Reitman Frankel assay) and AST activities using commercial kits (cat. nos. C009-2 and C010-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol.

*Effects of CAG, L-Arg, Sil and GA on OTA-induced hepatocyte injury*. The protective effects of CAG, L-Arg, Sil and GA on chicken hepatocyte injury was investigated *in vitro*. Hepatocytes were seeded in 96-well plates at a density of $5x10^5$ cells per well. Different batches of cells were then incubated with CAG, L-Arg, Sil and GA at concentrations of 0.1, 1, and 10 $\mu$g/ml for 24 h at 37˚C. Following incubation, the supernatant was discarded, and the cells were exposed to OTA concentrations of OTA 1 $\mu$g/ml for 24 h at 37˚C. Cell viability was determined using the MTT assay. The activities of AST and ALT in cell culture supernatants were detected using commercial kits, according to the manufacturer's

Table I. Primer sequences of target genes for reverse transcription-quantitative polymerase chain reaction.

| Target gene | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| β-actin | ATGTGGATCAGCAAGCAGGAGTA | TTTATGCGCATTTATGGGTTTTGT |
| Caspase-3 | CTGAAGGCTCCTGGTTTA | TGCCACTCTGCGATTTAC |
| Bax | GTGATGGCATGGGACATAGCTC | TGGCGTAGACCTTGCGGATAA |
| Bcl-2 | ATCGTCGCCTTCTTCGAGTT | ATCCCATCCTCCGTTGTCCT |

Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X.

protocol (cat. nos. C009-2 and C010-2; Nanjing Jiancheng Bioengineering Institute).

*Superoxide dismutase (SOD) activity, and glutathione (GSH) and malondialdehyde (MDA) levels*. Cells were then incubated with CAG, L-Arg, Sil and GA at concentrations of 1 $\mu$g/ml for 24 h at 37˚C. Following incubation, the supernatant was discarded, and the cells were exposed to OTA for 24 h at a concentration that induces death of 50% of the hepatocytes at 37˚C. Following treatments, hepatocytes were washed twice with 300 $\mu$l PBS (pH 7.4). The cell supernatants were used to measure SOD activity and the levels of GSH and MDA using SOD (Superoxide Dismutase assay kit; WST-1 method), GSH (reduced glutathione assay kit) and MDA (malondialdehyde assay kit; TBA method) kits (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's protocol.

*Flow cytometric analysis of apoptosis by annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining*. Hepatocytes were seeded in 96-well plates at a density of $5x10^5$ cells per well. Different batches of cells were then incubated with CAG, L-Arg, Sil and GA at concentrations of 1 $\mu$g/ml for 24 h at 37˚C. Following incubation, the supernatant was discarded, and the cells were exposed to OTA concentrations of OTA 1 $\mu$g/ml for 24 h at a concentration that induces death of 50% of the hepatocytes at 37˚C. In order to evaluate apoptosis, cells were treated as above and subjected to staining with an Annexin V-FITC/PI Apoptosis Detection kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's protocol. The apoptosis rates were analyzed by flow cytometry Flowjo V10.0.7; FlowJo LLC, Ashland, OR, USA).

*Relative quantification of apoptosis-associated gene expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Hepatocytes were seeded in 96-well plates at a density of $5x10^5$ cells per well. Different batches of cells were then incubated with CAG, L-Arg, Sil and GA at concentrations of 1 $\mu$g/ml for 24 h at 37˚C. Following incubation, the supernatant was discarded, and the cells were exposed to OTA concentrations of OTA 1 $\mu$g/ml for 24 h at a concentration that induces death of 50% of the hepatocytes at 37˚C. Total RNA from the six groups i) the control group, ii) OTA group, iii) OTA-treated hepatocytes pretreated with CAG, iv) OTA-treated hepatocytes pretreated with L-Arg, v) OTA-treated hepatocytes pretreated with Sil and vi) OTA-treated hepatocytes pretreated with GA) was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.). cDNA synthesis was performed with 1 $\mu$g total RNA using a cDNA synthesis kit (HIScipt™Q RT SuperMIX for qPCR, R123-01; Vazyme, Piscataway, NJ, USA; 42˚C for 15 min, 85˚C for 2 min). The mRNA expression levels of caspase-3, B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X (Bax) and β-actin were quantified by qPCR (AceQ® qPCR SYBR® Green Master Mix; Vazyme; Stage 1: Reps: 1, 95˚C for 5 min; Stage 2: Reps: 40, 95˚C for 10 sec, 60˚C for 30 sec; Stage 3: Reps: 1, 95˚C for 15 sec, 60˚C for 60 sec, 95˚C for 15 sec; CFX96 Real-time PCR Detection System; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primer sequences used are listed in Table I. The relative expression of target genes was normalized to β-actin. Data were calculated using the $2^{-\Delta\Delta Cq}$ method where $\Delta\Delta Cq=(C_{q,target}-C_{q,actin})$ treatment-$(C_{q,target}-C_{q,actin})$ control (31).

*Statistical analysis*. All the experiments were repeated three times. Data are presented as the mean ± standard deviation. Tukey's post hoc test of one-way analysis of variance was used for statistical comparisons. Graphs were plotted using GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Cytotoxicity of OTA in primary chicken hepatocytes*. Compared with control hepatocytes (Fig. 1A), the cell number and density decreased markedly and ruptured and necrotic hepatocytes were present in the supernatant in hepatocytes treated with different concentrations of OTA (Fig. 1B-F). The inhibitory concentration 50 ($IC_{50}$) was a standard to determine the dose of OTA that induced liver injury. The $IC_{50}$ was estimated from Fig. 2A. Results of MTT assays revealed that the cell viabilities were 51.33±4.27 and 47.28±4.42% when hepatocytes were exposed to 1 and 2 $\mu$g/ml OTA, respectively (Fig. 2A). As demonstrated in Fig. 2B and C, the activities of ALT and AST were dose-dependently increased compared with control cells following treatment with different concentrations of OTA. However, the activities of ALT and AST were significantly increased at a dose of 1 $\mu$g/ml OTA compared with the control group ($P<0.05$). These results indicated that OTA induced hepatocellular injury and the optimum injury dose was 1 $\mu$g/ml as it was the lowest dose to induce significant increases in ALT and AST.

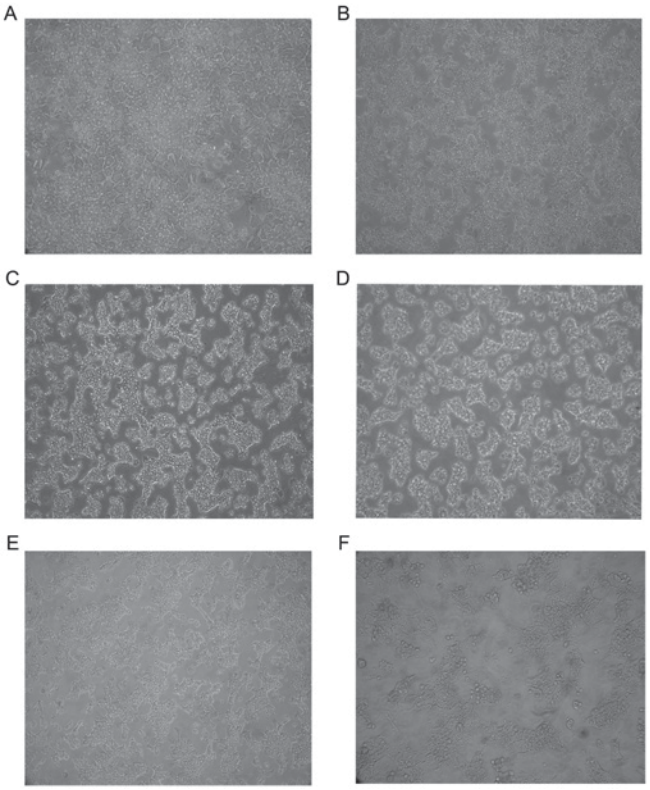


Figure 1. Morphological characterization of hepatocytes exposed to different concentrations of OTA. The hepatocytes were observed by using an inverted phase contrast microscope (magnification, x200). (A) Normal hepatocytes cultured for 24 h. Hepatocytes treated with OTA at (B) 0.25 $\mu$g/ml, (C) 0.5 $\mu$g/ml, (D) 1 $\mu$g/ml, (E) 2 $\mu$g/ml and (F) 4 $\mu$g/ml). OTA, ochratoxin A.

Table II. Effects of four hepatoprotective agents on the activities of ALT and AST in cell culture supernatants of OTA-treated hepatocytes.

| Group | ALT (U/l) | AST (U/l) |
|---|---|---|
| Control | 14.36±2.85 | 11.53±2.29 |
| OTA (1 $\mu$g/ml) | 32.19±4.14[a] | 23.38±1.45[a] |
| CAG ($\mu$g/ml) | | |
| 0.1 | 21.30±4.10[c] | 20.47±4.73 |
| 1 | 15.15±3.25[c] | 18.61±3.29 |
| 10 | 14.46±5.55[c] | 19.23±3.04 |
| L-Arg ($\mu$g/ml) | | |
| 0.1 | 17.56±4.57[c] | 18.54±4.19 |
| 1 | 20.94±2.76[c] | 16.43±3.14[b] |
| 10 | 17.02±2.19[c] | 18.08±2.20[b] |
| Sil ($\mu$g/ml) | | |
| 0.1 | 11.80±3.79[c] | 17.68±0.36[c] |
| 1 | 18.40±5.76[c] | 11.40±0.67[c] |
| 10 | 21.63±4.00[c] | 16.81±3.63[c] |
| GA ($\mu$g/ml) | | |
| 0.1 | 19.36±5.22[c] | 14.28±1.19[c] |
| 1 | 18.49±3.06[c] | 11.08±1.03[c] |
| 10 | 14.71±2.36[c] | 13.32±3.19[c] |

Hepatocytes were pretreated with CAG, L-Arg, Sil and GA prior to exposure to OTA for 24 h. Values are presented as the mean ± standard deviation (n=3). [a]P<0.01 vs. control; [b]P<0.05 and [c]P<0.01 vs. OTA group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; OTA, ochratoxin A; CAG, compound ammonium glycyrrhizin; L-Arg, L-arginine; Sil, silymarin; GA, glucurolactone.

*Effects of CAG, L-Arg, Sil and GA on OTA-induced hepatocellular injury*. As demonstrated in Fig. 2D-G, the cell viability was decreased compared with the control group when exposed to OTA for 24 h. However, the cell viabilities increased significantly when hepatocytes were pretreated with CAG, L-Arg, Sil and GA at concentrations of 0.1, 1 and 10 $\mu$g/ml in comparison with the OTA-only group (Fig. 2D-G). CAG and GA dose-dependently increased cell viability (Fig. 2D and G). However, the cell viabilities following treatment with L-Arg and Sil at a concentration of 10 $\mu$g/ml were lower than at the other concentrations (Fig. 2E and F). In addition, it was demonstrated that OTA treatment increased ALT and AST activities in the cell culture supernatant of hepatocytes, compared with control cells (P<0.01; Table II). In comparison with the OTA group, CAG, L-Arg, Sil and GA decreased the ALT activities (P<0.01; Table II); however, CAG had no significant effect on the AST activity induced by OTA.

*Effects of CAG, L-Arg, Sil and GA on SOD activity, and GSH and MDA levels*. Treating liver cells with 1 $\mu$g/ml OTA for 24 h reduced the SOD activity and GSH levels (Fig. 3A and B), while OTA treatment resulted in an increase in MDA (Fig. 3C), compared with control cells. Pretreatment with CAG, L-Arg, Sil and GA (1 $\mu$g/ml) for 24 h resulted in a significant increase in SOD activity and GSH levels (P<0.05; Fig. 3A and B) and a decrease in MDA levels (P<0.05; Fig. 3C), compared with the OTA-only group. Sil exhibited the largest significant differences compared with the OTA treatment group. These results indicate that Sil may exhibit an enhanced antioxidation activity compared with the other three hepatoprotective agents employed in the current study.

*Expression of apoptosis-associated genes*. The mRNA expression levels of caspase-3 increased following OTA treatment, compared with control cells, and decreased following pretreatment with CAG, L-Arg, Sil and GA, compared with the OTA-only group (Fig. 3D). The mRNA expression levels of Bcl-2 decreased compared with control cells following OTA treatment, and increased following pretreatment with CAG, L-Arg, Sil and GA in OTA-treated cells (Fig. 3E). Although the mRNA expression levels of Bax increased compared with control cells following OTA treatment, and significantly decreased following treatment with CAG and Sil in OTA-treated cells, Bax levels were not significantly altered in OTA-treated cells following pretreatment with L-Arg and GA (Fig. 3F).

*Effects of CAG, L-Arg, Sil and GA on OTA-induced apoptosis*. The apoptosis rate (only the Q3 quadrant) was determined in chicken hepatocytes by flow cytometry, and the results demonstrated that exposure to OTA induced a significant increase in apoptosis compared with control cells (Fig. 4A

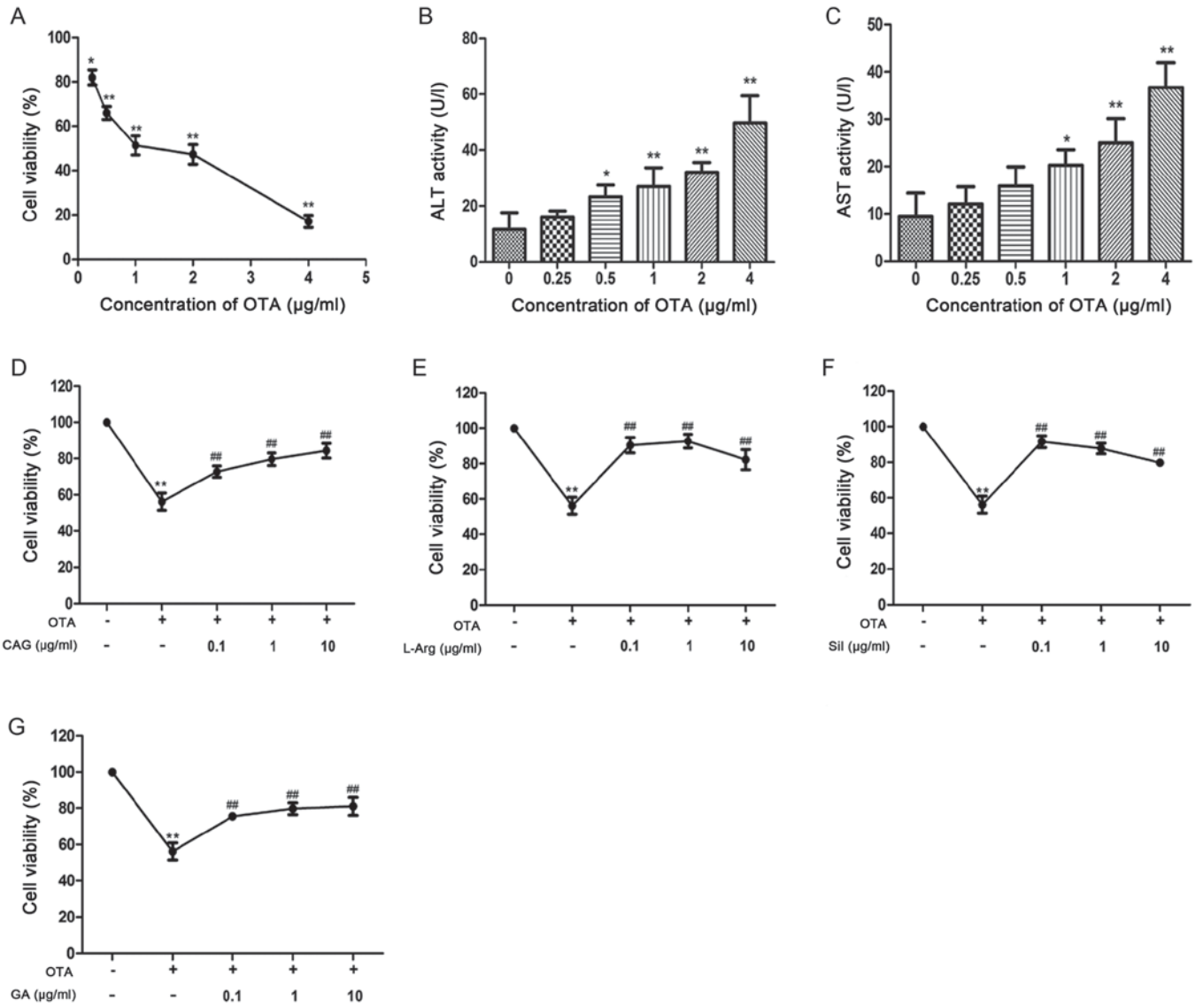


Figure 2. OTA-induced effects on cell viability and liver injury markers. (A) Dose-response effect of OTA on cell viability. (B) ALT and (C) AST activity was measured in the culture medium of untreated hepatocytes and hepatocytes treated with various concentrations of OTA. The effects of various concentrations of (D) CAG, (E) L-Arg, (F) Sil and (G) GA on cell viability in hepatocytes treated with 1 $\mu$g/ml OTA. $^{*}$P<0.05 and $^{**}$P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. OTA group. OTA, ochratoxin A; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAG, compound ammonium glycyrrhizin; L-Arg, L-arginine; Sil, silymarin; GA, glucurolactone.

Table III. Early apoptosis rate in different treatment groups.

| Group | Apoptosis rate (%) |
|---|---|
| Control | 1.80±0.45 |
| OTA | 32.06±2.11[a] |
| CAG | 14.90±1.50[c] |
| L-Arg | 18.76±1.55[c] |
| Sil | 12.86±2.12[c] |
| GA | 27.50±2.16[b] |

Hepatocytes were pretreated with CAG, L-Arg, Sil and GA prior to exposure to OTA for 24 h. Values are presented as the mean ± standard deviation (n=3). [a]P<0.01 vs. control [b]P<0.05 and [c]P<0.01 vs. OTA group. OTA, ochratoxin A; CAG, compound ammonium glycyrrhizin; L-Arg, L-arginine; Sil, silymarin; GA, glucurolactone.

and B, and Table III; P<0.01). Following pretreatment with CAG, L-Arg, Sil and GA, the apoptotic rate decreased to 14.90±1.50, 18.76±1.55, 12.86±2.12 and 27.50±2.16%, respectively (Table III). Representative flow cytometry plots are given for CAG, L-Arg, Sil and GA pretreatment groups in Fig. 4C-F.

## Discussion

OTA is a mycotoxin contaminant of food that primarily leads to nephrotoxicity and hepatotoxicity (3,32). OTA has a stronger toxicity compared with the other mycotoxins, excluding aflatoxin (2). Li *et al* (33) reported that OTA was slightly more effective at reducing cell viability in HepG2 cells, with an effective concentration 50 ($EC_{50}$) of 37.30 $\mu$M, compared with zearalenone (ZEA), which had an $EC_{50}$ of 41.28 $\mu$M. In addition, OTA was demonstrated to reduce the cell viability of

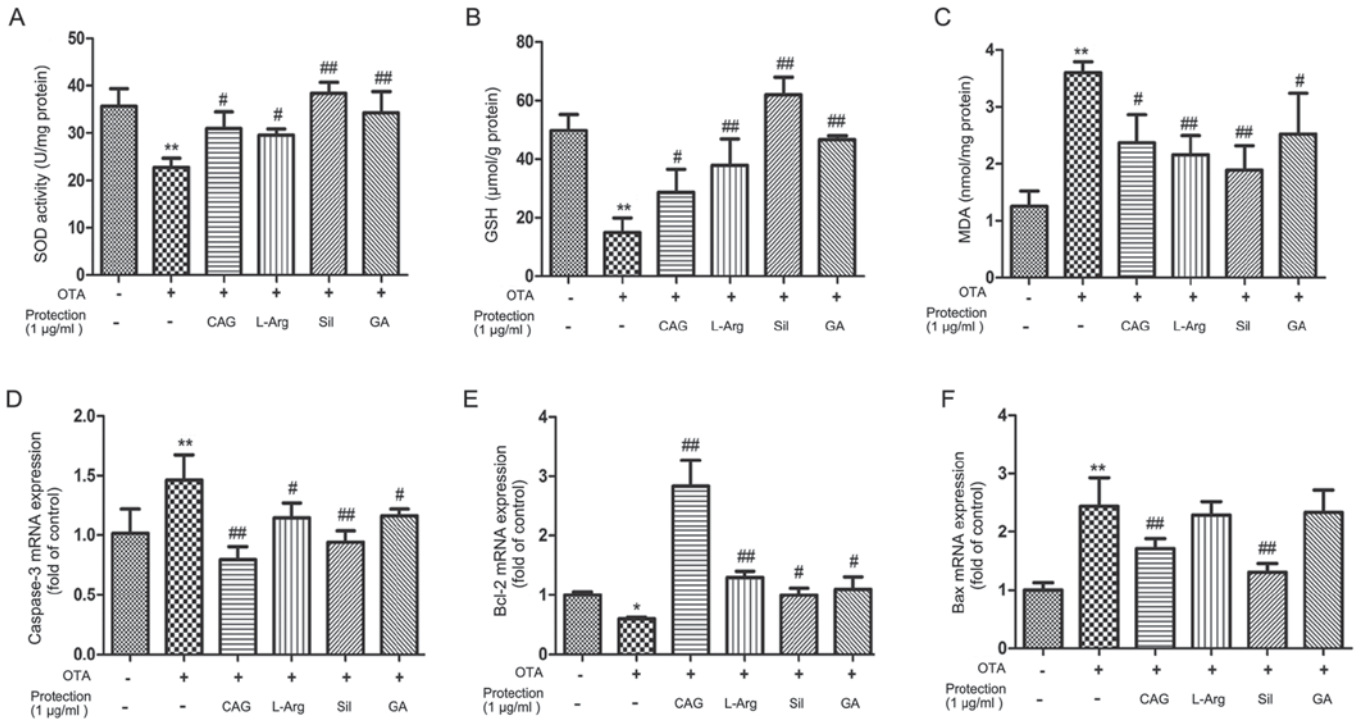


Figure 3. The effects of CAG, L-Arg, Sil and GA pretreatment on oxidative stress and cellular antioxidant enzymes. The levels of (A) SOD, (B) GSH and (C) MDA were measured in hepatocytes following treatment with 1 $\mu$g/ml OTA with or without 1 $\mu$g/ml CAG, L-Arg, Sil and GA. The effect of CAG, L-Arg, Sil and GA on OTA-induced alterations in the mRNA expression of hepatocellular genes associated with apoptosis, including (D) caspase-3, (E) Bcl-2 and (F) Bax. $^{*}$P<0.05 and $^{**}$P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. OTA group. CAG, compound ammonium glycyrrhizin; L-Arg, L-arginine; Sil, silymarin; GA, glucurolactone; SOD, superoxide dismutase; GSH, glutathione; MDA, malondialdehyde; OTA, ochratoxin A; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X.

KK-1 murine granular cells to a larger degree compared with ZEA, with an $EC_{50}$ ~7-fold lower compared with the EC50 for ZEA (34). These results indicated that OTA may exhibit a stronger effect than ZEA on cell viability. Klarić *et al* (35) demonstrated that the $IC_{50}$ of OTA and citrinin (CTN) in PK15 porcine kidney cells were 14.0±2.4 and 73.5±1.0 $\mu$M, respectively. Therefore, the toxicity of OTA was much higher than CTN. Wilk-Zasadna and Minta (36) demonstrated that rat embryo midbrain micromass cells exposed to OTA exhibited a dose-dependent reduction in viability, and the $IC_{50}$ was 2.52±0.062 $\mu$g/ml. In the present study, the $IC_{50}$ of OTA in chicken hepatocytes was estimated to be 1 $\mu$g/ml, which was lower than in OTA-treated rat embryo midbrain micromass cells (36). This indicates that chicken hepatocytes may be more susceptible to OTA.

Oxidative stress is characterized by an imbalance between pro-oxidant and antioxidant molecules, which subsequently results in damage to cells. Various methods are employed to assess the levels of oxidative stress, and these methods involve the quantification of products of peroxidation or antioxidants (37). In the present study, as an end-product of lipid peroxidation, which is among the processes implicated in oxidative stress-induced damage and is associated with mycotoxin-induced cytotoxicity, MDA was selected as a measure of oxidative stress and hepatocyte damage (38). By contrast, SOD and GSH are reported to protect host cells against oxidative damage by scavenging free radicals. Zheng *et al* (39) confirmed that OTA led to the induction of oxidative damage in HepG2 cells, while Klarić *et al* (40) demonstrated that OTA also induced marked oxidative stress in the porcine kidney. The present study demonstrated high levels of MDA, and decreased SOD activity and GSH concentration, in cell lysates collected from the OTA group, which indicated that OTA may have triggered oxidative damage to the cell membranes of hepatocytes. This concurs with the study by Klarić *et al* (40); however, a lower concentration was used in the present study compared with this previous study.

Liver damage induced by hepatotoxins may be a result of increased hepatocyte apoptosis, which is a form of programmed cell death. Apoptosis facilitates the removal of damaged cells (41). A previous study demonstrated that OTA-induced apoptosis was a result the activation of a mitochondrion-dependent pathway, where OTA led to increased ROS formation and decreased mitochondrial transmembrane potential through mitochondrial pore opening, subsequently allowing cytochrome c release and the downstream activation of caspases (42). El Golli Bennour *et al* (43) also demonstrated that exposure of human hepatocarcinoma cells to OTA led to the induction of caspase-dependent apoptosis via the mitochondrial pathway. In the present study, the apoptotic rate was determined by flow cytometry and measuring the expression of target genes associated with the mitochondrion-dependent apoptotic pathway by RT-qPCR. The apoptotic rate was 32.06±2.11% when the cells were exposed to 1 $\mu$g/ml OTA for 24 h. In addition, the mRNA expression of caspase-3 and Bax increased, while Bcl-2 decreased, compared with control cells. Therefore, OTA may induce chicken hepatocellular apoptosis by activating the mitochondrion-dependent pathway.

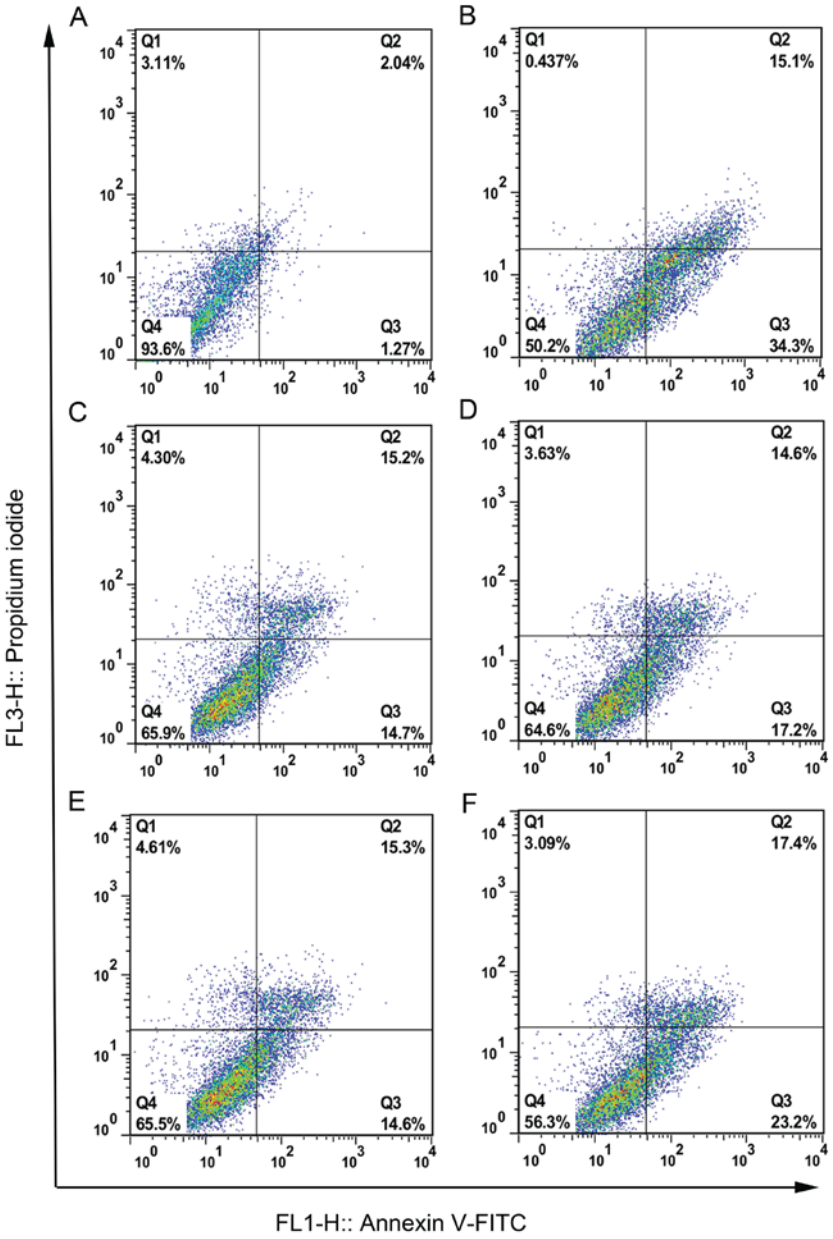


Figure 4. The effects of CAG, L-Arg, Sil and GA pretreatment on apoptosis in OTA-treated chicken hepatocytes. Representative flow cytometry plots are presented for (A) control group, (B) OTA group, (C) OTA-treated hepatocytes pretreated with CAG, (D) OTA-treated hepatocytes pretreated with L-Arg, (E) OTA-treated hepatocytes pretreated with Sil and (F) OTA-treated hepatocytes pretreated with GA. Q3 indicated early apoptotic cells. CAG, compound ammonium glycyrrhizin; L-Arg, L-arginine; Sil, silymarin; GA, glucurolactone; OTA, ochratoxin A; FITC, fluorescein isothiocyanate.

CAG, L-Arg, Sil and GA are commonly employed in the clinic as hepatoprotective agents. Glycyrrhizic acid is a triterpene saponin glycoside and the major bioactive compound of *Glyccyrhiza glabra* (liquorice) plant root extract, which is a member of the leguminosae family (44,45). In Japan, glycyrrhizic acid has been employed in the clinic for >20 years in patients suffering from chronic hepatitis (46). Among the 20 most numerous amino acids in mammals, L-Arg is considered to be a semi-essential or conditionally essential amino acid, depending on the developmental stage and health status of each individual (47). L-Arg is the precursor in nitric oxide synthesis (48). Sil is a flavonoid mixture extracted from *Silybum marium* (49). GA is a natural compound that functions as an essential structural component of the majority of connective tissues (50). The liver produces GA from glucose, which subsequently acts as an inhibitor of the B-glucuronidase enzyme, a metabolizer of glucuronides, which leads to increases in the blood levels of glucuronide. Glucuronides interact with various toxic compounds, including morphine and depot medroxyprogesterone acetate, and convert them to water-soluble glucuronide-conjugates, which allows them to be excreted via the urine. Yin *et al* (51) reported that pretreatment, and a combination of pre- and post-treatment, of hepatocytes with *Glycyrrhiza glabra* extract significantly reversed carbon tetrachloride-induced increases in lactate dehydrogenase, glutamate oxalate transaminase, glutamate pyruvate transaminase and MDA, and increased levels of SOD and glutathione peroxidase that were reduced by treatment with carbon tetrachloride. Shweta and Khanna (52) reported that L-Arg increased SOD and GSH levels in newborns. In the present

study, similar findings were obtained. Following pretreatment with Sil for 24 h, the cell viability increased, and the activity of ALT and AST decreased. Kumar *et al* (53) demonstrated that Sil liposomes prevented the paracetamol-induced decreases in GSH and SOD levels, and increases in MDA, which are responsible for the toxic effect of paracetamol. GA is commonly employed to protect the liver, however, there are few reports concerning the underlying hepatoprotective mechanism. The current study investigated the mechanism of GA in protecting the liver by using chicken primary hepatocytes. The results demonstrated that CAG, L-Arg, Sil and GA improved cell viability and inhibited the elevation of ALT. Arg, Sil and GA also decreased the activity of AST in supernatants, but CAG exhibit no effect on AST activity. The cell viability increased and ALT activity was decreased in a dose-dependent manner following treatment with CAG and GA. L-Arg exhibited an optimum protective effect at a lower dose. Sil had optimum function at a dose of 1 $\mu$g/ml. These observations indicated that CAG, Arg, Sil and GA may protect the viability of chicken hepatocytes. CAG, L-Arg, Sil and GA increased the levels of SOD and GSH, and decreased MDA levels, with Sil exhibiting the largest effect of the four agents. These results demonstrated that the four hepatoprotective agents employed in the present study may exhibit anti-oxidative effects in chicken hepatocytes.

Glycyrrhizic acid was reported to exhibit an antiapoptotic effect via the suppression of caspase-3, which may explain the hepatoprotective effect of glycyrrhizic acid (54). Glycyrrhizic acid has also been demonstrated to inhibit cytochrome c release into the cytoplasm from the mitochondria. Tuorkey (55) reported that Sil may protect cardiomyocytes against apoptosis induced by diabetes. The sarcoplasm of diabetic rats that received Sil treatment had an appearance that was similar to that of non-diabetic rats. There are few reports investigating the antiapoptotic effects of L-Arg and GA. In the present study, cell apoptosis rates were decreased when pretreated with CAG, L-Arg, Sil and GA, in comparison with the OTA group. The antiapoptotic ability of the four drugs was Sil>CAG>L-Arg>GA. These hepatoprotective drugs decreased the mRNA expression levels of caspase-3 and increased Bcl-2 expression, but no effects of L-Arg and GA were observed on Bax mRNA expression levels in OTA-treated cells. It was therefore concluded that the four hepatoprotective agents used in the current study exhibited an antiapoptotic effect in chicken hepatocytes. In addition, CAG and GA are likely to induce their effects through the mitochondrion-dependent pathway, while the exact hepatoprotective mechanisms of L-Arg and GA requires further research.

In conclusion, *in vitro* cell culture assays have contributed to OTA research by investigating the biochemical mechanisms of cytotoxicity. OTA may induce hepatotoxicity in chicken primary hepatocytes. The results of the current study indicate that oxidative stress and apoptosis may be implicated in OTA-induced hepatocellular injury. OTA is likely to mediate its effects through the mitochondrion-dependent apoptotic pathway. The present findings also demonstrated the hepatoprotective, antioxidant activities and antiapoptotic effects of CAG, L-Arg, Sil and GA in OTA-treated cultured hepatocytes of chickens. The present study demonstrates the mechanisms of OTA in chicken hepatocytes and that CAG, L-Arg, Sil and GA administration may be used an alternative therapy to treat or prevent acute hepatic damage.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

ZY designed, screened and optimized the formulation. FW and JT performed the experiments and wrote the paper. XG and RA did the preparation of the experiments. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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