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RESEARCH ARTICLE

Protective Effects of Glycyrrhizin on LPS and Amoxicillin/Potassium Clavulanate-Induced Liver Injury in Chicken

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ABSTRACT

Lipopolysaccharide (LPS) and amoxicillin/potassium clavulanate (APC) act synergistically to aggravate liver injury, and influence the pathological progress of liver injury. The protective effects of compound ammonium glycyrrhizin (CAG) on liver injury were investigated. The chickens were divided into 5 groups. Group I served as the control. Group II was administered with APC once a day for 3 consecutive days. Group III was injected with LPS. Group IV was administered with APC once a day for 3 consecutive days, and then injected with LPS when the third administration of APC was provided. Group V was pre-treated with CAG in drinking water for 3 days before the treatment of APC, and continued to the end of the experiment. Samples were collected at 6, 24 and 48 h after LPS injection. ALT, AST and malondialdehyde (MDA) levels were significantly increased at different degrees ($P<0.001$); superoxide dismutase (SOD) level was significantly reduced in Groups III and IV ($P<0.001$). The mRNA expression levels of nuclear factor-κB (NF-κB) and tumor necrosis factor-α (TNF-α) and levels of TNF-α and interleukin-1 (IL-1) were significantly increased in Groups III and IV at different degrees ($P<0.001$), but not in Group II. CD4+ and CD8+ T cells levels were significantly increased in Groups II and IV ($P<0.001$). Greater significant changes in these parameters were observed in Group IV when compared with Groups II and III. Meanwhile, the improvement of these parameters was due to CAG treatment. In conclusion, LPS plus APC can aggravate liver injury due to hepatic damage, oxidative stress, inflammation and immune injury, CAG may mitigate liver injury.

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INTRODUCTION

Under the intensive poultry-farming model, infections caused by gram-negative bacteria, especially conditional pathogenic *E. coli*, is the most troubling disease (Srinivasan *et al*., 2014; Bogusławska-Tryk *et al*., 2015; Kapakin *et al*., 2015). To control these infections, poultry animals are frequently administrated with antibiotics, especially bactericidal antibiotics. Antibiotics can promote the release of endotoxin when used to kill bacteria (Landman, 2015). When released into bloodstream, a large amount of endotoxin will cause liver microcirculation disorders, liver dysfunction, free radical injury and inflammation. Lipopolysaccharide (LPS), a major ingredient of endotoxin, has been used to establish liver injury model (Fan *et al*., 2012).

Recently, drug-induced liver injury (DILI) has attracted increasing attention in clinical medicine. Due to the high exposure rate, antibiotics are considered as a common cause of DILI (Leitner *et al*., 2010; Devarbhavi *et al*., 2014). Amoxicillin, especially in combination with potassium clavulanate, is commonly used to prevent and cure Gram-negative bacterial infections and has been found to be associated with hepatocellular and cholestatic liver injury in clinical medicine (Stine and Chalasani, 2015). However, in veterinary clinic, DILI has not gained extensive attention. In fact, liver injury symptoms are common in poultry-farming practice, which have caused low performance and even death. With the intensive poultry-farming model, chicken have been provided with antibiotics through administration in drinking water. To ensure adequate drugs ingested, animals are always prohibited to provide water for

several hours before drug administration, and a high dosage is also prepared.

Amoxicillin/potassium clavulanate (APC) can promote LPS release when used to kill bacteria (Landman 2001). In this study, the chickens were administrated with LPS plus APC individually or simultaneously to explore the causes and pathological progress of chicken liver injury. Glycyrrhizin, a saponin isolated from the licorice root, is known to be effective as an anti-inflammatory, anti-allergic, antioxidant, or immune-regulatory agent, and can protect cell membrane, or improve liver function. Compound ammonium glycyrrhizin (CAG) tablets containing glycyrrhizin, glycine and methionine as the bioactive, and injection containing glycyrrhizin, glycine and Cysteine Hydrochloride as the bioactive ingredients have been used for treating chronic hepatitis in clinical practice for more than 25 years (Orazizadeh *et al*., 2014). Based on these studies, the protective effect of CAG on chicken liver injury induced by LPS and APC was also explored.

MATERIALS AND METHODS

Preparation of LPS extract: Chicken pathogenic *E. coli* (Jiangsu Academy of Agricultural Science) was cultured at 37°C, followed by centrifugation at 3,000rpm for 20min. The pellets were washed 3 times with PBS, re-suspended in PBS to a concentration of 2.0×109 CFU/mL, disrupted by sonication. The supernatant was centrifuged at 5,000rpm for 10min and filtered through a 0.22μm filter. The LPS extract was boiled for 10 min, stored at 4°C.

Animals: One-day-old Hyline egg-type chickens, 50% females and 50% males, were obtained from Nanjing Tangquan Farm. Chickens were treated at a controlled temperature (24°C) under a 12 h light-dark cycle, fed with standard laboratory chow and water. Chickens were raised until the average weight was 1±0.2kg. This study followed the protocol approved by Animal Care and Use Committee of Nanjing Agricultural University.

Design of experiment: 120 chickens were randomly divided into 5 groups, 24 chickens in each group. Group I (control) without any treatments. Group II (APC) were intragastrically administered with APC (100mg/kg) once a day for 3 days. Group III (LPS) were intraperitoneally injected with LPS extract (4mL/kg) at the third day, and Group IV (APC+LPS) were administered with both APC and LPS extract. Group V (CAG+APC+LPS) were pre-treated with CAG (40mg/L in drink water) for 3 days before the same treatment of group IV. CAG was formulated in our laboratory, which containing ammonium glycyrrhizin, glycine methionine and matrix. The formula and preparation method have been granted by patent (Number: ZL 201110425509.7).

Eight chickens of each group were sampled at 6, 24, and 48 h after the last dose. Blood was collected from brachial vein, to obtain serum and anti-coagulated. one part of liver samples was frozen immediately in liquid nitrogen, stored at -80°C, the other were fixed with 4% buffered formaldehyde, processed for embedding in paraffin, then were stained with hematoxylin/eosin (HE) according to standard procedure.

ALT, AST, SOD, MDA and Cytokine levels: ALT and AST levels in serum, superoxide dismutase (SOD) and malondialdehyde (MDA) levels in liver were determined with corresponding detection kits, TNF-α and IL-1 content in liver were measured by ELISA kits (purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The protein concentrations in liver tissues were determined using bicinchoninic acid method.

RNA isolation and real-time RT-PCR: Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). First strand cDNA synthesis was performed with the following cycling: 25°C for 10min, 42°C for 30min and 85°C for 5min. PCR amplification of NF-κB and TNF-α fragments was performed using cDNA as template, each PCR contained 10μL SYBR Green premix (Vazyme Biotech Co., Ltd, Nanjing, China) and 0.4μL forward and reverse primers. Cycling conditions were 95°C for 5min, then 40 cycles of following: 95°C for 10s, 60°C for 30s. Negative controls without RNA were setup. Primers (Vazyme Biotech Co., Ltd) specific for NF-κB, TNF-α and β-actin were designed as described and synthesized for real-time PCR analysis (Zhao *et al*., 2013; Guo *et al*., 2014). The $2^{-\Delta\Delta Ct}$method was used to analyze RT-PCR data.

Flow cytometry: Lymphocytes in peripheral blood were separated by lymphocyte separation medium (Haoyang Biological Technology Co., Ltd, Tianjin, China). The cells were diluted to 10^5-10^6/mL in PBS, and divided equally into two tubes. Monoclonal antibody against CD4 and CD8 (Novus Biologicals, Colorado, USA) was added in the different tube. The subsequent working program was carried out according to standard procedure. The data was analyzed by FlowJo software 7.6.5.

Data analysis: Data were expressed as mean±SEM. Group comparisons were carried out by two-way ANOVA using GraphPad Prism 5 statistical software. $P<0.05$ was considered as the significant difference among groups.

RESULTS

The changes in levels of ALT, AST, SOD and MDA: As shown in Fig. 1, compared to the control group, ALT and AST activities were significantly increased at 48 h ($P<0.05$) in APC treatment group, and increased at 24 h ($P<0.05$) and 48 h ($P<0.001$) in LPS treatment group. MDA concentration was significantly increased and SOD activity was significantly decreased at 24 h ($P<0.05$) and 48 h ($P<0.001$) in LPS-induced injury group. In LPS plus APC group, the increased ALT and AST activities and MDA content as well as decreased SOD activity were observed. Although the activities of ALT and AST, and MDA levels in CAG treatment group were still higher than those in the control group, significant decreases ($P<0.001$) at 24 h and 48 h were observed when compared to LPS plus APC group. The SOD activity was significantly increased ($P<0.001$) at 48 h.

The changes in NF-κB and TNF-α mRNA expression and TNF-α and IL-1 content in liver: As shown in Fig. 2, compared to control group, NF-κB mRNA expression was significantly increased at 6 h (P<0.01), 24 h (P<0.001) and 48 h (P<0.001), TNF-α mRNA expression and IL-1 content were significantly increased (P<0.001) at 24 h and 48 h, TNF-α content was significantly increased at 24 h (P<0.05) and 48 h (P<0.001) in LPS-induced injury group. In APC treatment group, NF-κB mRNA expression was only significantly increased (P<0.001) at 48 h and IL-1 content was only significantly increased (P<0.05) at 6 h. Meanwhile, LPS plus APC induced more significantly increase of the fore indicators at 6 h to 48 h (P<0.001). Although the mRNA expression of NF-κB and TNF-α, and TNF-α and IL-1 contents in CAG treatment group were still higher than those in the control group, the mRNA expression of NF-κB and TNF-α significantly decreased at 6 h, 24 h and 48 h (P<0.01). TNF-α and IL-1 contents were significantly decreased (P<0.001) at 48 h, when compared to that in the LPS plus APC group.

The changes in CD4+ and CD8+ T cells in chicken blood: As shown in Fig. 3, compared to control group, CD4+ T cell population was significantly increased at 6 h (P<0.01), 24 h (P<0.05) and 48 h (P<0.001), and CD8+ T cell population was significantly increased at 24 h (P<0.05) and 48 h (P<0.01) in APC treatment group. But CD4+ and CD8+ T cell levels were not significantly increased in LPS treatment group. In LPS plus APC group, CD4+ and CD8+ T cell levels was significant increase in 24 h and 48 h (P<0.001). These changes exhibited a greater level than that in APC treatment group. In CAG treatment group, CD4+ T cell population was significantly decreased when compared to the LPS plus APC group.

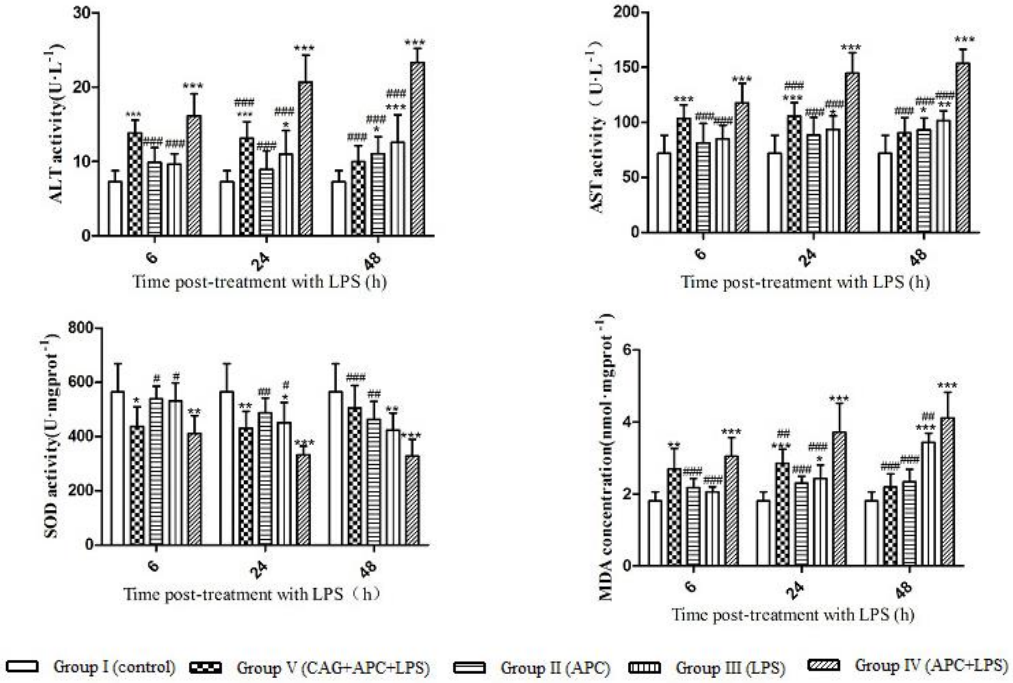

Fig. 1: The changes of ALT, AST, SOD and MDA values in different time points. *P<0.05, **P<0.01, and ***P<0.001 indicate significant differences between Group I and other groups. #P<0.05, ##P<0.01, and ###P<0.001 indicate significant differences between Group IV and other groups.

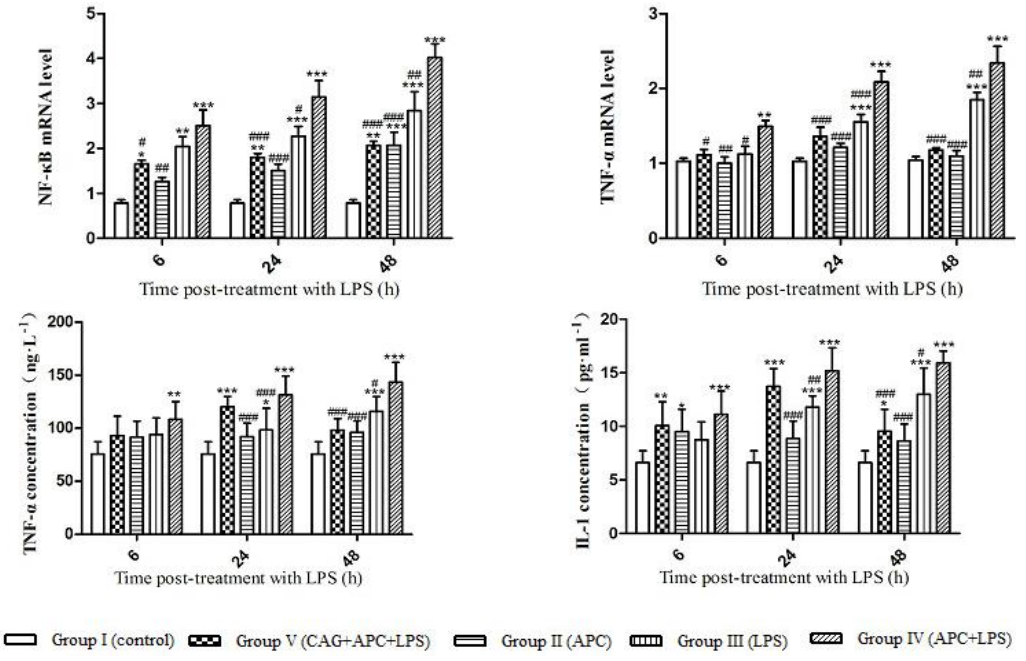

Fig. 2: The changes in mRNA expression of NF-κB and TNF-α and TNF-α and IL-1 contents in different time points. *P<0.05, **P<0.01, and ***P<0.001 indicate significant differences between Group I and other groups. #P<0.05, ##P<0.01, and ###P<0.001 indicate significant differences between Group IV and other groups.

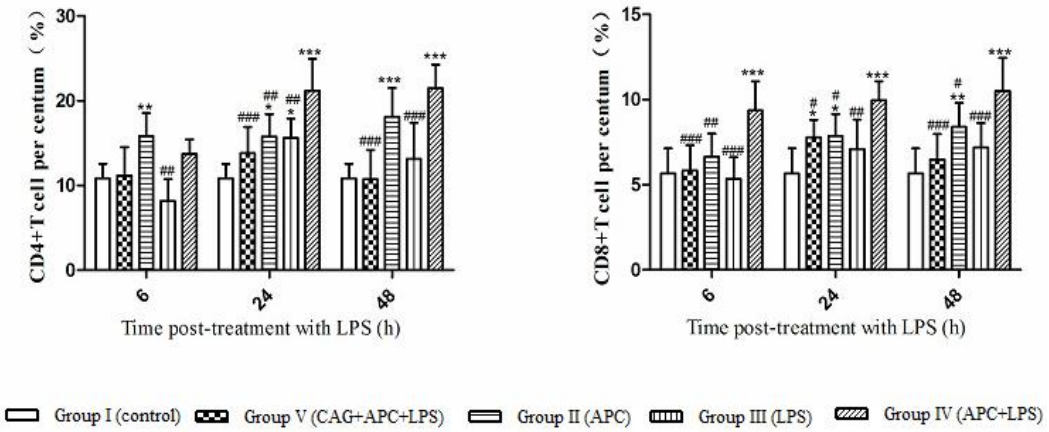

Fig. 3: The population changes of CD4+ and CD8+ T cells in different time points. *P<0.05, **P<0.01, and ***P<0.001 indicate significant differences between Group I and other groups. #P<0.05, ##P<0.01, and ###P<0.001 indicate significant differences between Group IV and other groups.

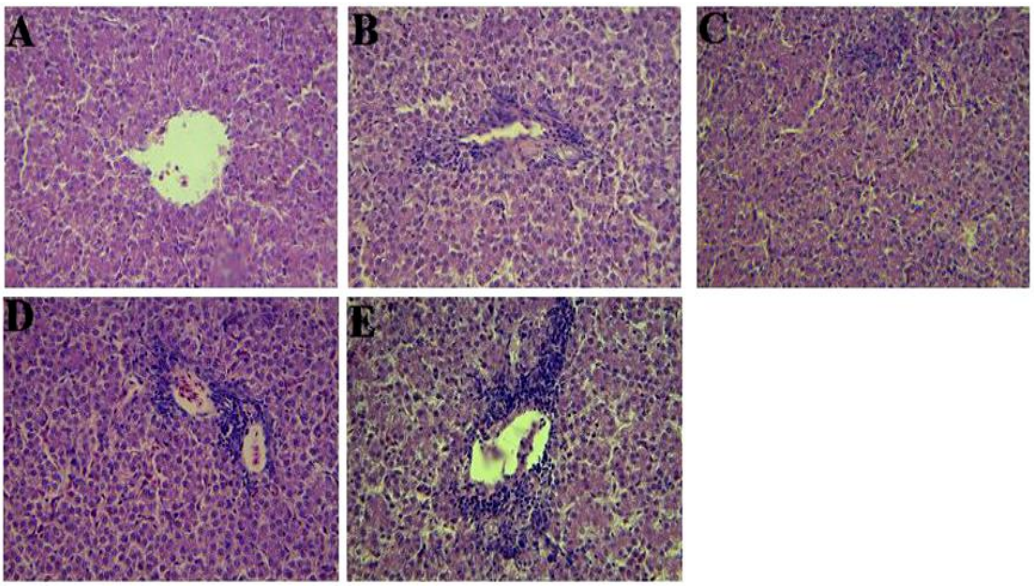

Fig. 4: Histopathological lesions in liver tissues of chickens, Sections were stained with hematoxylin/ eosin (HE), magnitude 400×. A: Group I (control); B: Group V (CAG+APC+LPS); C: Group II (APC); D: Group III (LPS); E: Group IV (APC+LPS).

Table 1: Primers used for amplification of *β-actin*, TNF-α, and NF-κB. Viability and Tm of primers was evaluated using NetPrimer program

Target genes	Forward (5'-3')	Reverse (5'-3')	Product (bp)
β-actin	Atgtggatcagcaagcaggagta	tttatgcgcatttatgggttttgt	126
TNF-α	Gcccttcctgtaaccagatg	acacgacagccaagtcaacg	82
NF-κB	Tcaacgcaggacctaaagacat	gcagatagccaagttcaggatg	162

The histopathological changes in chicken liver: As shown in Fig. 4, the hepatocytes in the control group had a plump cytoplasm and normal structure. In the LPS plus APC group, hepatic tubes were arranged haphazardly with severe congestion and infiltration of inflammatory cells, Hepatocytes had irregular contour with many vacuoles formed by fatty degeneration. In APC treatment group, hepatic lobule structure was normal, liver tubes arranged loosely with mild congestion, and the cytoplasm was reduced. In LPS treatment group, liver tubes were arranged in disorder with congestion and infiltration of inflammatory cells, but all the characteristics were not as serious as those in the LPS plus APC group. In CAG group, the damage to hepatic cells was substantially improved.

DISCUSSION

It is reported that APC accounts for 13-23% of DILI and is a leading cause of adverse hepatic events during hospitalization (Lucena *et al*., 2006; Björnsson *et al*., 2015). And Amoxicillin-treated *E. coli in vitro* exhibited a higher release level of LPS than untreated *E. coli*. In our study, compared to the control group, after APC or LPS treatment, ALT and AST activities were increased significantly. We also observed that the increased ALT and AST activities were more significant in LPS plus APC group. The pathological changes in liver tissue also showed that LPS plus APC induced more serious infiltration of inflammatory cells, fatty degeneration, necrosis, and cell rupture than in LPS or ACP-induced injury alone. Based on these results, we speculated that liver injury could be aggravated when APC and LPS were present simultaneously.

SOD is the first line defense antioxidant enzyme, which can remove oxygen free radicals. MDA is one of the final products of lipid peroxidation. MDA level is commonly known as the marker of oxidative stress and antioxidant status (Tsikas *et al*., 2016). In our study, the non-balance of reduction-oxidation was observed. Compared to the control group, the changes in SOD activity and MDA content in liver tissue homogenate were not significant in APC treatment alone group. But the increase in MDA concentration and the decrease in SOD activity were significant (P<0.001) at 6 h to 48 h in LPS plus APC treatment group. These results showed that the presence of LPS could greatly improve the risk of APC-induced oxidative stress.

NF-κB is a dimeric transcription factor that plays an important role in the modulation of cellular immune, inflammatory and proliferative responses (Braz *et al*., 2010). The expression of many pro-inflammatory mediators, e.g. TNF-α and IL-1, is regulated by NF-κB (French *et al*., 2016). TNF-α and IL-1 are the well-characterized cytokines in inflammatory process of liver injury (Santos *et al*., 2015). TNF-α, the first multifunctional cytokine produced from monocytes and macrophages during trauma or infection, can elicit the inflammatory cascade and contribute to aggravate liver injury (Kim *et al*., 2015; Yao *et al*., 2016).

It has been reported that drug exposure during periods of inflammation can increase an individual's susceptibility to toxicity (Kim *et al*., 2011; Maruf and O'Brien, 2014). In our study, NF-κB mRNA expression was significantly increased ($P<0.001$) at 48 h and IL-1 content was significantly increased ($P<0.05$) at 6 h in APC treatment group when compared to the control group. Thus, we speculate that the inflammatory effect induced by APC may play an important role in the process of APC-induced liver injury of chicken.

LPS is known to induce the production of several inflammatory and chemotactic cytokines (Chen *et al*., 2012). Consistent with previous reports, the mRNA expression levels of NF-κB and TNF-α and the content of TNF-α and IL-1 were increased after treatment with LPS alone. But the mRNA expression levels of NF-κB and TNF-α and the content of TNF-α and IL-1 were significantly increased during 6 h to 48 h ($P<0.001$) in LPS plus APC treatment group. The changing tendencies of TNF-α and IL-1 levels in liver were consistent with their mRNA expression levels in liver. The results suggest that the presence of APC could greatly improve the risk of LPS-induced inflammatory reactions.

Both cellular immunity and humoral immunity responses are involved in the DILI process (Devarbhavi *et al*., 2014). Several studies have shown that the expression of activation markers on the surface of T-lymphocytes was up-regulated by clavulanic acid alone or amoxicillin/clavulanic acid *in vitro* (Yazici *et al*., 2015). T-lymphocytes, especially CD4 phenotype, are the key regulators in initiating ischemia- and reperfusion-induced liver inflammation (Choi *et al*., 2015). The drug reactivation causes damage to liver tissues by an immune allergic reaction, and the destruction is primarily caused by CD8+ cytotoxic T lymphocytes. In our study, APC treatment also promoted a significant increase in CD4+ and CD8+ T cell subpopulations. But CD4+ and CD8+ T cells were not significantly increased in LPS-induced liver injury group in our study, suggesting that LPS perhaps has weak effect on the regulation of CD4+ and CD8+ T cells. LPS plus APC could induce more significant increase in the population of CD4+ T and CD8+ T cells. This may be due to the release of LPS-promoted pro-inflammatory cytokines such as TNF-α and IL-1, which play an important role in regulating the adaptive immune response in T cell subsets (Sims and Smith 2010; Vigne *et al*., 2011).

CAG possess beneficial pharmacological activities including anti-ulcerative effect, anti-inflammatory activity and antioxidant activity, which is known to be effective as a hepatic protective drug (Orazizadeh *et al*., 2014; Wang *et al*., 2013). In this study, compared to LPS plus APC group, CAG preventive treatment can significantly decrease ALT and AST activities and MDA content and increase SOD activity to prevent the damage to hepatic cells and enhance free radical-scavenging capacity. CAG also could markedly suppress the mRNA expression of NF-κB and TNF-α and reduce the release of TNF-α and IL-1 to alleviate the liver inflammatory responses. CAG treatment can decrease CD4+ and CD8+ T cell levels induced by LPS plus APC. These results suggest that administration with CAG before LPS plus APC treatment is beneficial to the protection of liver. However, the activities of ALT, AST and SOD, and the level of MDA in CAG treatment group were significantly different from those in the control group at 6 h and 24 h, we speculate that there still has room for the improvement by adjusting the dose regimen although the level of indexes mentioned above was significantly improved at 48 h, or maybe it is partially blocking the inflammatory mediators, and the exact reason needs further study. It was reported that CAG inhibited the inflammatory of LPS-induced acute lung injury in mice (Shi *et al*., 2010). The results in this study also suggested that CAG may regulate the inflammatory process to produce a protective effect of chicken liver injury. SOD and MDA play an important role in the oxidative stress process, and MDA is toxic molecule and biological marker of oxidative stress (Tsikas *et al*., 2016). TNF-α is a key factor that contributes to the triggering of an inflammatory cascade involving the induction of cytokines such as INF-γ and IL-1β (Lu *et al*., 2016). We have demonstrated that CAG can inhibit the increase in liver tissues levels of SOD, MDA, NF-κB, TNF-α and IL-1 in LPS and APC induced liver injury in chicken, which were speculated was the mechanism of action for its anti-oxidative and anti-inflammatory activity. The mechanism of action for its anti-inflammatory activity. Thus, the exact mechanism and clinical application needs further investigation.

Conclusions: In conclusion, LPS plus ACP may aggravate chicken liver injury through synergistic damage to hepatocytes, oxidative stress, inflammatory response and immune injury. CAG may be useful for treating liver injury through improving these characteristic parameters. Based on these results, to alleviate the poultry liver injury induced by LPS plus antibiotics, more attention should be given to rational application of antibiotics in poultry-farming practice.

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Authors' contribution: ZY contributed to design the whole experiment design and obtained the funding. FG and ZZ executed the preparation of crude LPS extract, analyzed the samples and statistical analyses. XL JT HL involved in Animals housing and sample collection. All

authors involved in discussing the contents of the manuscript and agreed to publication.

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