Protective role of *Salvia miltiorrhiza* Polysaccharides on Florfenicol Induced Oxidative and Apoptotic Injury of Liver in Broilers

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Research Article

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Abstract

Salvia miltiorrhiza Polysaccharides (SMPs) can alleviate liver injury in mice, but there are few reports on liver injury of broilers, especially the liver injury caused by antibiotics. To explore the hepatoprotective effects of SMPs against florfenicol (FFC) induced broilers liver injury, the broilers were treated with FFC and SMPs. The results showed SMPs could significantly inhibit the decrease of weight gain and the increase of liver index induced by FFC ($P < 0.05$). SMPs could significantly reduce the contents of Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) in serum and the malondialdehyde (MDA), nitric oxide (NO) and inducible nitric oxide synthase (iNOS) in liver tissues ($P < 0.05$), also significantly increased the content of total protein (TP) in serum and superoxide dismutase (SOD), catalase (CAT) in liver tissues ($P < 0.05$). QPCR and western bolt results showed that SMPs significantly increased the mRNA and protein expression of cytochrome P4501A1 (CYP1A1), cytochrome P4502H1 (CYP2H1), nuclear factor-erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1) and NAD(P)H dehydrogenase quinone-1 (NQO-1) in liver tissue, also significantly reduced the rate of hepatocyte apoptosis and the mRNA and protein expression of p53, cytochrome-C (CytC), caspase-3 in liver tissue ($P < 0.05$). The results demonstrated that SMPs can inhibit the oxidative stress in hepatocytes by regulating the related proteins in Nrf2 pathway, thereby reducing the apoptosis of hepatocytes, and protecting liver injury.

1 Introduction

Drug induced liver injury (DILI) refers to the direct toxicity of the drug itself or its metabolites to the liver, or the liver injury caused by allergic reaction to the drug or its metabolites after animal exposure to conventional or high-dose drugs (Donato and Tolosa 2019; Iorga et al. 2017; Shen et al. 2019). It has reported that antibiotics are the most common among all the factors would cause drug-induced liver injury, such as penicillins, Chloramphenicols and macrolides (Björnsson 2017). Many studies had demonstrated the hepatotoxicity of antibiotics in human and animal (Björnsson 2017; Ferrajolo et al. 2017; Pan et al. 2019; Stine and Chalasani 2015), which include FFC. FFC is a broad-spectrum animal antibiotic (Varma et al. 1986). It has a good antibacterial effect on a variety of gram-negative and positive bacteria (Shin et al. 2005). It has been reported that high dose of FFC not only enhances the antibacterial effect, but also aggravates the liver injury (Li et al. 2018; Yun et al. 2020). Our previous studies also found that FFC can significantly inhibit the early weight gain of chicks, damage the hematopoietic function of chicks, and damage the liver function of chicks (Han et al. 2020).

In recent years, some research reported that Salvia miltiorrhiza (a traditional Chinese medicine) has a good effect on alleviate liver injury (Nagappan et al. 2019; Shi et al. 2020; Wu et al. 2019), SMPs is one of the important effective components in Salvia miltiorrhiza. Modern studies have found that SMPs also has the protective effect of liver injury (Song et al. 2008; Sun et al. 2011). Our previous research found that SMPs can inhibit inflammatory pathways TRL4/MyD88, NF-κB and chemokine CXCL-10 to reduce the release of inflammatory factors, so as to inhibit the inflammatory response, and then alleviate immune liver injury (Han et al. 2019b; Han et al. 2018; Wang et al. 2019). In addition, SMPs can

effectively regulate oxidative stress in the liver of mice, inhibit apoptosis of hepatocyte, and then alleviate liver injury (Han et al. 2019b; Wang et al. 2019). At the same time, we also found that SMPs can improve liver drug enzyme activity in liver, reduce oxidative stress of liver cells, so that, SMPs also had a good alleviation effect on chicken liver injury caused by CCL_4 (Han et al. 2019a). In conclusion, SMPs has good remission effect on various types of liver injury, and has the potential to be developed into liver protective drugs.

In this experiment, we take broilers as the experimental object to study the effects of SMPs on FFC induced oxidative stress and apoptosis in broiler liver. We provide a new insight for the development of traditional Chinese medicine, which is to alleviate the toxic and side effects of antibiotics.

2 Materials And Methods

2.1 Drugs and Reagents

SMPs (purity ≥ 95%) were purchased from SHENGYAKAI Biological Technical Co. Ltd (Hunan, China), which consisted of five different monosaccharides, including mannose, rhamnose, arabinose, glucose and galactose, in the molar ratio of 2.24:2.35:1.27:0.98:1.11.

AA broilers were purchased from Hebei Dawu Company (Baoding, China). FFC (purity ≥ 10%) were purchased from Shenniu Biological Technical Co. Ltd (Shandong, China). Commercial kits for AST, ALT, MDA, SOD, CAT, GSH, NO and iNOS were obtained from the Jiancheng Bioengineering Institute (Nanjing, China). Polyclonal caspase-3, CytC, P53(Abcam, Cambridge, UK); Polyclonal Nrf2, HO-1, NQO-1 (Bioss Antibodies, Beijing, China) and β-actin (A5441, Sigma, Saint Louis, MO, USA) were used for the western blot assays. Other chemicals used in these experiments of analytical grade were from commercial sources.

2.2 Experimental grouping

A total of 120 healthy 1-day-old chickens were randomly divided into 4 groups, i.e., control Group, FFC group, FFC + SMPs group and SMPs group, of 30 chicks each. From the age of day 1, the chickens in FFC group and FFC + SMPs group were gavaged with FFC solution at a dose of 0.15 g/L, and chickens in control group were given an equal volume of water. Starting from the first day treated with FFC, the chickens in FFC + SMPs group were also added 5 g/L of SMPs in their drinking water in same time. Chickens in SMPs group were added 5 g/L of SMPs in their drinking water from day 1 to day 5 of the experiment. The chickens were fed with normal diet. Ten chickens in each group were sacrificed on 5th and 21st days, and fasted for 12 h before the sacrifice, respectively. Blood was collected from the inferior wing vein of chickens, then obtained the liver immediately. All the experimental protocols were approved by the Animal Care and Use Committee of Hebei Agricultural University prior to the initiation of the study (Permission number: AUH-2020138).

2.3 Weight gain and liver index were measured

We recorded the initial body weight, 5-day-old weight and 21-day-old weight of each chicken in each group, and calculated the weight gain of broilers at 5-day-old and 21-day-old; and their livers were weighed to calculate the liver index, and the liver index = liver weight (g) / body weight (g) × 100%.

2.4 Histology

The liver samples were fixed in 4% paraformaldehyde buffer for at least 24 hours. 5 μm sections were dewaxed in xylene, rehydrated in gradient diluted ethanol solution, and stained with hematoxylin and eosin (H&E). The sections were evaluated by a microscope (CXK41, Olympus Corporation, Tokyo, Japan). According to the degree of necrosis and coagulation in the central and focal areas of the liver, the severity of liver injury was classified from 1 to 5 (Jiang et al. 2017).

2.5 The contents of biochemical markers in serum were detected.

The blood samples were placed in the coagulation promoting tube, placed in the refrigerator at 4 ℃ for 1 hour, and then the serum was separated by low-speed centrifugation. Serum levels of ALT, AST, TP and Alb using a semi-automatic biochemical analyzer (Microlab 300, The Netherlands).

2.6 The contents of oxidative stress markers in liver tissue were detected.

The 10% (wt/vol) liver tissue homogenate was prepared, centrifuged at 4 ℃ for 15 min at the rate of 4000×g, and the supernatant was extracted, then the contents of MDA, CAT, NO, iNOS, GSH, and SOD in tissue homogenate supernatant by commercial assay kits (Nanjing Jiancheng), Protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Cwbiotech, Beijing, china).

2.7 RT-qPCR

The RNA was extracted from liver using Promega total RNA extraction Kit (Promega) and cDNA was obtained by reverse transcription. The transcription levels of CYP1A1, CYP2H1, Nrf2, HO-1, NQO-1, p53, CytC and caspase-3 mRNA were detected by real-time quantitative PCR. The Primers were designed and synthesized by Takara (Dalian, China), and primers sequences were CYP1A1, F: AGGACGGAGGCTGACAAGGT, R: CAGGATGGTGGTGAGGAAGAG; CYP2H1, F: ATCCCCATCATTGGAAATGT, R: ACAGCACCACTCGTAGCCAT; Nrf2, F: CACCCAGCTCACCTGGTATAGTTC, R: TGTGCTTACTTCAGCCAGGTTGTC; HO-1, F: AGTCTCCAACGCCACCAAGTTC, R: AGCTTCTGCAGCGCCTCAA; NQO-1, F: GAA CCCCGAGTGCTTTGTCT, R: CCGCTTCAATCTTCTTCTGCTC; P53, F: CCATTGCTGGAA CCCACTGA, R: CTGCCAGTTGCTGTGATCCTC; CytC, F:TTAGGCAGCACTGCAATATACA CCA, R: GCAGCAGTAGCAACCTGAAACATC; Caspase-3, F: GGACTCTGGAATTCTGCC TGATG, R: CCGTGCCTGAACGAGATGAC; β-actin, F:ATTGTCCACCGCAAATGCTTC,R: AAATAAAGCCATGCCAATCTCGTC. The samples were pre denatured at 95 ℃ for 120 s, followed by 45 cycles, including 5 s at 95 ℃ and 30 s at 64 ℃. Using β-actin as internal reference gene, the relative transcription level of each gene was calculated by $2^{-\Delta\Delta CT}$.

2.8 Western Blotting

Total protein was extracted from chicken liver in control group, FFC group and FFC + SMPs group with protein extraction kit (cwbiotech, Beijing, China). The protein concentration was determined by BCA method. A mount of 40 μg protein extract from each sample was denatured in boiling water for 5 min, and loaded on SDS PAGE gel for electrophoresis and transferred to the nitrocellulose membrane (Carlsbad). After blocking the nonspecific binding sites, Caspase-3, CytC, P53 (Abcam, Cambridge, USA) and Nrf2, HO-1, NQO-1 (BIOS antibody, Beijing, China) were incubated for 12 hours in 4 ℃, and then stained with nitroblue tetrazolium chloride (NBT) / 5-bromo-4-chloro-3-indole-phosphate (BCIP) test kit (Solarbio, Beijing, China).

2.9 statistical analysis.

SPSS21.0 software (IBM, Armonk, NY) was used to test the normality and homogeneity of variance. One-way ANOVA was used to compare the average difference among the groups. LSD test was used for homogeneity of variance and dunnett-13 test was used for variance heterogeneity. The experimental data are expressed as mean ± SD. The difference was significant when $P < 0.05$.

3 Results

3.1 Effect of SMPs on weight gain and liver index of Broilers

Compared with the control group, FFC significantly decreased the weight gain and increased the liver index at 5 and 21 days of age ($P < 0.05$), while compared with FFC group, SMPs significantly increased the weight gain of broilers at 5 and 21 days of age ($P < 0.05$), and significantly inhibited the increase of liver index at 5 and 21 days of age ($P < 0.05$) (Table 1).

Table 1
Average weight gain and liver index of broilers

Group	weight gain(g)		Liver index (%)	
	d5	d21	d5	d21
Control	156.34 ± 13.97	1028.34 ± 55.05	2.01 ± 0.54	2.47 ± 0.55
SMPs	161.38 ± 14.32	1120.90 ± 81.34*	2.03 ± 0.36	2.34 ± 0.22
FFC	103.34 ± 21.67*	652.74 ± 38.55*	2.57 ± 0.73*	3.12 ± 0.5*
FFC + SMPs	139.96 ± 13.07#	922.74 ± 37.76*#	1.93 ± 0.42#	2.34 ± 0.41#
*$P < 0.05$, compared with the control group; #$P < 0.05$, compared with the FFC group.				

3.2 Effect of SMPs on serum biochemical markers

At the age of 5 and 21 days, the AST and ALT contents in serum of FFC group were significantly higher than those of control group ($P < 0.05$) (Fig. 1a and Fig. 1b), and the TP content in serum of FFC group were significantly lower than that of control group ($P < 0.05$) (Fig. 1c); in addition, the contents of serum AST and ALT in FFC + SMPs group were significantly lower than those in FFC group ($P < 0.05$) (Fig. 1a and Fig. 1b); and the contents of serum TP in FFC + SMPs group was significantly higher than that in FFC group ($P < 0.05$). As for ALB content in serum, there was no significant difference between each group at 5 days old and 21 days old ($P > 0.05$) (Fig. 1d).

3.3 Effect of SMPs on Liver Histopathology

Of the control group (A) and SMPs group (B), histological architecture of the liver was clear in d5 and d21. Of the FFC group (C), the cytoplasm was deeply stained, A lot of inflammatory cells were t infiltrated in the portal area, the central meridian hepatocytes were swollen and degenerated, necrotic of the model group. while FFC + SMPs group showed less damage of liver cells, indicating that the herbal ingredient alleviated the injury (Fig. 2a and Fig. 2b). Furthermore, the liver injury score showed that SMPs improved the FFC-induced liver injury (Fig. 2c).

3.4 Effect of SMPs on markers of oxidative stress in liver tissue

At 5 and 21 days of age, in broilers treated with FFC alone, we found a significant induction of oxidative stress in livers with a marked increase of MDA, iNOS, and NO ($P < 0.05$) (Fig. 3d, Fig. 3e and Fig. 3f), and the contents of SOD and CAT in liver tissues were significantly decreased ($P < 0.05$) (Fig. 3a and Fig. 3b). Compared with FFC group, the contents of MDA, NO and iNOS of broilers liver tissue in FFC + SMPs group were significantly decreased ($P < 0.05$) (Fig. 3d, Fig. 3e and Fig. 3f), while the contents of CAT and SOD were significantly increased ($P < 0.05$) (Fig. 3a and Fig. 3b); however, there was no significant difference in GSH content in liver tissues of broilers at 5 and 21 days of age ($P > 0.05$) (Fig. 3c).

3.5 Effect of SMPs on the contents and mRNA transcription of CYP1A1 and CYP2H1 in liver

We measured the contents of CYP1A1 and CYP2H1 in liver by ELISA kit and mRNA transcription of CYP1A1 and CYP2H1 in liver by RT-qRCR, we found that at 5 and 21 days of age, the content and the mRNA transcription level of CYP1A1 in FFC group were significantly lower than the control group ($P < 0.05$); while compared with FFC, SMPs could significantly increase the content of CYP1A1 and the transcription level of CYP1A1 mRNA in liver tissue of broilers ($P < 0.05$) (Fig. 4). At 5 days of age, the content of CYP2H1 and the transcription level of CYP2H1 mRNA in the liver of broilers in FFC group were significantly lower than those in control group, SMPs group and FFC + SMPs group; However, there was no significant difference in the content of CYP2H1 and the transcription level of CYP2H1 mRNA between each group at 21 days of age ($P > 0.05$) (Fig. 4).

3.6 Effect of SMPs on Nrf2 pathway protein and mRNA transcription in broilers liver

As shown in Fig. 5a, RT-qPCR analysis showed a lower expression level of Nrf2, HO-1 and NQO-1 mRNA in FFC group than in control group. However, the decrease in the expression of Nrf2, HO-1 and NQO-1 mRNA caused by FFC was abrogated up on SMPs treatment. Next, We detected the protein expression of Nrf2, HO-1 and NQO-1 in broilers liver tissues, the results as Fig. 5b and Fig. 5c showed: compared with control group, FFC significantly reduced protein expression levels of Nrf2, HO-1 and NQO-1 in the liver at age of days 5 and 21 ($P < 0.05$); however, compared with FFC group, 5 g/L SMPs significantly increased Nrf2 and HO-1 protein expression levels at age of days 5 and 21 ($P < 0.05$), and significantly increased the NQO-1 protein expression levels only at age of days 5 ($P < 0.05$).

3.7 Effect of SMPs on apoptosis protein and mRNA transcription in chicken liver

As shown in Fig. 6a, we detected the hepatocyte apoptosis of broilers in each group at age of 5 days by TUNEL test, and found that compared with the control group, FFC could significantly increase hepatocyte apoptosis, and the hepatocyte apoptosis rate after intervention with FFC + SMPs was significantly lower than that of FFC alone treatment group ($P > 0.05$). Next, we detected the mRNA transcriptional level of p53, cytc and caspase-3 in broiler liver tissues, we found that compared with the control group, FFC could significantly increase the mRNA transcription levels of p53, cytochrome C (CytC) and Caspase-3 in liver of broilers at 5 and 21 days of age ($P < 0.05$) (Fig. 6b); 5 g/L SMPs significantly inhibited the expression of p53, CytC and caspase-3 mRNA in liver induced by FFC ($P < 0.05$) (Fig. 6b). We also detected the protein expression of P53,CytC and Caspase-3 in broilers liver tissues, the results showed that at age of 5 days, FFC could significantly increase the expression of p53 and caspase-3 protein in liver of broilers ($P < 0.05$); However, the protein expression levels of p53 and Caspase-3 in liver of broilers treated with 5 g/L SMPs and FFC were significantly lower than those of FFC alone ($P < 0.05$), and there was no significant difference in the expression of CytC protein in the liver of broilers among the groups ($P > 0.05$) (Fig. 6c). While at age of 21 days, compared with the control group, FFC significantly increased the expression of p53 and CytC protein in liver of broilers ($P < 0.05$); however, the expression levels of p53 and CytC protein in group of combination with 5 g/L SMPs and FFC were significantly lower than those in FFC alone group ($P < 0.05$), but there was no significant difference in the expression level of Caspase-3 in the liver of broilers among each groups ($P > 0.05$) (Fig. 6d).

4 Discussion

Our previous studies found that florfenicol (0.15 g/L) had certain damage effects on growth performance, hematopoietic and liver function of broilers within 7 days of age (Han et al. 2020). In this study, we explored whether SMPs can alleviate florfenicol induced liver toxicity in broilers. We found that: at 21 days of age, 0.15 g/L FFC still significantly inhibited the weight gain of broilers, while the weight gain of broilers treated with SMPs combined with FFC was significantly higher than that of treated with FFC alone. The results showed that SMPs could alleviate the growth inhibition of broilers induced by FFC.

The activity of ALT and AST in serum are specific indexes to the degree of liver injury (Ben Abdennebi et al. 2011; Hu et al. 2014). In the current study, SMPs reduced the high levels of ALT and AST in serum elevated by FFC, the results indicate that SMPs can significantly alleviate the injury of hepatocytes. Liver is also the synthesis site of total protein and albumin in serum, the content of total protein and albumin in serum can reflect the function of liver (Swart et al. 1988). In this study, different concentrations of SMPs treatment increased serum total protein and albumin levels in varying degrees, indicating that SMPs have a role in relieving liver injury.

More and more evidences show that oxidative stress plays an important role in drug-induced liver injury (Ezhilarasan 2018; Gao 2012). In order to detect whether SMPs can alleviate liver injury by inhibiting oxidative stress of hepatocytes. In this experiment, we detected the markers of oxidative stress, MDA, NO and iNOS, as well as the key antioxidant enzymes SOD, CAT and GSH (Qu et al. 2019). The results showed that after SMPs intervention, the content of MDA, NO and iNOS decreased, and the levels of SOD, CAT and GSH in liver tissue of broilers increased, which indicated that the antioxidant capacity of the body was improved, and the stress defense function was activated, thus alleviating the liver cell damage of broilers.

There is evidence that Nrf2 signaling pathway, as a coordination factor, plays an important role in the antioxidant stress system (Palliyaguru et al. 2016). By inducing and regulating the constitutive and inducible expression of phase II detoxification enzymes and antioxidant enzymes, it is beneficial to improve the body's oxidative stress state, promote cell survival and maintain cell redox homeostasis (Motohashi and Yamamoto 2004; Surh and Na 2008). Our results showed that SMPs could improve florfenicol induced liver injury in broilers by activating the expression of Nrf2 and its downstream proteins. In addition, Nrf2, as a key molecule in oxidative stress, can regulate the expression of downstream HO-1, NQO-1 and other proteins, and plays an important role in regulating intracellular fat accumulation (Leung et al. 2003; Yang et al. 2012). Theoretically, the expression of NQO-1 and HO-1 will increase when the body is subjected to oxidative stress, thus reducing oxidative stress (Gonzalez-Burgos et al. 2012). Our results show that SMPs can increase the expression of HO-1 and NQO-1, which indicates that SMPS can enhance the antioxidant capacity of cells and regulate the lipid metabolism of hepatocytes.

Oxidative stress is also one of the inducing factors of apoptosis (Egnatchik et al. 2014). In order to detect the effect of SMPs on liver cell apoptosis. We detected the apoptosis rate of hepatocytes by TUNEL test. It was found that SMPs could significantly inhibit the increase of hepatocyte apoptosis rate induced by FFC, and Western bolt and QPCR results also indicated SMPs can significantly inhibit the expression of Pro-apoptotic protein p53, CytC and caspas-3 in the liver, indicating that SMPs can inhibit the excessive apoptosis induced by FFC and restore the normal function of liver. Previous studies have shown that dysfunction of mitochondria in hepatocytes leads to ROS accumulation. Excessive ROS further damages mitochondrial DNA, protein and phospholipids and induces hepatocyte apoptosis (Riedl and Shi 2004). CytC and some other caspase activating factors are released into the cytoplasm from the mitochondrial

membrane space, which changes the permeability of mitochondrial membrane and further activates the caspase cascade reaction (Riedl and Shi 2004), this result is consistent with our experimental results.

5 Conclusion

SMPs against FFC-induced liver injury by attenuating oxidative stress and apoptosis through the Nrf2/HO-1 signaling pathway, thus reduce the content of AST and ALT in serum and restore the normal tissue structure and protein synthesis function of liver.

Declarations

Ethical Approval

All the experimental protocols were approved by the Animal Care and Use Committee of Hebei Agricultural University prior to the initiation of the study (Permission number: AUH-2020138).

Conflict of interest

No author has declared a conflict of interests with respect to this study.

Consent to Participate

Not applicable.

Consent to Publish

All co-authors agreed to publish this article.

Authors Contributions

Wanyu Shi and Yongzhan Bao conceived and designed the study, and they rigorously revised the manuscript. Xiao Wang, Yuqing Cui and Chao Han participated in the design of the experiment, conducted most of the experiments and analyzed the results. They also co-wrote the manuscript. Yumeng Geng helped collect samples and participated in the qPCR experiment. Di Zhang and Wei Liu participated in the H&E and Western blot experiments. All authors read and approved the final manuscript.

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Competing Interests

The authors have no conflicts of interest.

Availability of data and materials

The data sets supporting the results of this article are included within the article and its additional files.

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Figures

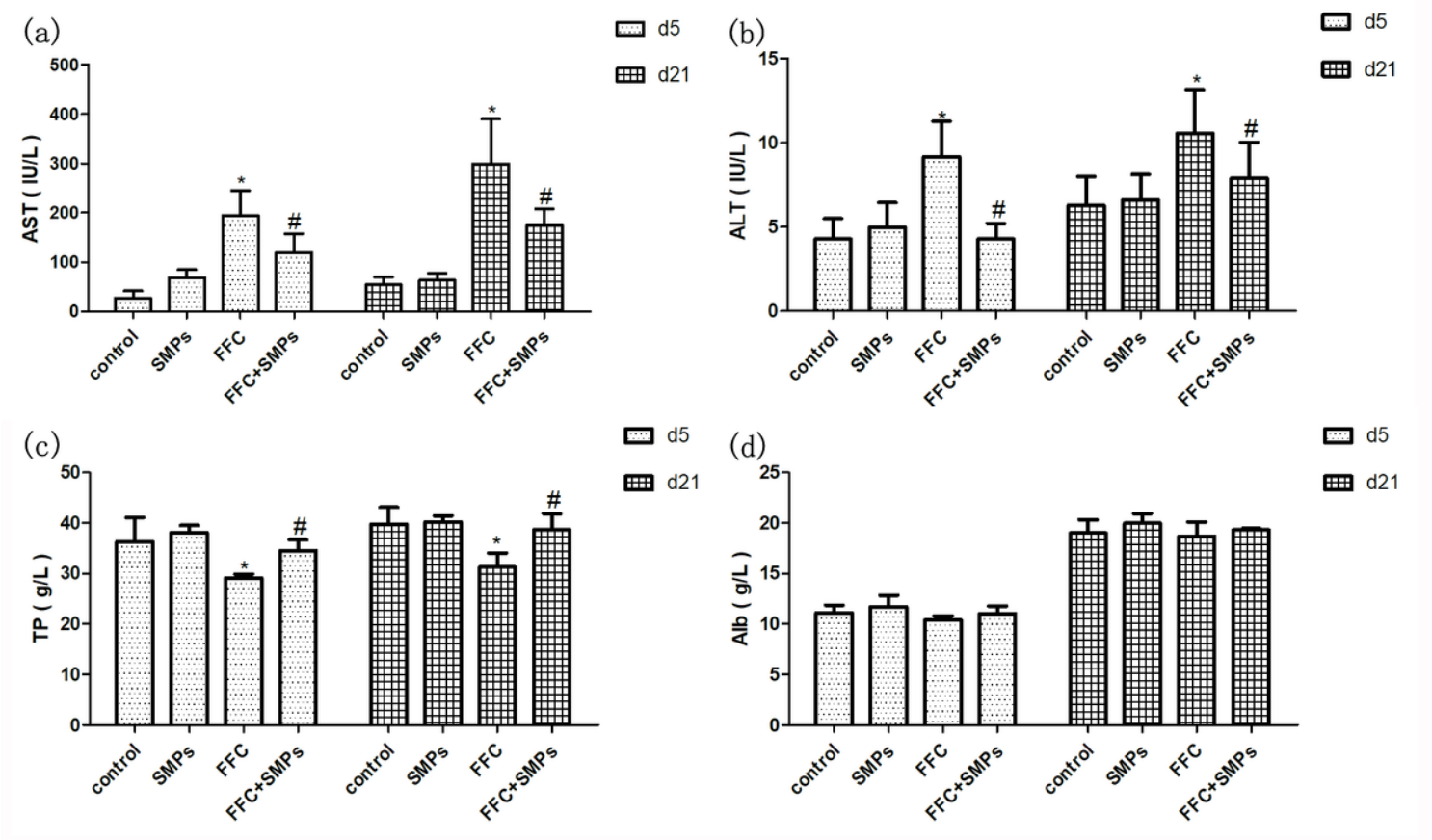

Figure 1

The expression of liver function markers in broilers serum, (a): the AST content; (b) the ALT content; (c): the TP content; (d): the ALb content. *P < 0.05, compared with the control group; #P < 0.05, compared with the FFC group.

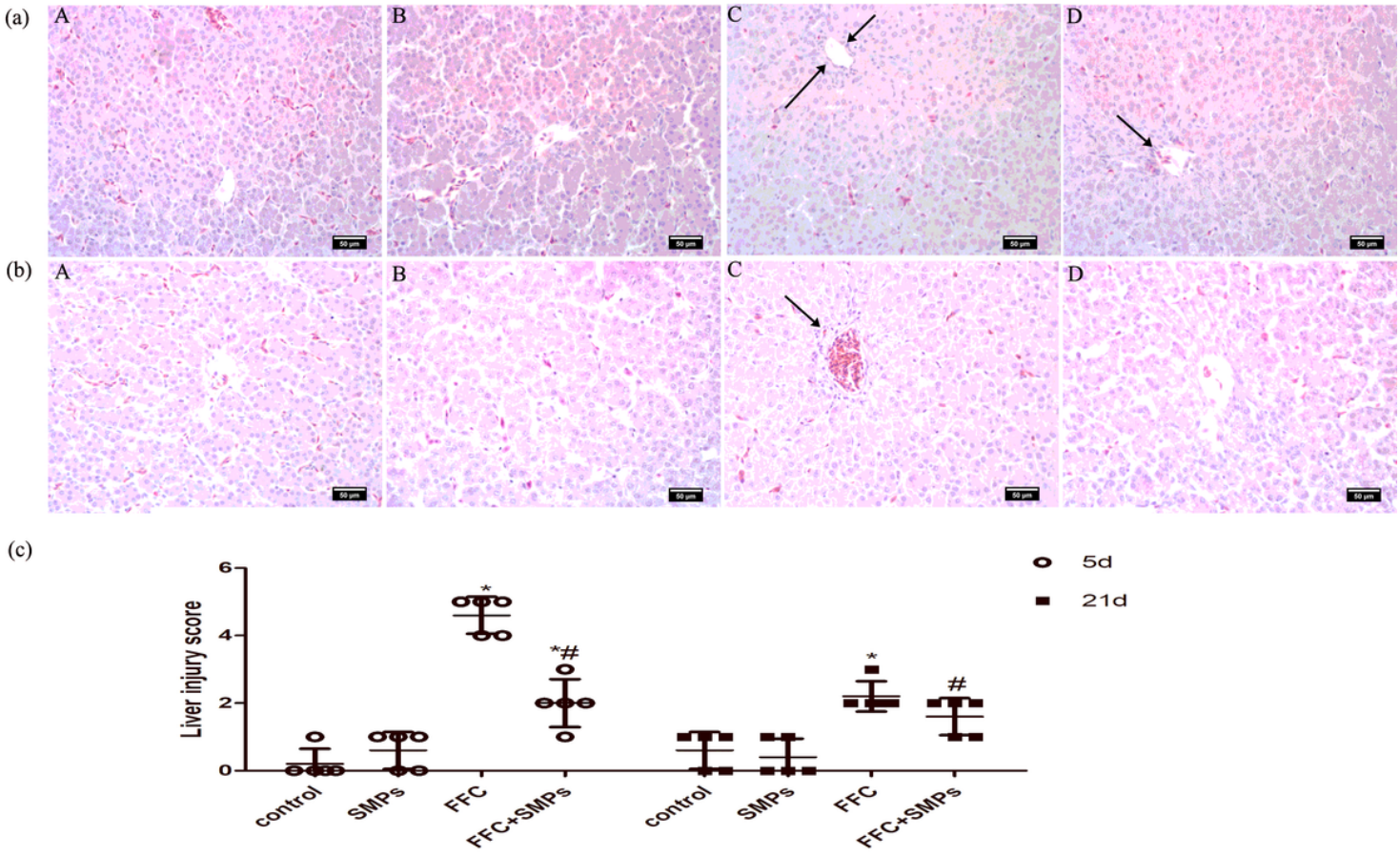

Figure 2

The effects of SMPs on histopathological changes on the liver in (a) d5 and (b) d21; (c) the severity of liver injury was analyzed using the liver injury scoring system in FFC-induced chicks. A is control group, B is SMPs group, C is FFC group, group D is FFC+ SMPs group. *P < 0.05, compared with the control group; #P < 0.05, compared with the FFC group. Arrow head donated central veins and highlight liver injury/necrosis.

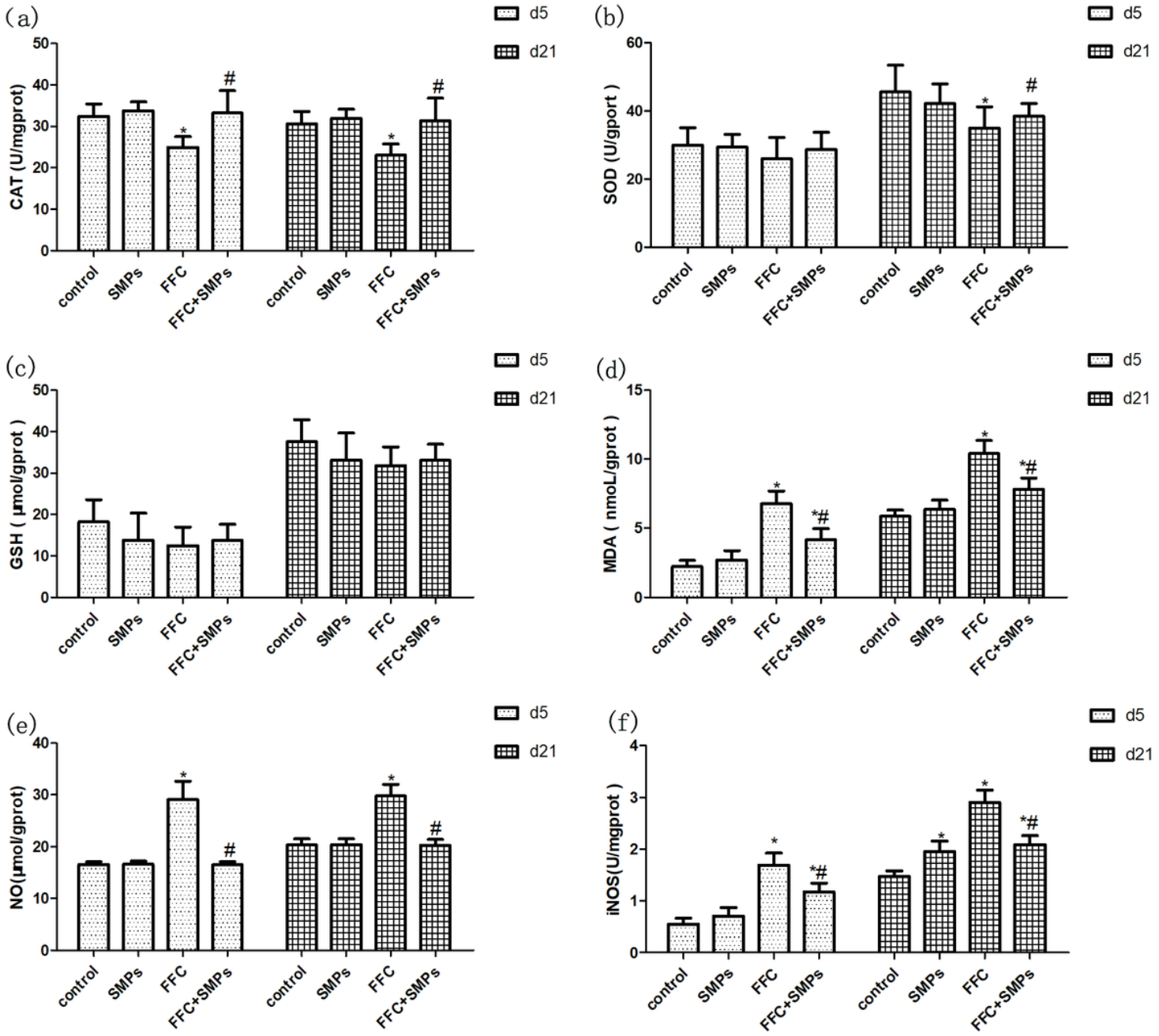

Figure 3

The expression of oxidative stress markers in liver tissue, (a): the CAT content; (b) the SOD content; (c): the GSH content; (d): the MDA content; (e): the NO content; (f): the iNOS content. *P < 0.05, compared with the control group; #P < 0.05, compared with the FFC group.

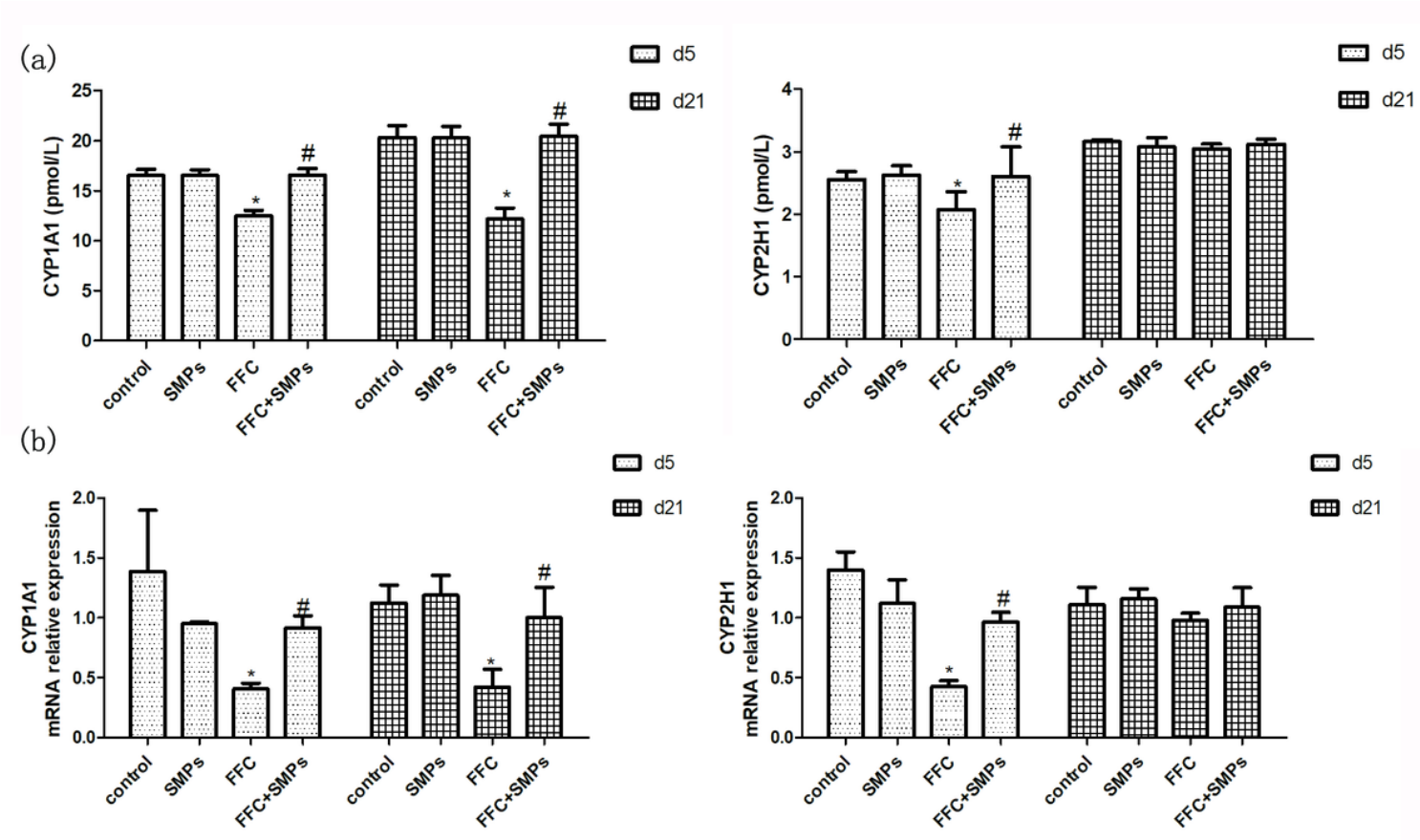

Figure 4

Activity of liver drug enzymes in broiler liver tissues, (a): the contents of CYP1A1 and CYP2H1; (b) the mRNA transcriptional levels of CYP1A1 and CYP2H1. *P < 0.05, compared with the control group; #P < 0.05, compared with the FFC group.

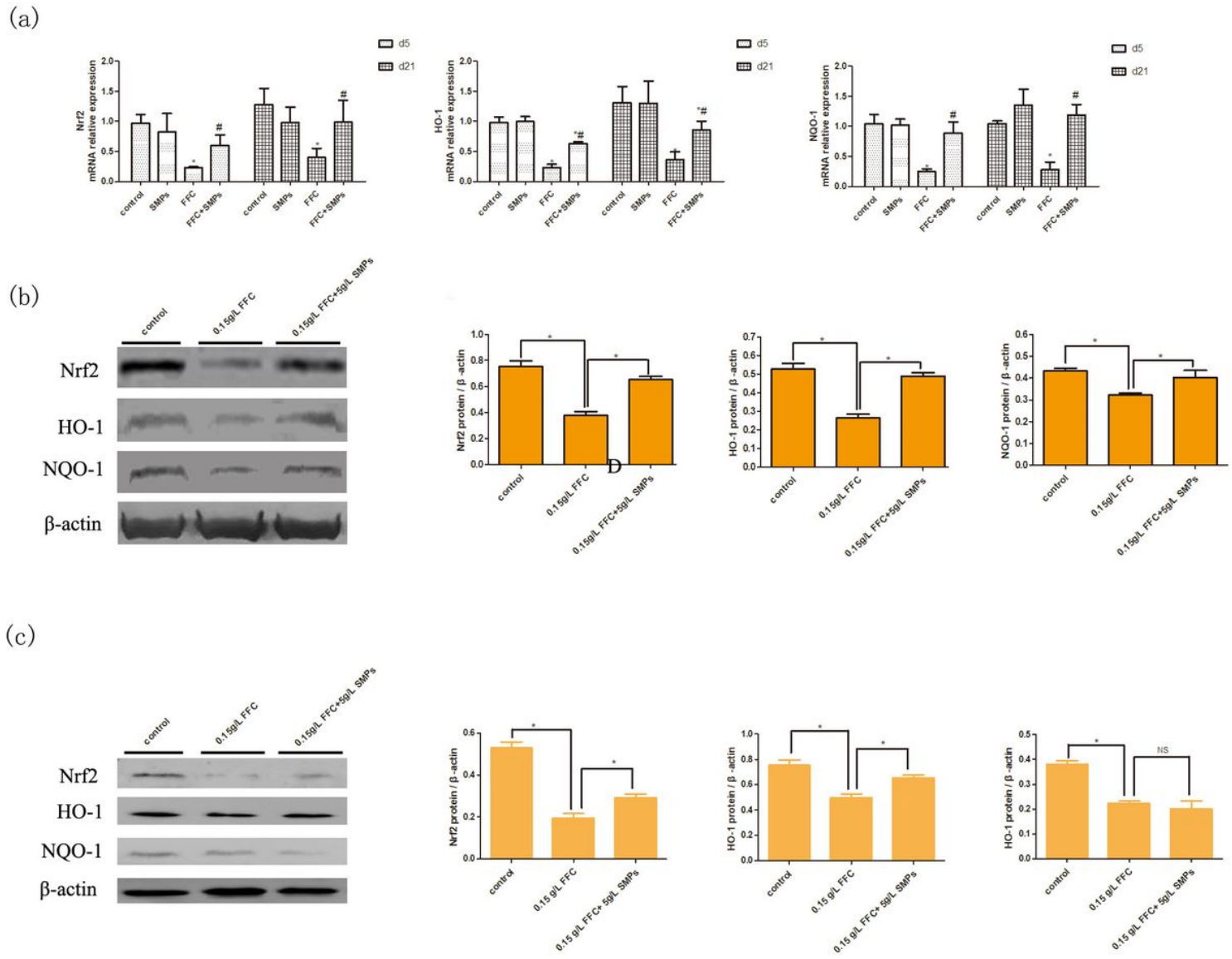

Figure 5

The key molecules expression of Nrf2 pathway in broilers liver, (a) mRNA transcription level of Nrf2, HO-1 and NQO-1 in liver tissue ; (b) protein expression level of Nrf2, HO-1 and NQO-1 in liver tissue at age of days 5; (c) protein expression levels of Nrf2, HO-1 and NQO-1 in liver tissue at age of days 21*P < 0.05, compared with the control group; #P < 0.05, compared with the FFC group.

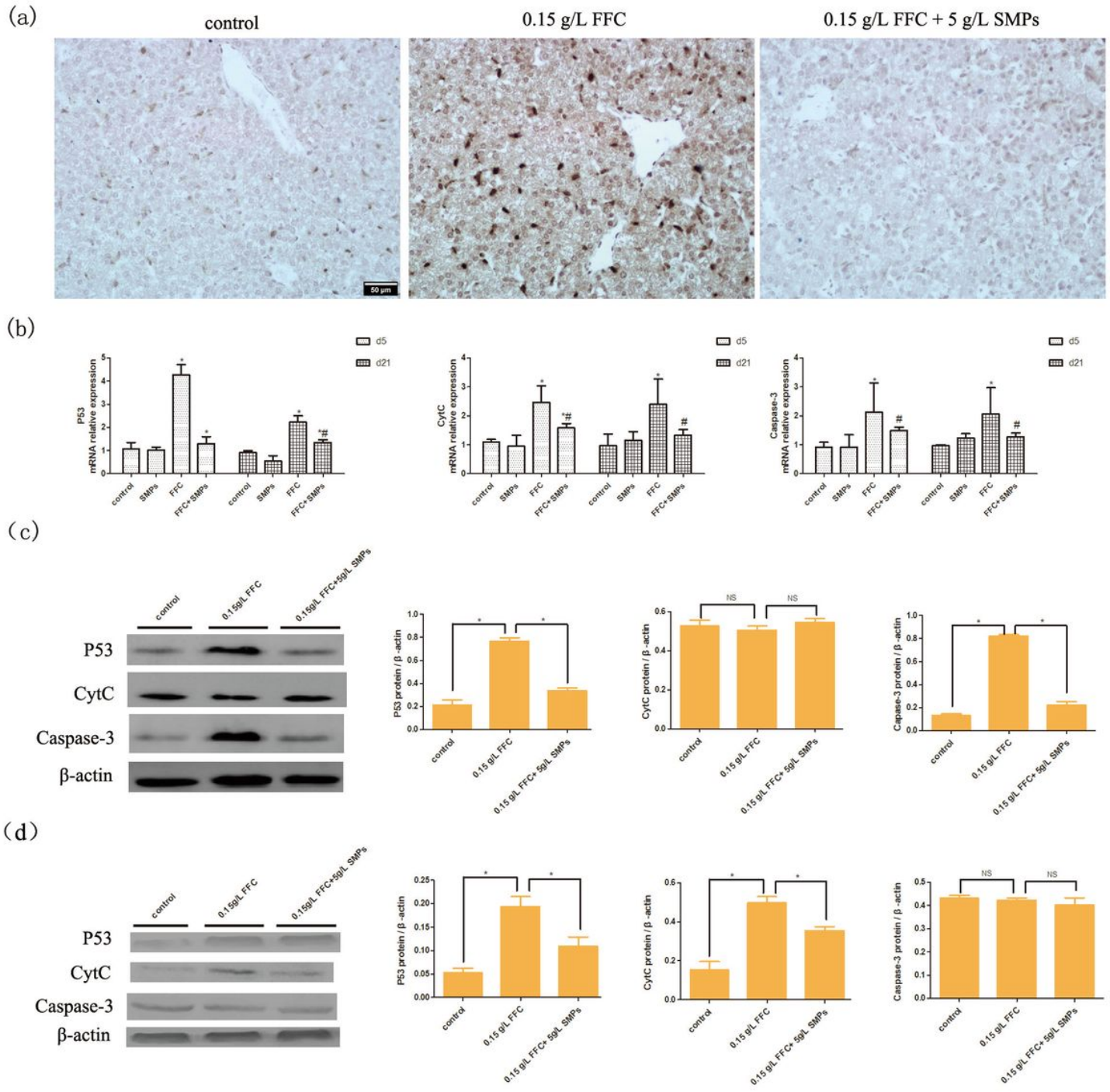

Figure 6

The apoptosis of broilers liver, (a): hepatocyte apoptosis rate detected by TUNEL test at age of days 5; (b) mRNA transcription level of p53, cytc and Caspase-3 in liver tissue of broilers at age of days 5 and 21; (c) protein expression level of p53, cytc and Caspase-3 in liver tissue at age of days 5; (d) protein expression levels of p53, cytc and Caspase-3 in liver tissue at age of days 21. *P < 0.05, compared with the control group; #P < 0.05, compared with the FFC group.