

Protective effect of andrographolide against concanavalin A-induced liver injury

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Abstract This study was designed to investigate the hepatic protective effect and the molecular mechanisms of andrographolide in concanavalin A-induced liver injury model. Results showed that andrographolide (Ag) attenuated concanavalin A (Con-A)-induced liver injury and inhibited hepatocyte apoptosis. Further results showed that oxidative stress response genes were significantly elevated during the pathogenesis induced by Con-A. Meanwhile, gadolinium chloride and *N*-acetyl-L-cysteine (NAC) treatment, which inactivates Kupffer cells or reduces reactive oxygen species, respectively, prevented the liver injury. So the messenger RNA levels of the oxidative response genes mentioned above were detected, and the following results showed that Ag treatment reduced their expression. Besides, serum lactate dehydrogenase and myeloperoxidase activity was significantly reduced by Ag. Finally, Ag treatment did not further reduce serum tumor necrosis factor- α production compared with NAC treatment alone. Thus, our results indicate that Ag prevents Con-A-induced liver injury and reduced the hepatic oxidative stress response. The hepatic protective effect of Ag indicates that Ag supplementation may be beneficial in the treatment of immune-mediated liver injury.

Keywords Andrographolide · Con-A · Liver injury · Apoptosis · ROS

Abbreviations

Con-A	Concanavalin A
ROS	Reactive oxygen species
TNF- α	Tumor necrosis factor- α
Q-PCR	Quantitative reverse transcription real-time PCR
GdCl ₃	Gadolinium chloride
NAC	<i>N</i> -acetyl-L-cysteine
LDH	Lactate dehydrogenase
MPO	Myeloperoxidase
NOS	Nitric oxide synthase
iNOS	Inducible nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
PS	Phospholipid phosphatidylserine
FITC	Fluorescein isothiocyanate
RNS	Reactive nitrogen species

Introduction

It is well known that severe hepatic injury can be induced after concanavalin A (Con-A) administration into mice. This experimental hepatitis model was first reported in 1992, and it is widely used nowadays for the investigation of immune-mediated acute liver injury (Tiegs et al. 1992; Wang et al. 2009; Adams et al. 2010). This model can be induced by intravenous injection of Con-A via the tail, subsequently T and NKT cells are activated, producing various inflammatory cytokines and chemokines, such as tumor necrosis factor- α (TNF- α), IFN- γ , IL-4, MCP-1, etc. (Ajuebor et al. 2003; Ojiro et al. 2010). These factors recruit and activate more immune cells which would attack

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hepatocytes, leading to severe liver injury. A recent report (Nakashima et al. 2008) has shown that production of superoxide and reactive oxygen species from the Con-A-activated Kupffer cells plays a critical role in this hepatitis.

Reactive oxygen species (ROS) are very small reactive molecules including oxygen ions and superoxide anions, and their reactivity is due to the presence of unpaired valence shell electrons. ROS, natural byproduct of the normal metabolism of oxygen, have important roles in cell signaling. However, they can be increased dramatically due to environmental or xenobiotics stresses (e.g., ultraviolet and heat exposure, or acetaminophen overdose, etc. (Das et al. 2010)), which cause severe damage to cell structures. This cumulates into a situation known as oxidative stress. ROS are involved in liver damage induced by many different causes, including alcohol, virus infection, lipid alterations, carbohydrate metabolism, and xenobiotics. Regulating ROS production plays a crucial role in the progression of liver damage (Loguercio et al. 2001). Furthermore, increased ROS are related to mitochondrial dysfunction, because the impairment of mitochondrial function is a key event in ROS signaling and subsequent activation of apoptosis (Kirkland and Franklin 2003). So the cellular enzymes such as superoxide dismutase 1 (SOD1) and reducing agents such as glutathione are involved in apoptosis by regulating the ROS levels (Carmody and Cotter 2001).

According to the reports above, antioxidant therapy would be a realistic approach in preventing Con-A-induced liver injury, and herbal medicine would be a valuable pool for the drug discovery (Ghosh et al. 2011). Based on our previous results (Qin et al. 2006; Jin et al. 2011), andrographolide (Ag) can be a potential agent in attenuating Con-A-induced liver injury. Ag is a labdane diterpene lactone isolated from the leaves of the *Andrographis paniculata* plant (Koteswara Rao et al. 2004). *A. paniculata* has long been used as herbal medicine in Asian countries and in Scandinavia. Ag has many types of bioactivity, such as anticancer (Li et al. 2007; Ji et al. 2009; Lin et al. 2011), antioxidative (Akowuah et al. 2009; Pekthong et al. 2009; Chern et al. 2011), antidiabetic and anti-inflammatory activity (Abu-Ghefreh et al. 2009; Lee et al. 2011). Although there are reports on the hepatocyte-protective effect of Ag both in vitro and in vivo (Handa and Sharma 1990; Visen et al. 1993; Trivedi et al. 2007; Kondo et al. 2011), the detailed mechanisms were not yet presented.

Materials and methods

Animals

C57BL/6 male mice, 8–10 weeks of age, were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (SLAC Co. Ltd.,

Shanghai, China). Mice were housed in the animal facilities of the Shanghai Institute of Endocrine and Metabolic Disease, Shanghai Jiao Tong University School of Medicine, under pathogen-free conditions according to the Institutional Animal Care and Use Committee guidelines. Mice were fed ad libitum a standard laboratory chow diet provided by SLAC Laboratory Animal Co. Ltd.

Treatment of animals

Mice (five to ten mice per group) were injected via the caudal vein with a single dose (15 mg/kg of body weight) of Con-A (Vector Laboratories, CA, USA) to produce the hepatitis model. In control-treated animals, only the carrier solution (PBS) was injected. Mice were injected once since 8:00 am sequentially, and liver tissues were removed at indicated time points after neck dislocation. For each animal, the left lobe of the liver was collected for histological purposes, and the right lobe was collected for quantitative reverse transcription real-time pCR (Q-PCR) and DNA extraction. Ag (Sigma Chemical Co., MO, USA, 98% purity) was dissolved in 10% dimethyl sulfoxide (DMSO) (v/v) with saline at different concentrations and intraperitoneally injected 2 h before Con-A injection; control mice were injected with 10% DMSO. To inactivate the Kupffer cells, mice were injected intravenously via the caudal vein with 10 mg/kg body weight gadolinium chloride (GdCl₃) (Sigma Chemical Co.) 24 h before Con-A administration. To reduce ROS, 100 mg/kg body weight *N*-acetyl-L-cysteine (NAC) (Sigma Chemical Co.) was intraperitoneally injected 1.5 h before Ag treatment or injected at indicated times as shown in Fig. 6.

Serum biochemistry determination

Blood was obtained at indicated time points after Con-A injection. Alanine aminotransferase (ALT) levels were determined using the ALT detection kit (Yihua Medical Science & Technology, Shanghai, China). Serum lactate dehydrogenase (LDH) activity was determined using the LDH detection kit (Kehua Bio-engineering, Shanghai, China). Serum nitric oxide synthase (NOS) and myeloperoxidase (MPO) activity was determined using the NOS and MPO detection kits, respectively (Jiancheng Bio-engineering, Jiangsu, China). All the procedures were performed according to the manufacturer's instructions.

Liver histopathology

For observation of hepatic morphological changes during pathogenesis, livers were removed after neck dislocation and fixed in 4% phosphate-buffered paraformaldehyde (Sigma Chemical Co.), then embedded in paraffin. Tissue

sections (5 μ m) were prepared, stained with hematoxylin & eosin (H&E) (Baso Diagnostics, Taiwan, China) or stained with Annexin V-FITC detection kit (Beyotime Institute of Biotechnology, Zhejiang, China), and examined under Olympus BX51 light microscopy with an Olympus DP71 Digital Camera (Olympus Imaging Corp., Tokyo, Japan). Images were analyzed with Image Pro Plus 6.0 Software (Media Cybernetics, MD, USA). A total of ten tissue sections were analyzed for each animal.

DNA fragmentation assay

For semi-quantitative determination of DNA fragmentation, the pattern of low weight DNA from liver cells was analyzed on an agarose gel electrophoresis. Fragmented DNA was isolated using a genomic DNA extraction kit (Beyotime Institute of Biotechnology, Zhejiang, China) according to the manufacturer's instruction. The eluants containing DNA pellets were subsequently electrophoresed on a 2% agarose gel, and ethidium bromide staining was performed. A DL2000 molecular weight marker was used for gel electrophoresis (TaKaRa, Shiga, Japan). Gel images were captured with the Syngene G:BOX Imaging System (Syngene System, Cambridge, UK).

Reverse transcription and real-time quantitative polymerase chain reaction assay

Total RNA was isolated from liver tissues using the standard TRIzol method (Invitrogen, CA, USA) according

to the manufacturer's instructions. Concentration and purity of all RNA samples were determined. RNA purity and concentration were measured using the NanoDrop[®] Spectrophotometer ND-1000 (Thermo Scientific, MA, USA). RNA was stored at -80°C before use. First-strand complementary DNA (cDNA) synthesis was performed for each RNA sample using Promega Reverse Transcription System (Promega Corp., WI, USA). Oligo-dT was used to prime cDNA synthesis. Q-PCR was performed for determination of hepatic messenger RNA (mRNA) expression of related genes during Con-A-induced liver injury. Q-PCR was performed by using TaKaRa SYBR Master Mix (TaKaRa) on Light-Cycler480 (Roche Applied Science, Basel, Switzerland). PCR conditions included an initial holding period at 95°C for 5 min, followed by a two-step PCR program consisting of 95°C for 5 s, and 60°C for 20 s for 40 cycles. Data were collected and quantitatively analyzed through LightCycler480 Software (Release 1.5.0). Primers for those genes were selected according to Primer Bank (<http://pga.mgh.harvard.edu/primerbank>), except for RPLP0 from Tateishi et al. (2009), and the sequences are listed in Table 1. Relative quantitation analysis of gene expression data was conducted according to the $2^{-\Delta\Delta\text{Ct}}$.

Cytokine assay

Plasma concentration of TNF- α was determined 2 h after Con-A injection, respectively, using specific enzyme-linked immunosorbent assay kits (R&D Systems, MN, USA) according to the manufacturers' instruction.

Table 1 Description of gene and target gene primers used in the Q-PCR assay

Symbol ^a (official symbol)	Official full name	Entrez gene ID	Primer Bank ID	Reverse primer
36B4 (Rplp0)	Ribosomal protein, large, P0	11837	(Reference)	F: 5' GAAACTGCTGCCTCACATCCG R: 5' CTGGCACAGTGACCTCACACG
Glut1 (Slc2a1)	Solute carrier family 2 (facilitated glucose transporter), member 1	20525	22094111a1	F: 5' CAGTTCGGCTATAAACTGGTG R: 5' GCCCCGACAGAGAAGATG
iNOS (Nos2)	Nitric oxide synthase 2, inducible	18126	6754872a1	F: 5' GTTCTCAGCCCAACAATACAAGA R: 5' GTGGACGGGTGCGATGTCAC
HIF-1 α (Hif1a)	Hypoxia-inducible factor 1, alpha subunit	15251	2821940a1	F: 5' ACCTTCATCGGAACTCCAAAG R: 5' ACTGTTAGGCTCAGGTGAACT
SOD1 (Sod1)	Superoxide dismutase 1, soluble	20655	12805215a1	F: 5' AACCAGTTGTGTTGTGTCAGGAC R: 5' CCACCATGTTTCTTAGAGTGAGG
HO-1 (Hmox1)	Heme oxygenase (decycling) 1	15368	6754212a1	F: 5' AAGCCGAGAATGCTGAGTTCA R: 5' CCGTGTAGATATGGTACAAGGA
COX2 (Ptgs2)	Prostaglandin-endoperoxide synthase 2	18126	31981525a1	F: 5' TGAGCAACTATTCCAAACCAGC R: 5' CACGTAGTCTTCGATCACTATC

F forward primer, R reverse primer

^a Symbol: gene symbol used in this paper; official symbol: gene symbol from <http://www.informatics.jax.org/mgihome/nomen>

Statistical analysis

All the results were expressed as mean \pm SE. Statistical comparisons between two groups were made using Student's *t* test after analysis of variance by GraphPad Prism 5 (GraphPad Software, Inc. CA, USA). The level of significance was set at $\alpha=0.05$. All the tests were two-sided.

Results

Effects of Ag on Con-A-induced liver injury

Because serum ALT activity is a sensitive and mostly used index for evaluating liver injury, the serum ALT activity was determined with or without Ag treatment after Con-A injection. To determine whether Ag treatment protects Con-A-induced liver injury, mice were treated with increasing dose of Ag 2 h before Con-A injection. Serum was collected 8 h after Con-A injection. As shown in Fig. 1a, without Ag treatment, serum ALT activity was significantly elevated 8 h after Con-A injection; 10-mg/kg Ag treatment did not reduce serum ALT activity. However, serum ALT activity was significantly reduced when mice were treated with 30 or 100 mg/kg Ag ($p<0.01$). To confirm the protective effect of Ag after Con-A injection, mice liver

sections were observed through a microscope after H&E staining. As shown in Fig. 1c, necrotic areas were observed 8 h after Con-A injection compared with Fig. 1b. However, as shown in Fig. 1d–f, necrotic areas were diminished by increasing concentration of Ag treatment. Besides, lesions were scored blindly by a pathologist, as shown in Supplementary Fig. S1. These results suggest that Ag attenuates liver injury induced by Con-A intravenous injection via the tail.

Effects of Ag on Con-A-induced hepatocyte apoptosis

Because Con-A intravenous injection is well known to cause apoptosis of hepatocytes, the protective effect of Ag on Con-A-induced apoptosis in hepatocytes was examined. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a phospholipid-binding protein that has a high affinity for PS and therefore binds to cells with exposed PS. Since externalization of PS occurs in the earlier stages of apoptosis, fluorescein isothiocyanate (FITC)-labeled-Annexin V staining can identify apoptosis (Martin et al. 1995). Liver sections from all treatments were stained using FITC-Annexin V detection kit as mentioned in the “Materials and methods”. As

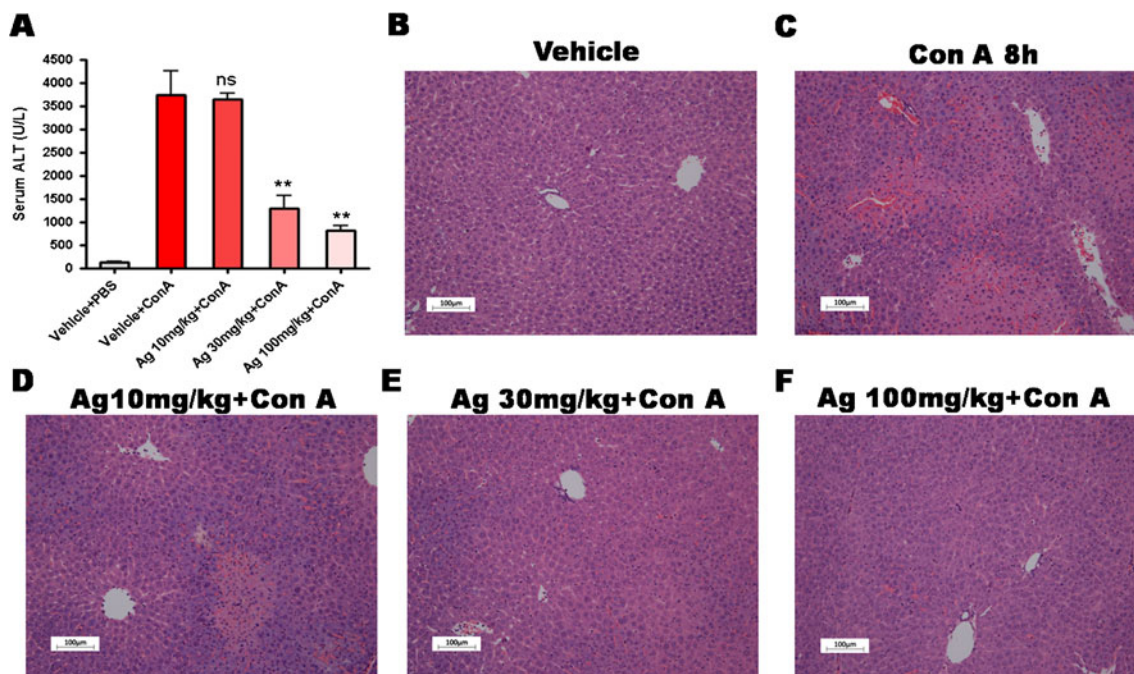


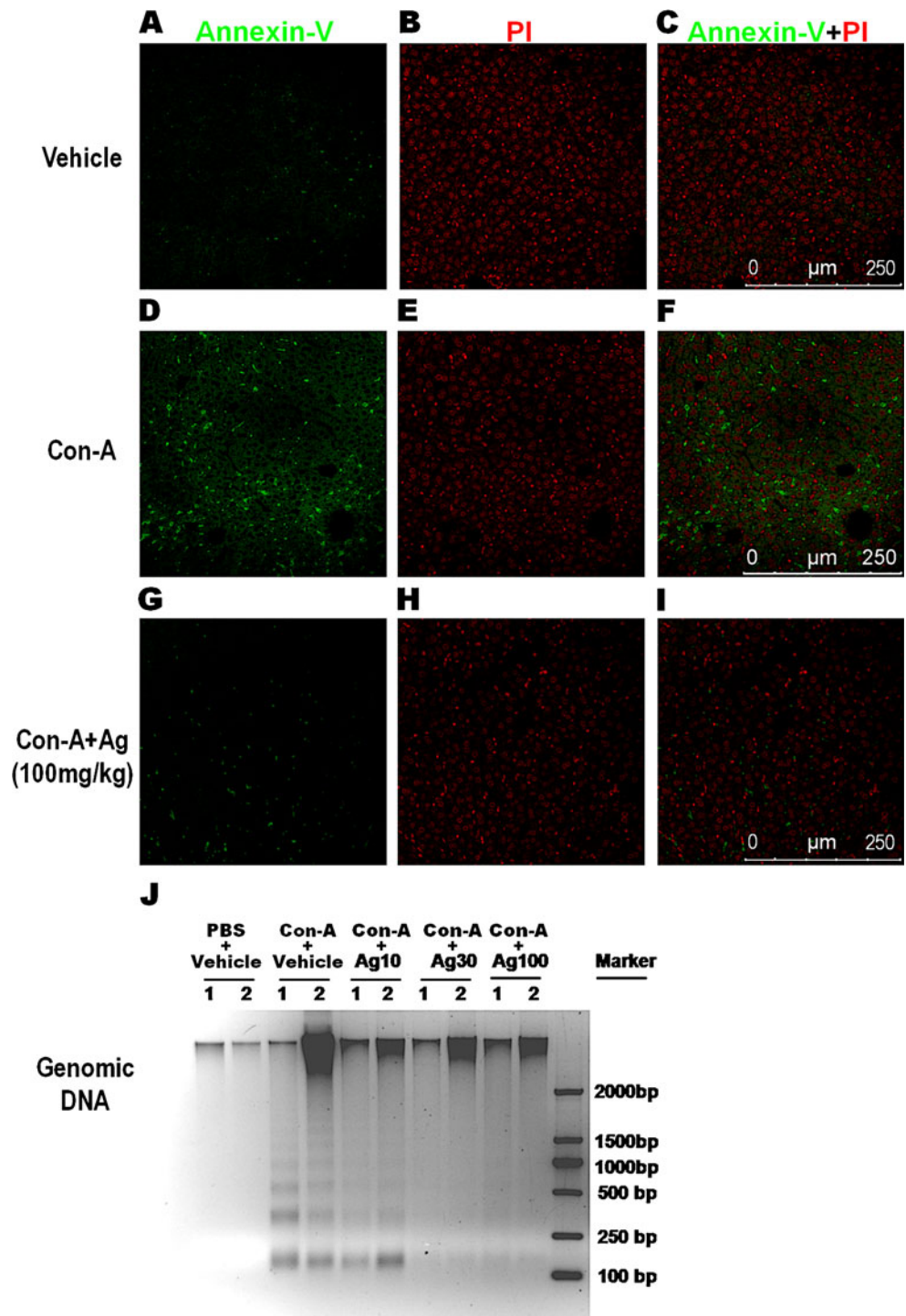
Fig. 1 Ag treatment attenuated Con-A-induced liver injury in a dose-dependent manner. Mice were treated with vehicle or increasing dose of Ag before they received a Con-A (15 mg/kg) injection via the tail vein. **a** Serum ALT activity from different groups was examined 8 h after Con-A injection. **b–f** Representative photomicrographs are

shown depicting H&E staining of liver sections from different groups. Results are shown as mean \pm SE, and *p* value stands for comparisons between groups of Con-A injection alone and Ag treatment before Con-A injection. Original magnification $\times 200$. ** $p<0.01$ ($n=5$ per group)

shown in Fig. 2d–f, sections from Con-A-treated mice showed that apparently more FITC-positive hepatocytes were observed compared with vehicle-treated mice shown in Fig. 2a–c. However, apparently fewer hepatocytes were positively stained when treated with 100 mg/kg Ag, as shown in Fig. 2g–i. Because genomic DNA fragmentation is another marker of apoptosis, it was analyzed in liver tissues

of differentially treated mice (Trautwein et al. 1998). As also shown in Fig. 2j, genomic DNA fragmentations were observed 8 h after Con-A injection. However, treatment with Ag apparently reduced the DNA fragmentation in a dose-dependent manner, which was in accordance with the results mentioned above. The results suggest that Ag prevents hepatocyte apoptosis induced by Con-A injection.

Fig. 2 Ag treatment attenuated hepatocyte apoptosis in Con-A-induced liver injury. **a–i** Representative fluorescence photomicrographs are shown depicting Annexin V-FITC staining of liver sections from differentially treated groups 8 h after Con-A injection. *Green*: Annexin V-FITC staining for apoptotic cells; *red*: PI (propidium iodide) staining for nucleus. Original magnification $\times 200$. **j** Hepatic DNA fragmentation analysis from differentially treated groups 8 h after Con-A injection. Genomic DNAs from differentially treated mice livers were extracted and run on agarose gel electrophoresis, respectively. Result was shown with *black–white* color reversed, and the Con-A-induced DNA ladder in *black* was easily observed. 1 and 2 represent genomic DNA from different tissues ($n=5$ per group)



Hepatic oxidative stress response induced by Con-A

Oxidative stress is one of the key factors involved in immune-mediated liver injury in some disease models as previously described, and ROS produced from Con-A-activated Kupffer cells plays a critical role in this model (Nakashima et al. 2008), which should be a key subject for investigation. Consequently, the detailed role of oxidative stress in the pathogenesis of Con-A-induced liver injury and the subsequent protective effect of Ag during the process were investigated. As shown in Fig. 3a–d, cyclooxygenase-2 (COX2) (Lee et al. 2006), Glut1 (Kozlovsky et al. 1997), hypoxia-inducible factor-1 α (HIF-1 α) (Gorlach and Bonello 2008), and heme oxygenase-1 (HO-1) (Aggeli et al. 2011) represent the most important markers of oxidative stress response, so the hepatic mRNA levels were examined during Con-A-induced pathogenesis. Results show that mRNA levels of these genes were significantly increased after Con-A

injection. Moreover, Kupffer cell inactivation by GdCl₃ or ROS reduction by NAC significantly reduced serum ALT level through the liver injury progression, as shown in Fig. 3e. These results suggest that oxidative stress has played very important roles in the pathogenesis of Con-A-induced liver injury.

Effects of Ag on Con-A-induced oxidative stress response

Previous reports and results above suggest that oxidative stress participates in Con-A-induced liver injury. But whether oxidative stress is involved in the protective effect of Ag in Con-A-induced liver injury is still unknown. To prove this possibility, enzyme activity related to oxidative stress in mice was determined in this study. LDH catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD⁺. Serum LDH activity is often used to evaluate liver injury, especially injury induced by oxidative stress (Kodai et al. 2007). MPO

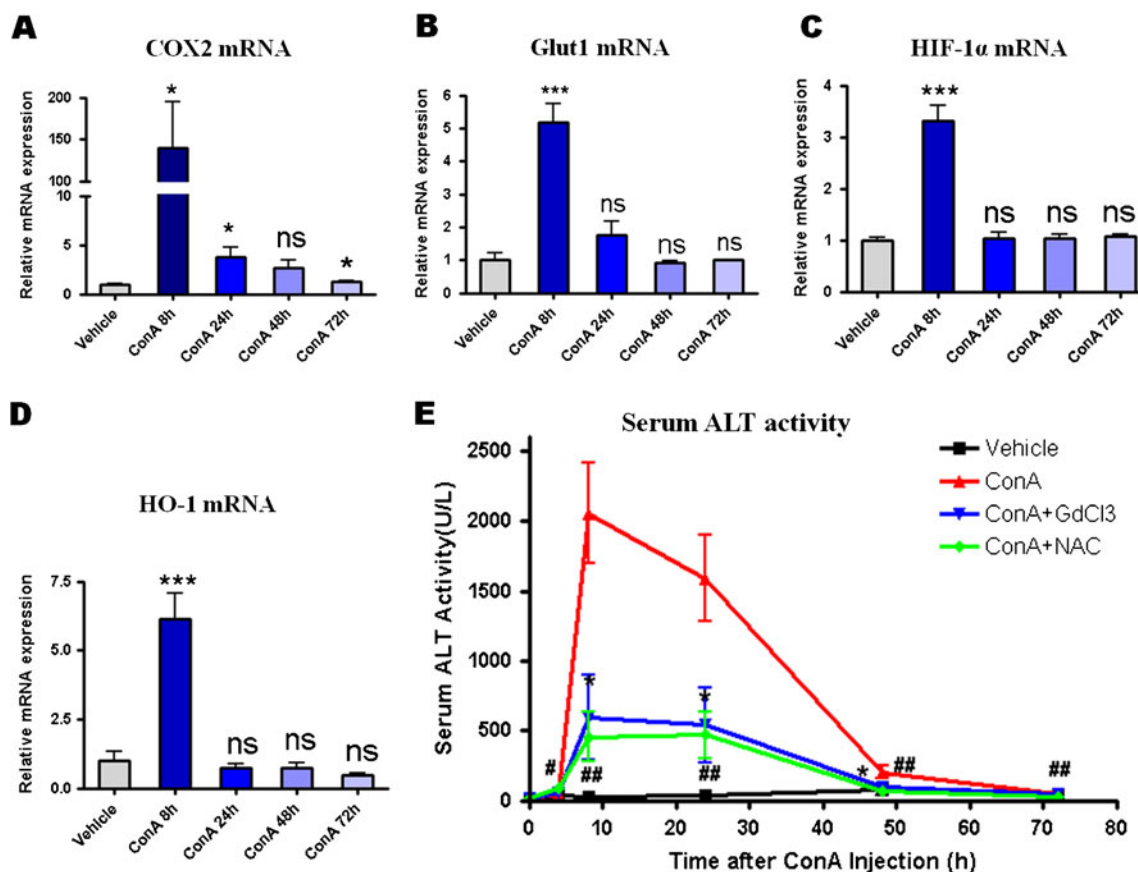


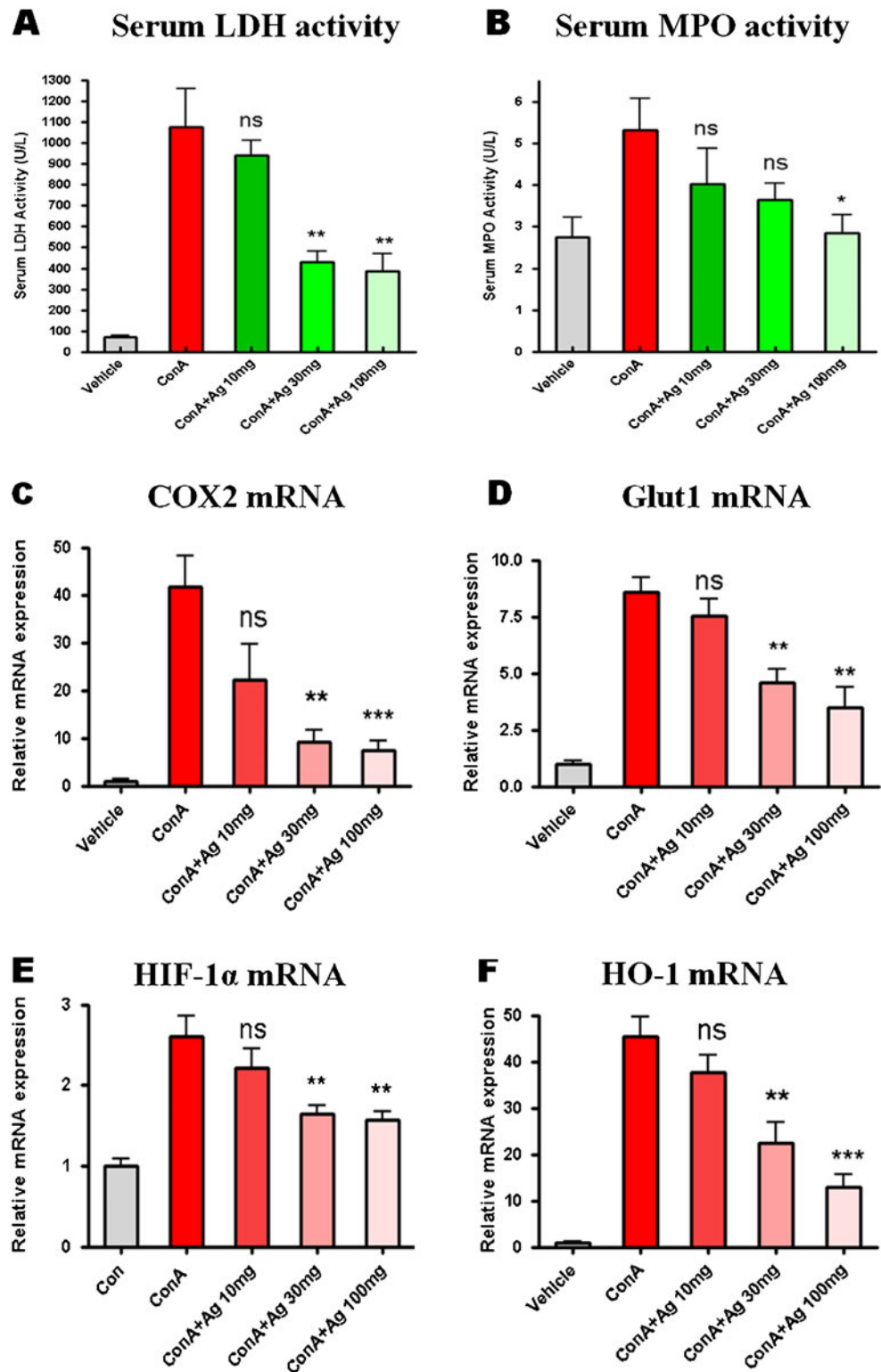
Fig. 3 Macrophage ROS production was involved in Con-A-induced liver injury. **a–d** Mice received a Con-A (15 mg/kg) injection via the tail vein, and COX2, Glut1, HIF-1 α , and HO-1mRNA expression levels were analyzed at different time points after Con-A injection respectively. * p <0.05, *** p <0.001 compared with vehicle group. n =5–6 per group. **e** Mice were treated with GdCl₃ or NAC before they

received a Con-A (15 mg/kg) injection via the tail vein. Serum ALT activity was analyzed between groups at indicated time points. * p <0.05 GdCl₃ pretreated compared with Con-A injected alone, ## p <0.01 NAC pretreated compared with Con-A injected alone. (n =6 per group in **a–d** and n =8 per group in **e**)

is a peroxidase most abundantly present in activated neutrophils, which was secreted to liberate oxygen radicals, so MPO activity is recognized as an important marker for hepatic oxidative stress (Sener et al. 2006). As shown in Fig. 4a, b, serum LDH and MPO activity was significantly

elevated after Con-A injection, and Ag treatment significantly reduced their activity in a dose-dependent manner. Moreover, hepatic mRNA levels of COX2, Glut1, HIF-1 α , and HO-1 were significantly reduced after Ag treatment in a dose-dependent manner as well, compared with Con-A

Fig. 4 Ag treatment reduced ROS production and attenuated hepatic oxidative stress. Mice were treated with vehicle or increasing dose of Ag before they received a Con-A (15 mg/kg) injection via the tail vein. **a, b** Serum LDH activity and MPO activity were analyzed 8 h after Con-A injection. **c–f** Mice were treated with vehicle or increasing dose of Ag before they received a Con-A (15 mg/kg) injection via the tail vein, and COX2, Glut1, HIF-1 α , and HO-1 mRNA expression levels were analyzed at 8 h after Con-A injection respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with Con-A injection alone ($n = 5$ per group)



injection alone, as shown in Fig. 4c–f. Besides, hepatic protein levels of COX2, Glut1, HIF-1 α , and HO-1 8 h after Con-A injection were checked by western blot, and the results indicated a different expression style, except for COX2, as shown in Fig. S2. The inconsistency of the results might be attributed to the differential expression profile at different time points in this disease model, in which liver was seriously damaged. The damage could have a certain effect on mRNA translation. The mRNA expression data suggest that Ag treatment significantly reduces hepatic oxidative stress induced by Con-A.

Effects of Ag on Con-A-induced alteration of SOD1 and NOS expression and activity

Reduced SOD1 expression leads to accumulation of ROS that cannot be cleared, which might explain the oxidative stress-induced liver injury after Con-A injection. So SOD1 was examined in this study. As shown in Fig. 5a, SOD1

mRNA level was significantly reduced after Con-A injection alone. To further elucidate the detailed antioxidative effect of Ag, both the hepatic mRNA levels of SOD1 and serum activity of SOD were checked. As shown in Fig. 5b, c, compared with Con-A treatment alone, Ag treatment not only restored the hepatic mRNA level of SOD1, but also increased the serum activity of SOD in a dose-dependent manner. These results suggest that Ag treatment significantly restores both the expression and activity of hepatic SOD1 that were reduced by Con-A.

Inducible nitric oxide synthase (iNOS) is a stress-inducible form of NOS that produces reactive nitrogen species (RNS) for eliminating pathogens. However, over-produced RNS increases oxidative stress in local tissues (Muriel 2009). As shown in Fig. 5d, hepatic iNOS mRNA levels were significantly increased 8 h after Con-A injection, which would cause elevated RNS production, and with Ag treatment, the iNOS mRNA level was significantly reduced in a dose-dependent level. Moreover,

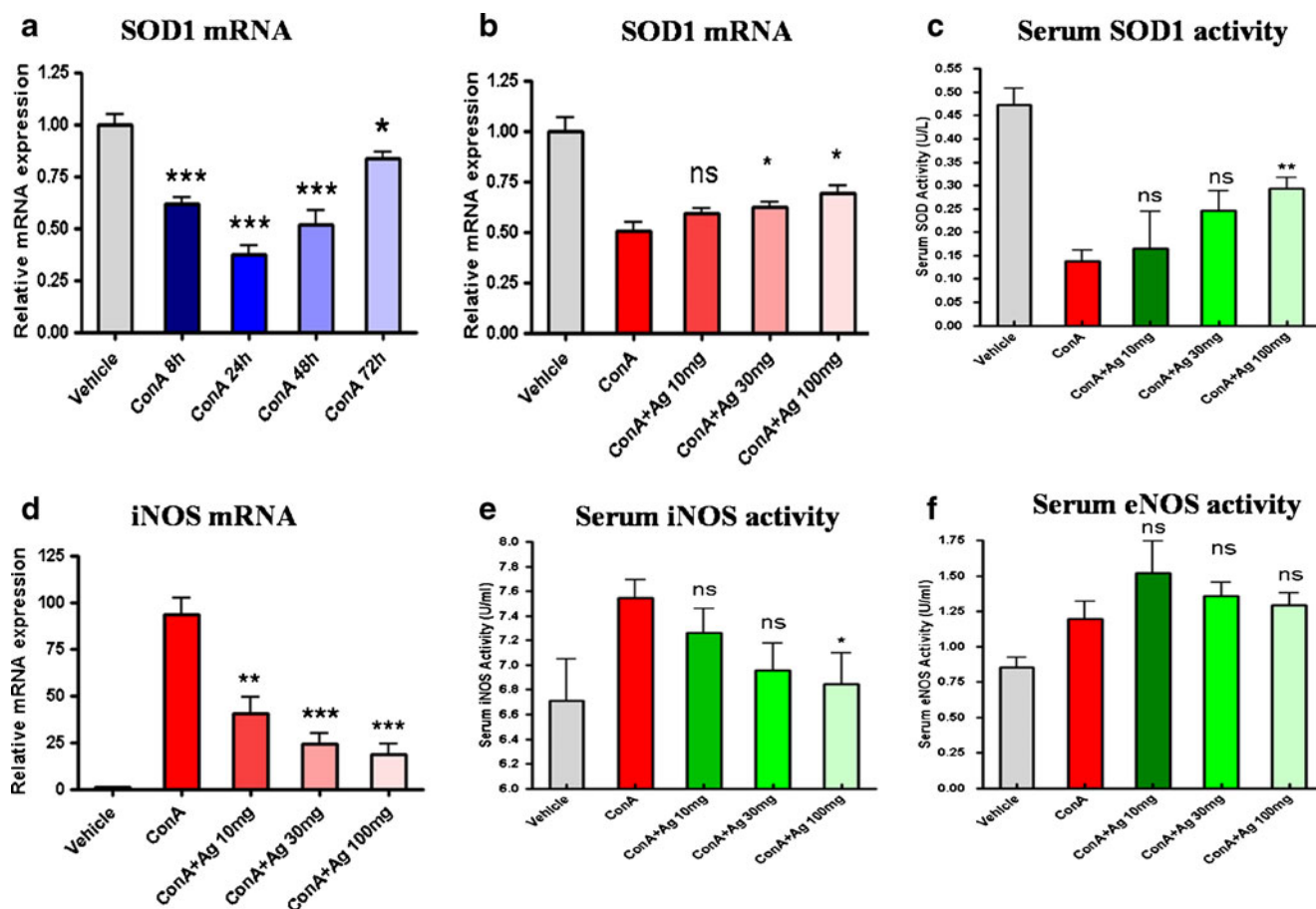


Fig. 5 Ag treatment restored SOD1 activity and reduced serum iNOS activity. **a** Mice received a Con-A (15 mg/kg) injection via the tail vein, and SOD1 mRNA expression levels were analyzed at different time points after Con-A injection. * $p < 0.05$, *** $p < 0.001$. **b, d** Mice were treated with vehicle or increasing dose of Ag before they received a Con-A (15 mg/kg) injection via the tail vein, and SOD1 and iNOS

mRNA expression levels were analyzed at 8 h after Con-A injection respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with Con-A injection alone. **c, e, f** Mice were likewise and serum SOD activity and NOS (including iNOS and eNOS) activity were analyzed at 8 h after Con-A injection, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with Con-A injection alone ($n = 6$ in **a** and $n = 5$ in **b–f** per group)

as shown in Fig. 5e, f, Ag treatment significantly reduced serum iNOS activity in a dose-dependent manner, while no significant effect of serum endothelial nitric oxide synthase (eNOS) activity was observed. These results suggest that Ag significantly inhibits hepatic iNOS mRNA expression and activity to reduce oxidative stress induced by RNS.

Effects of Ag on ROS-mediated TNF- α production induced by Con-A

The results above indicate that Ag attenuated Con-A-induced liver injury and reduced hepatic oxidative stress. To further elucidate whether oxidative stress was involved in the protective effect of Ag in Con-A-induced liver injury, ROS produced by Con-A was cleared by NAC before Ag treatment. Then serum TNF- α level was determined. As shown in Fig. 6, serum TNF- α levels were significantly reduced after NAC treatment compared with Con-A treatment alone. Also Ag treatment significantly reduced serum TNF- α level in comparison with Con-A treatment alone. However, when mice were treated with NAC 1.5 h before Ag treatment, serum TNF- α level was not further reduced compared with NAC treatment alone. The result suggests that the protective effect of Ag on Con-A-induced liver injury is mediated by reducing oxidative stress.

Discussion

This study shows for the first time that Ag prevents Con-A-induced oxidative stress which would lead to liver injury. The protective role of Ag on Con-A-induced liver injury

was proven at the beginning of this study. Further serum biochemistry and liver histological observations confirmed the protective role of Ag. Then Kupffer cells inactivated by GdCl₃ and ROS reduced by NAC showed attenuated ALT activity induced by Con-A, which led us to examine the role ROS plays in this injury model. Those results show that the protective role of Ag could depend on oxidative stress. Our findings reveal that oxidative stress is involved in Con-A-induced liver injury, and Ag shows a protective effect through reducing hepatic oxidative stress.

Intravenous injection of Con-A causes liver-specific accumulation of Con-A, which activates immune cells located inside the liver (Gantner et al. 1995). Activated Kupffer cells produce and release ROS, which are known to be the main effectors of hepatotoxicity induced by either lipopolysaccharide injection (Liu et al. 1995) or by ischemia reperfusion injury (Jaeschke et al. 1992). Besides, a previous report has demonstrated that the increase of CD68⁺ Kupffer cells and their ROS release are the main mechanisms of Con-A-induced hepatitis (Nakashima et al. 2008). Excessive and persistent ROS production can induce hepatic oxidative injury, which leads to hepatocyte apoptosis. Oxidative stress response or hepatocyte apoptosis will further increase immune cell accumulation and activation, which leads to a number of necrosis areas (Osburn et al. 2008). To further elucidate the detailed role of oxidative stress in Con-A-induced liver injury as well as in the protective effect of Ag, mice were injected with Con-A, and hepatic mRNA levels of oxidative stress response genes were examined. As shown in Fig. 3, the genes were significantly elevated during the pathogenesis, particularly 8 h after Con-A injection. The results indicate that oxidative stress could be evoked after Con-A injection. Further, mice were treated with GdCl₃ or NAC before Con-A injection, as shown in Fig. 3. Reduced serum ALT activity suggested that liver injury was significantly attenuated by GdCl₃ or NAC treatment during Con-A-induced pathogenesis. These results confirm the critical role of ROS production by Kupffer cells in Con-A-induced liver injury (Nakashima et al. 2008). The results above lead us to prove the possibility that Ag could protect Con-A-induced liver injury through reducing hepatic oxidative stress. We demonstrated here that not only serum LDH and MPO activity but also the oxidative stress-induced gene expression was significantly elevated after Con-A injection, but significantly reduced by Ag pretreatment in a dose-dependent manner, as shown in Fig. 4. These results indicate that Ag reversed liver injury and reduced hepatic oxidative stress.

We further asked why there was excessive oxidative stress after Con-A injection. One possibility is that they were not effectively reduced in time. SOD1 is widely considered to be the major intracellular antioxidant enzyme

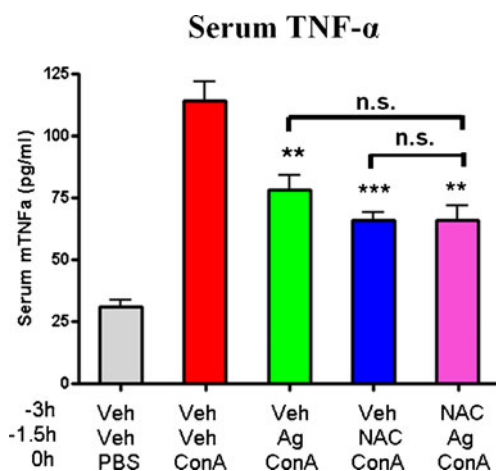


Fig. 6 Ag treatment attenuated serum TNF- α production through a ROS-dependent manner. Mice were divided and treated in sequence as indicated in the graph with vehicle, NAC (100 mg/kg), and/or Ag (100 mg/kg). Serum TNF- α levels were analyzed 2 h after Con-A injection, respectively. ** p <0.01, *** p <0.001 compared with Con-A injection alone (n =6 per group)

in mammals, which catalyzes the conversion of superoxide anion into H_2O_2 and protects cells from oxidative injury (Lei et al. 2006). So SOD1 expression and activity were investigated after Con-A injection. As shown in Fig. 5a, hepatic mRNA expression of SOD1 was significantly reduced after Con-A injection. Besides, serum SOD activity was also significantly reduced as shown in Fig. 5c. The results suggest that the reduced expression and activity of SOD1 is responsible for the accumulation of ROS in liver tissue induced by Con-A. The results in Fig. 5b, c show that Ag treatment retrieved both hepatic SOD1 mRNA expression and serum SOD1 activity in a dose-dependent manner. These results suggest that Ag attenuates Con-A-induced liver injury by reducing oxidative stress partly through restoring SOD1 activity.

iNOS is a stress-inducible form of NOS that produces RNS to eliminate pathogens and can be activated by oxidative stress. Increased iNOS is effective in pathogen defending, but it increases organic oxidative stress at the same time, and excessive RNS production may cause tissue damage (Muriel 2009). Both hepatic mRNA expression level and serum activity of NOS were checked in this study. As shown in Fig. 5d–f, both hepatic mRNA expression and serum activity of iNOS were significantly elevated after Con-A injection, while serum activity of the constitutive expression form of NOS, eNOS, was not changed. However, Ag treatment reduced hepatic iNOS mRNA expression and reduced serum iNOS activity without affecting eNOS activity. These results indicate that Con-A can induce both ROS and RNS, which synergistically increase hepatic oxidative stress and induce liver injury; Ag treatment reduces RNS production by reducing iNOS activity and further protects liver from excessive oxidative stress-induced hepatocyte apoptosis.

To further confirm the role of oxidative stress in mediating the protective effect of Ag on Con-A-induced liver injury, a widely used antioxidant, NAC, was applied before Ag treatment (Koeppel et al. 1996; Schulz et al. 2007). As shown in Fig. 6, when Con-A-induced ROS were reduced by NAC treatment, Ag treatment did not further reduce serum TNF- α production compared with NAC treatment alone. The results suggest that reducing TNF- α production by Ag is dependent on reduction of oxidative stress.

Taken together, we further confirm that oxidative stress is involved in Con-A-induced liver injury and that Ag protects mice from Con-A-induced liver injury through reducing oxidative stress. Besides, the results suggest that Ag can be further studied as a potential therapy for immune-mediated acute liver injury in the future.

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