Ecotoxicology and Environmental Safety 191 (2020) 110239

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Florfenicol induces oxidative stress and hepatocyte apoptosis in broilers via Nrf2 pathway

Chao Han[a,1], Yuanyuan Wei[a,1], Yuqing Cui[a], Yumemg Geng[a], Yongzhan Bao[a,b,**], Wanyu Shi[a,b,*]

[a] College of Traditional Chinese Veterinary Medicine, Agriculture University of Hebei, Baoding, 071001, China
[b] Hebei Provincial Engineering Center for Chinese Veterinary Herbal Medicine, Baoding, 071001, China

ARTICLE INFO

Keywords:
Florfenicol
Oxidative stress
Apoptosis
Liver injury
Broilers

ABSTRACT

In order to explore the mechanism of liver injury induced by florfenicol (FFC) in broilers, one hundred and twenty broilers were randomly divided into six groups, twenty broilers in each group. Except for control group, the other five groups were given different doses of FFC (0.15 g/L, 0.3 g/L, 0.6 g/L, 1.2 g/L and 1.8 g/L) in drinking water. After five days of continuous use, blood was collected from the subpterional vein and the chickens' liver were obtained. Chicken weight gain and liver indices were calculated; blood routine analysis was performed; the oxidative stress and apoptosis of hepatocytes was detected. The results showed that compared with the control group, except for 0.15 g/L FFC, the other doses of FFC significantly decreased the weight gain, white blood cell (WBC) and platelet (PLT) contents in blood, 0.3 g/mL FFC and 1.8 g/L FFC significantly reduced the content of hemoglobin (RGB) ($P < 0.05$); all doses of FFC significant decreased red blood cell (RBC) increased Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) contents in serum of chickens ($P < 0.05$), and significantly decreased the contents of albumin (ALB) and total protein (TP) in serum ($P < 0.05$), but had no significant effect on alkaline phosphatase (ALP) contents($P > 0.05$). FFC significantly increased malondialdehyde (MDA) content in serum and liver tissues, but decreased glutathione (GSH), Superoxide dismutase (SOD) and catalase (CAT) content ($P < 0.05$), and significantly inhibited the mRNA transcription and protein expression of antioxidant proteins nuclear factor-erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1) and NAD(P)H dehydrogenase quinone-1 (NQO-1)($P < 0.05$). FFC also inhibited the content and the transcription level of cytochrome P4501A1(CYP1A1) and CYP2H1 in liver ($P < 0.05$). At the same time, FFC significantly promoted the apoptotic rate of hepatocytes and the mRNA transcription and protein expression of caspase-3 and caspase-6 ($P < 0.05$). With the increase of FFC concentration, liver injury became more and more serious, which affected liver function in chickens by inhibiting enzyme activity in Nrf2-ARE pathway to increase oxidative stress and promoting apoptotic protein expression to accelerate hepatocyte apoptosis.

1. Introduction

Florfenicol (FFC) is a broad-spectrum antimicrobial agent for veterinary chloramphenicol developed successfully in the late 1980s (Syriopoulou et al., 1981). Its mechanism is to interfere with the synthesis of bacterial proteins (Sidhu et al., 2014). It has the characteristics of rapid absorption, wide distribution in vivo, long half-life and no aplastic anemia is widely used in animal breeding (Fraunfelder et al., 1982). It is often used in treating infections caused by a variety of pathogens, including *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, Streptococcus, Pasteurella multocida and Mycoplasma pneumoniae (Shin et al., 2005; Wisselink et al., 2006). With the extensive use of FFC in livestock and poultry farming, the toxic and side effects of FFC have attracted more and more attention. The drug resistance of FFC has become more and more serious, such as the emergence and increasing severity of multi-drug-resistant pathogenic bacteria such as Haemophilus influenzae, Xanthomonas aureus, which can coexist with humans and animals (Li et al., 2015; Osman et al., 2019; Verner-Jeffreys et al., 2017). Moreover, the damage of FFC to animal tissues and organs has been reported continuously, including the damage to hematopoietic and immune functions, such as inhibition of macrophage phagocytosis (Bretzlaff et al., 1987), lymphocyte

* Corresponding author. College of Traditional Chinese Veterinary Medicine, Agriculture University of Hebei, 2596# Le Kai South Street, Baoding, 071001, China.
** Corresponding author. College of Traditional Chinese Veterinary Medicine, Agriculture University of Hebei, 2596# Le Kai South Street, Baoding 071001, China.
E-mail addresses: baoyongzhan2006@126.com (Y. Bao), shiwanyu2010@126.com (W. Shi).
[1] equal contributors.

https://doi.org/10.1016/j.ecoenv.2020.110239
Received 11 October 2019; Received in revised form 26 December 2019; Accepted 21 January 2020

proliferation (Shuang et al., 2011), immune response (Khalifeh et al., 2009) and hematopoietic suppression (Hassanin et al., 2014). In addition, the abuse of FFC can also cause hepatotoxicity and nephrotoxicity (Shah et al., 2016). When the liver of animals is damaged, the detoxification ability of animals would decrease, resulting in the residue of antibiotics in animal-derived food even exceeding the standard (Bjornsson, 2017). Long-term consumption of florfenicol-rich meat, eggs and milk, which will lead drug resistance and hepatotoxicity to endanger human health and cause major public safety and health problems (Donoghue, 2003; Paige et al., 1997); remains a major challenge confronting contemporary.

In the process of broiler feeding, 1-day-old broilers were often fed with FFC in their feedstuff or drinking water to resist intestinal bacterial infection and to enhance the immunity of chickens, thereby improving the survival rate and growth rate of chickens (Pozniak et al., 2017). And in this process, Overuse of FFC in chicken farming constantly exists, so the toxicity effects of FFC on broilers are inevitable. It has been found that FFC can cause moderate bone marrow cell dysplasia and toxicity (Hassanin et al., 2014). And other research has reported that FFC can promote the apoptosis of chicken hepatocytes in vitro (Li et al., 2018), but the toxicity of FFC to broiler liver has not been reported. Therefore, to determine whether the therapeutic dose of FFC has toxicity effect on chicken liver, and further to guide the scientific use of FFC in broilers production, we evaluated FFC-induced toxicity and side effects to broliers hepatocytes. The blood and tissue samples obtained from broilers treated or untreated with a therapeutic dose of FFC (0.15 g/L), we determined the levels of ALT, AST, ALP, TP and Alb in serum, the changes of oxidative indices in serum and liver tissue, the contents of CYP1A1 and CYP2H1 in liver tissue, and the changes of Nrf2 pathway protein and caspase protein in oxidative regulation pathway.

2. Materials and methods

2.1. Animal

A total of 120 chickens (Arbor Acres broilers) were purchased from Hebei Dawu Agricultural Group Poultry Company Ltd (Baoding, China).

2.2. Drugs and reagents

FFC (purity ≥ 95%) were purchased from Shenniu Biological Technical Co. Ltd (Shandong, China). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Commercial kits for detecting chicken cytochromeP450 1A1(CYP1A1) and CYP2H1 were purchased from Shanghai hengyuan Biotech CO.,Ltd (Shanghai china). Other chemicals used in these experiments of analytical grade were from commercial sources.

2.3. Experimental design

A total of 120 one-day-old chicks were housed in a clean well-ventilated room and kept under good sanitation and hygienic management. Feed and water were available ad libitum. After an acclimatization period of one day, chicks (average body weight = 42.34g ± 1.3 g/chick) were randomly allotted into 6 groups (20 chicks per group). FFC was given orally in drinking water at 0.0 g/L for group A (control group), FFC at 0.15 g/L (Recommended dosage of Chinese Veterinary Pharmacopoeia in chick) for group B, FFC at 0.3 g/L for group C, FFC at 0.6 g/L for group D, FFC at 1.2 g/L for group E, FFC at1.8 g/L for group F. The FFC treatment was from day 1 to day 5 of age consecutively and chicks in all groups were fed with basal diet (purchased from Hebei Dawu Agricultural Group Poultry Company Ltd). At day 6 of age, chickens were sacrificed following euthanization with sodium pentobarbital. Blood samples were collected from the vein and the liver of the chickens was obtained. All the experimental protocols were approved by the Animal Care and Use Committee of Agricultural University of Hebei prior to the initiation of the study.

Table 1
Amounts of weight gain and Liver index of each group.

Groups	weight gain(g)	Liver index (%)
control	119.81 ± 16.49	4.641 ± 0.363
0.15 g/L FFC	68.31 ± 19.15*	4.088 ± 0.301
0.3 g/L FFC	47.70 ± 12.14**	4.773 ± 0.572
0.6 g/L FFC	40.69 ± 11.02**	4.849 ± 0.695
1.2 g/L FFC	19.96 ± 12.84**	5.572 ± 0.406**
1.8 g/L FFC	16.49 ± 8.91**	6.350 ± 1.195**

$^{**}P < 0.01$, Compared with the control group; $^{*}P < 0.05$, compared with the control group.

2.4. Amount of body weight gain and relative liver weight

Each group of chickens were recorded their initial body weight, weighed each group again after feeding to 5-day-old chickens, and then the differences were calculated between the weight of 1-day-old chickens and 5-day-old chickens as the amount of weight gain of each group. In each group, 10 chickens were randomly selected and executed, and relative liver weight was expressed as a percentage of body weight. Relative liver weight = liver weight (g)/body weight (g) × 100% (Zhang et al., 2013).

2.5. Hematology analysis

The whole blood samples of chickens were taken into anticoagulant tube, and then detected by Automatic Hematological analyzer BC-5000vet (Mindray Medical International, Shenzhen, China). The number of red blood cells (RBC), white blood cells (WBC), hemoglobin (RGB) and platelet (PLT) in the blood of chickens in each group were recorded.

2.6. Biochemical parameter measurement

At Day 6, the blood samples were collected from 10 birds of each group. The blood samples were left to coagulate at room temperature. The serum was separated by centrifugation of coagulated blood at 3000 rpm for 15 min. The clear serum was kept in a freezer (−20 °C) until use to detect the serum total protein (Yatzidis, 1977), albumin (Doumas et al., 1972), ALP (Sodium phenylene phosphate colorimetric method), ALT and AST (Reitman and Frankel, 1957), superoxide dismutase (SOD) (Nishikimi et al., 1972), glutathione(GSH), catalase (CAT) (Goth, 1991), lipid peroxidation (MDA) (Buege and Aust, 1978).

2.7. ELISA analysis

The liver tissue of each group was made into 10% tissue homogenate, and the supernatant was taken after centrifugation. The contents of CYP1A1 and CYP2H1 in the supernatant of liver homogenate were measured by ELISA kit (Shanghai hengyuan Biotech CO.,Ltd. Shanghai china).

2.8. Quantitative real-time PCR

RNA was extracted from liver by total RNA extraction kit (Promega, Beijing, China), and the gene was retrieved. The transcription levels of CYP1A1, CYP2H1, Nrf2, HO-1, NQO-1, Capase-3 and Caspase-6 in livers were detected by real-time quantitative fluorescent PCR. The primers were designed and synthesized by Takara (Dalian, China), The primer sequences are shown as follows, CYP1A1 Forward: A GGACGG AGGCTGACAAGGT and Reverse: CAGGATGGTGGTGAGGAAGAG; CYP2H1 Forward: ATCCCCATCATTGGAAATG and Reverse: TCGTAG

Table 2
Hematological analysis.

Groups	WBC (10^9/L)	RBC (10^{12}/L)	RGB (g/L)	PLT (10^{12}/L)
control	231.65 ± 6.70	1.99 ± 0.12	115.50 ± 7.19	90.20 ± 3.45
0.15 g/LFFC	215.78 ± 6.46*	1.96 ± 0.14	119.13 ± 7.38	87.70 ± 2.50
0.3 g/L FFC	219.51 ± 11.16*	1.75 ± 0.12*	112.00 ± 3.85*	84.40 ± 1.85*
0.6 g/L FFC	213.79 ± 11.24*	1.69 ± 0.34*	119.50 ± 10.32	79.50 ± 4.00*
1.2 g/L FFC	214.40 ± 6.49*	1.90 ± 0.12	118.75 ± 7.40	78.75 ± 5.40*
1.8 g/L FFC	196.95 ± 18.15**	1.51 ± 0.08**	99.75 ± 16.61*	69.50 ± 8.30**

$^{**}P < 0.01$, Compared with the control group; $^{*}P < 0.05$, compared with the control group.

CCATAC.

AGCACCAC; Nrf2 Forward: CACCCAGCTCACCTGGTATAGTTC and Reverse: TACTTCAGCCAGGTTGTCTGTGCT; HO-1 Forward: AACGCC ACCAAGTTCAG.

TCTCC and Reverse: AGCTTCTGCAGCGCCTCAA; NQO-1 Forward: GAACCC.

CGAGTGCTTTGTCT and Reverse: CCGCTTCAATCTTCTTCTGCTC; caspase6.

Forward: TTAGGCAGCACTGCAATATACACCA and Reverse: GCAG CAGTAGCA.

ACCTGAAACATC; caspase3 Forward: GGACTCTGGAATTCTGCCTG ATG and Reverse: CCGTGCCTGAACGAGATGAC; β-actin Forward: ATTGTCCACCGC AAATGCTTC and Reverse: AAATAAAGCCATGCCAA TCTCGTC. The pre-denaturation treatment was 95 ˚C 120 s and 45 cycles were extended, including 95 ˚C 5 s and 64 ˚C 30 s. Then 72 ˚C 30 s. The internal reference gene is beta-actin, and the relative transcriptional level of each gene is calculated by $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct$ = (Ct (target, test) -Ct (reference, test)) - (Ct (target, calibrator)- Ct (reference, calibrator)).

2.9. Western blotting

The total protein extracted from the liver of chickens was extracted by Cwbiotech (Beijing, China), and the protein concentration was measured by bicinchoninic acid (BCA) method. The same amount of protein extract was separated into 10% polyacrylamide gel (Life Technologies, Carlsbad, CA, USA) and transferred to the nitrocellulose membrane. After blocking the non-specific binding sites, the membrane was incubated with antibodies against caspase-3, caspase-6 (abcam, Cambridge, USA) and Nrf2, HO-1, NQO-1 (Bioss Antibodies, Beijing, China) for 12 h in a low temperature shaker at 4 ˚C, nitroblue tetrazolium choride (NBT)/5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) kit was used for color rendering (Solarbio, Beijing, China).

2.10. TUNEL reaction

Pretreatment of paraffin-embedded tissue slices: the tissue slices were placed in a dyeing vat and washed twice with xylene for 5 min each time. Anhydrous alcohol (I) and (II) were used for 2 min, and then down to 95%, 80%, 70% and 50% alcohol for 5 min respectively. Tissue slices were placed in 0.01M sodium citrate buffer solution (pH6.0) and heated to boiling in a microwave oven. The power was cut off at intervals of 5–10 min and repeated 1–2 times. Then wash with distilled water. Wash twice with PBS for 5 min each time. Use filter paper carefully to absorb the excess liquid around the tissue on the slide. Instantly add terminal deoxynucleotidyl transferase (TDT) enzyme buffer on the slice and place it at room temperature for 1–5 min. Next, carefully absorb the excess liquid around the slice, immediately drop 50 mL TDT enzyme reaction liquid on the slice, and react 1 h in a wet box at 37 ˚C. (Note: Negative staining control, add the reaction liquid without TDT enzyme). The slices were placed in the dyeing vat, and the washing and termination buffer which had been preheated to 37 ˚C was added. The slides were held at 37 ˚C for 30 min. The slides were gently lifted and laid down every 10 min to make the liquid agitate slightly. Tissue sections were washed with PBS three times, 5 min each time. Two drops of peroxidase labeled antibodies were directly added to the sections and reacted at room temperature for 30 min in a wet box. Wash 3 times with PBS, 5 min each time. Freshly prepared 50–100 μL DAB solution (Boster Biological Technology co.ltd, Wuhan, China) was directly dripped onto tissue sections and colored at room temperature for 3–6 min. Wash 3 times with PBS, 5 min each time, 2 min with hematoxylin, and then rinse with tap water for a while. Xylene was dehydrated three times, 2 min each time. After sealing and drying, the experimental results were observed and recorded under optical microscope.

2.11. Statistical analysis

Statistical analysis was performed by using SPSS 19.0 (IBM Corp., Armonk, NY, USA). All data were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's test was used for multiple comparisons, and $P < 0.05$ was considered as significant compared with different groups.

3. Results

3.1. Effects of FFC on weight gain and relative liver weight in chickens

Compared with the control group, FFC at 0.15 g/L significantly reduced the weight gain of chickens ($P < 0.05$), while the other doses of FFC extremely significantly reduced the weight gain of chickens ($P < 0.01$). At the same time, the relative liver weight of chickens ($P < 0.01$) were significantly increased in groups of 1.2 g/L FFC and 1.8 g/L FFC compared with the control group (Table 1).

3.2. Effects of FFC on RBC, WBC, RGB and PLT in blood of chickens

As showed in Table 2, when compared with control group, FFC in each dose group significantly reduced the count of WBC in blood ($P < 0.05$ or $P < 0.01$); except 0.15 g/L FFC group and 1.2 g/L FFC group. The content of RBC in blood of the other three FFC groups were significantly decreased ($P < 0.05$ or $P < 0.01$); however, only 0.3 g/L FFC and 1.8 g/L FFC significantly reduced the content of RGB ($P < 0.05$); the contents of PLT in blood of groups of 0.3 g/L FFC, 0.6 g/L FFC, 1.2 g/L FFC and 1.8 g/L FFC were significantly lower than the control group ($P < 0.05$ or $P < 0.01$);

3.3. Effects of FFC on biochemical parameters in chickens

Compared with the control group, FFC significantly increased the levels of AST and ALT in serum of chickens ($P < 0.05$). However, there was no significant difference ($P > 0.05$). in the levels of ALP in serum of chickens between each group. The results showed that the total protein content in the serum of chickens treated with FFC was significantly lower than that in the control group ($P < 0.05$). At the same time, FFC at 1.2 and 1.8 g/L significantly decreased the serum albumin content in comparison with the control group ($P < 0.05$) (Fig. 1a).

As showed in Fig. 1b, compared with the control group, FFC

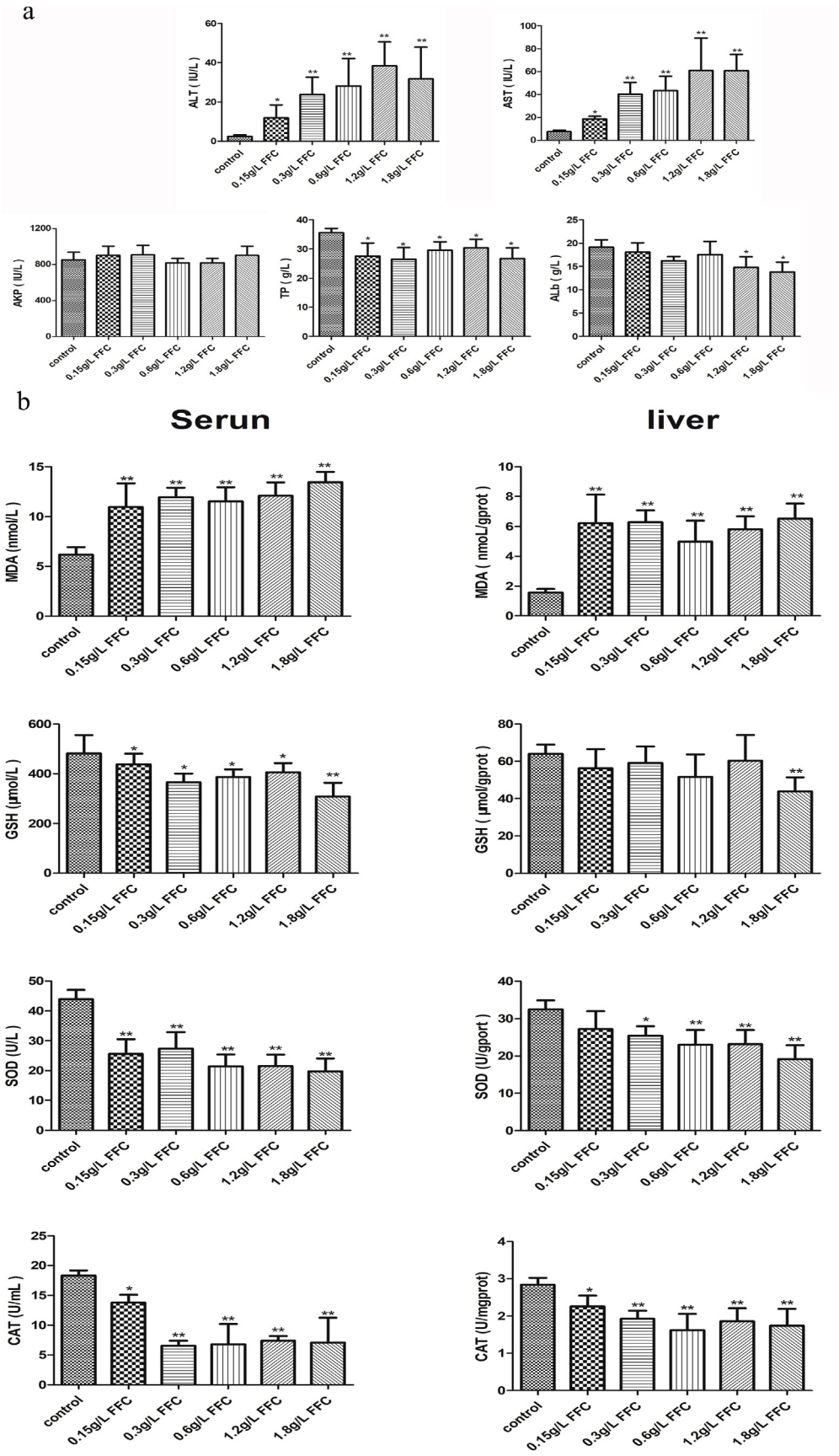

Fig. 1. Contents of ALT, AST, AKP, TP and Alb in chicks serum (a) and MDA, GSH, SOD and CAT levels in serum and liver tissue of chicks (b) in each group.**P < 0.01, Compared with the control group; *P < 0.05, compared with the control group.

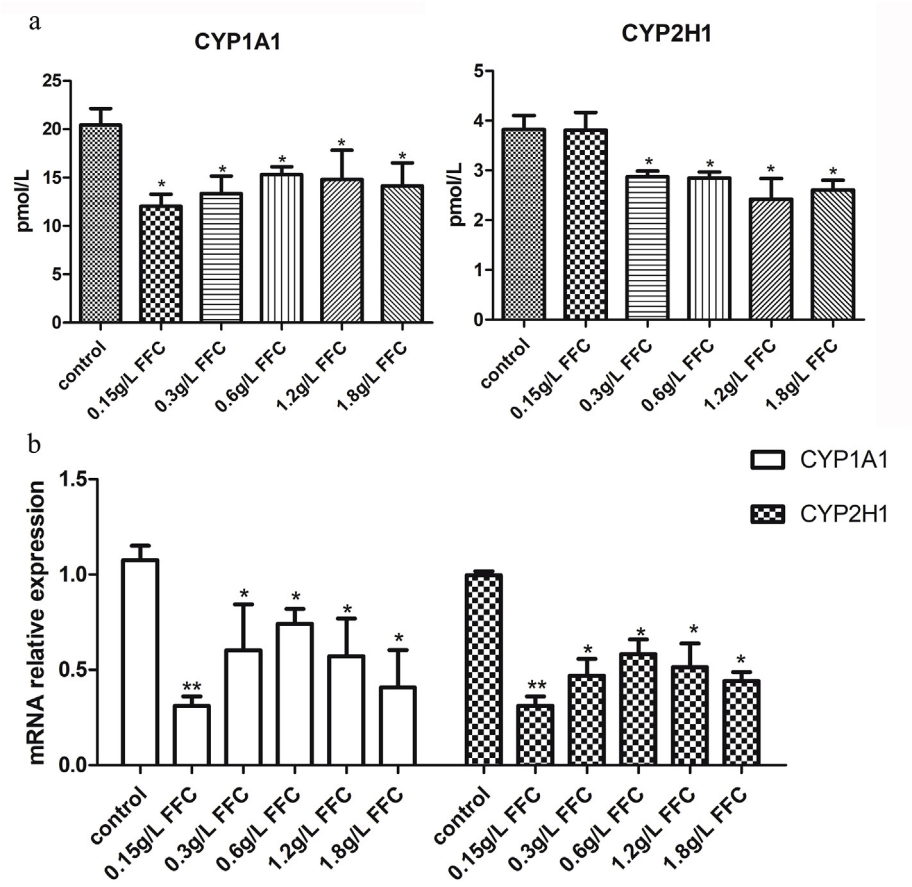

Fig. 2. CYP1A1 and CYP2H1 levels (a)and mRNA transcriptional levels (b) in liver tissues of chicks in each group. $^{**}P < 0.01$, Compared with the control group; $^{*}P < 0.05$, compared with the control group.

significantly increased MDA content in serum and tissues of chickens ($P < 0.05$), and FFC significantly decreased the contents of GSH, SOD and CAT in serum of chickens ($P < 0.05$ or $P < 0.01$). At the same time, the contents of GSH, SOD and CAT in liver tissues were determined. Compared with the control group, only 1.8 g/L of FFC significantly reduced the contents of GSH in liver, while the contents of SOD in the 0.15 g/LFFC group were not different from those in the control group ($P > 0.05$). While the other doses of FFC significantly reduced the contents of SOD and CAT in liver ($P < 0.05$ or $P < 0.01$).

3.4. Effects of FFC on the contents of CYP1A1 and CYP2H1 and the transcription level of the hepatic drug enzymes in chickens

All FFC groups significantly decreased the content of CYP1A1 in chicken liver ($P < 0.05$) (Fig. 2a); compared with control group. At the same time, except 0.15 g/L FFC group, the CYP2H1 contents of liver tissue in other FFC groups were also significantly decreased ($P < 0.05$) (Fig. 2a). mRNA transcriptional level of CYP1A1 and CYP2H1of chicken liver in each FFC groups were significantly lower than that in the control group ($P < 0.01$ or $P < 0.05$) (Fig. 2b).

3.5. Effects of FFC on transcriptional levels of Nrf2 pathway protein and mRNA in chicken liver

As shown in Fig. 3a and b, compared with control group, 0.15 g/mL FFC, 0.3 g/mL FFC and 0.6 g/mL FFC significantly reduced the transcription of Nrf2 gene in liver ($P < 0.05$ or $P < 0.01$). Except group B, the protein expression of Nrf2 in liver of other FFC groups were significantly lower than that of control group ($P < 0.05$ or $P < 0.01$). Compared with the control group, the levels of HO-1 mRNA transcription and protein expression in chicken liver were significantly decreased in all doses of FFC ($P < 0.05$). while there was no significant difference in the levels of NQO-1 mRNA transcription but the protein expression was significant different in chicken liver between 1.2 g/L FFC group and control group. However, the protein expression and the mRNA transcription levels of NQO-1 gene in other FFC group (0.15 g/L FFC, 0.3 g/L FFC, 0.6 g/L FFC and 1.8 g/L FFC) were significantly decreased when compared with control group ($P < 0.05$ or $P < 0.01$).

3.6. Effects of FFC on Caspase-3, Caspase-6 protein and RNA transcription in chicken liver tissues

Caspase is a family of cysteine proteases closely associated with apoptosis. As shown in Fig. 4a and b, compared with the control group, the levels of Caspase-3 gene transcription in chicken liver were significantly increased in all FFC groups ($P < 0.05$), and except 0.15 g/L group, the expression of caspase-3 protein in chicken liver was significantly increased in other FFC groups ($P < 0.05$ or $P < 0.01$). All dosages of FFC significantly increased the transcription and protein expression levels of caspase-6 mRNA in the liver of chicks ($P < 0.05$ or $P < 0.01$).

3.7. Effect of FFC on apoptosis of chicken liver tissue

As shown in Fig. 5, the number and distribution of apoptotic cells in the liver of chickens in the control group were small and sparse, while the number of apoptotic cells in the liver of chickens in each FFC treatment group increased gradually, and the distribution area and apoptotic cells were all significantly increased. The percentage of

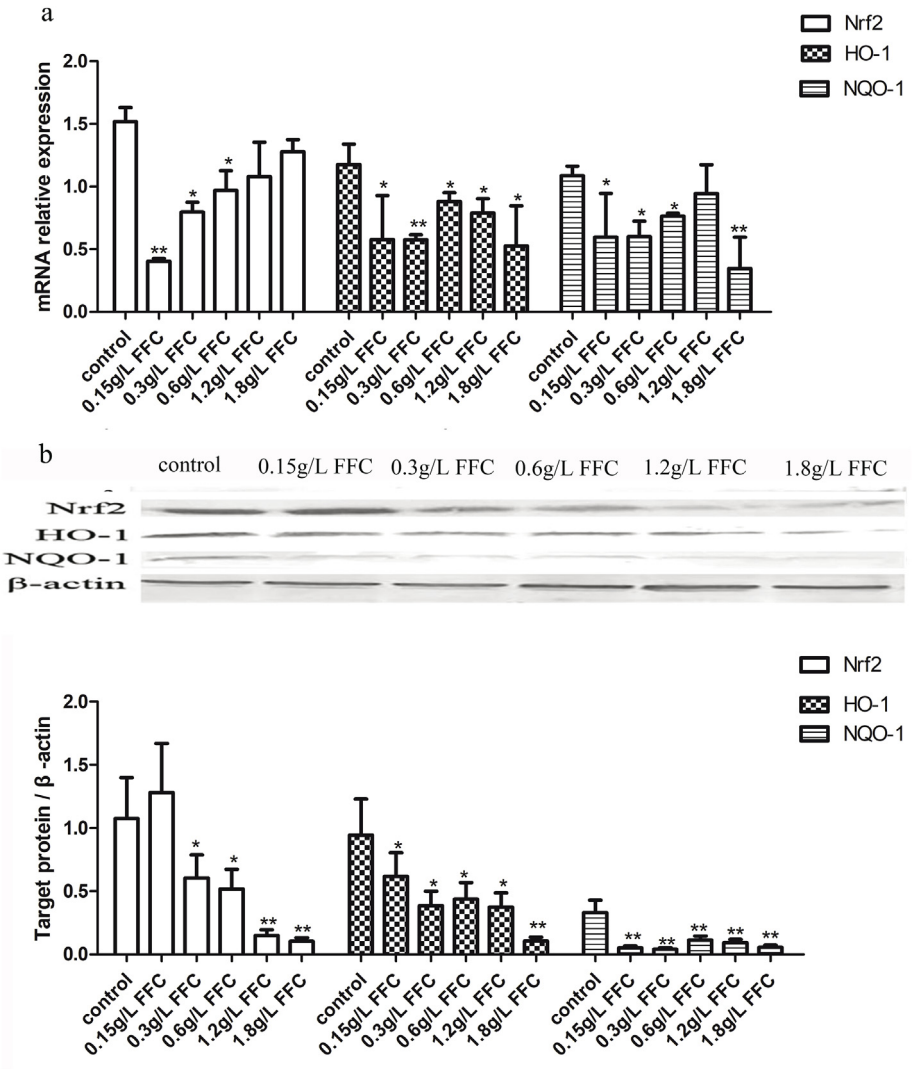

Fig. 3. Nrf2, HO-1 and NQO-1 mRNA transcriptional levels (a) and protein expression (b) in liver tissues of chicks in each group, $^{**}P < 0.01$, Compared with the control group; $^{*}P < 0.05$, compared with the control group.

apoptotic cells in the each FFC group was significantly higher than that in the control group ($P < 0.05$ or $P < 0.01$).

4. Discussions

Chloramphenicol can permanently bind to the 50S subunit of ribosome to inhibit protein synthesis (Hanekamp and Bast, 2015), and its metabolites and residues in vivo are long-term and difficult to eradicate, leading to long-term liver and kidney toxicity. Therefore, these antibiotics are only allowed to treat specific diseases (Picco et al., 2001). However, as an alternative to chloramphenicol, FFC has similar efficacy and less side effects in the treatment of animal diseases, which makes it the preferred drug in veterinary medicine field (Skolimowski et al., 1983). FFC is a fluorinated derivative of chloramphenicol with similar properties, but its parent compound has no adverse and adverse effects on animals (Pentecost et al., 2013). However, in recent years, the toxic and side effects of FFC (including normal therapeutic dose) have also been widely reported (Hu et al., 2016; Zhang et al., 2019). Our study found that FFC significantly inhibited chicken weight gain and increased liver index. In addition, FFC also reduced the number of red blood cells, white blood cells and platelets in the blood of chickens, indicating that FFC can also damage the hematopoietic function of chickens. And the influence of FFC on the body weight, RBC, and PLT were dose-dependent. This result is consistent with previous research results (Hassanin et al., 2014).

Our study also found that FFC significantly increased the levels of ALT and AST in chicken serum. ALT and AST are intracellular enzymes of liver cells. The increase of serum levels means that the integrity of liver cells was impaired (Ben Abdennebi et al., 2011). Our results showed that FFC damaged the integrity of liver in chickens. At the same time, as a site of serum protein synthesis, FFC significantly inhibited the blood of chickens. The content of total protein and albumin in the serum indicated that FFC inhibited the function of hepatic synthetic protein.

Oxidative stress is caused by excessive reactive oxygen species (ROS) and electrophiles produced by cells, and excessive ROS can induce free radical chain reaction, destroy cell biological macromolecules such as protein, lipid and DNA, and induce a series of habitual diseases (Kohen and Nyska, 2002). In order to control ROS level and prevent ROS accumulation, the body has formed a complex antioxidant defense system, in which nuclear factor-erythroid 2-related factor 2 (Nrf2) is an important redox-sensitive transcription factor (Khor et al., 2011), which can improve the oxidative stress state, promote cell survival and maintain the redox homeostasis of cells by inducing and regulating the constitutive and inducible expression of phase II detoxifying enzyme and antioxidant enzyme in cells (Motohashi and Yamamoto, 2004; Surh and Na, 2008). Our results showed that FFC induced the transcription of Nrf2 in liver tissue and the expression of Nrf2 protein. In addition,

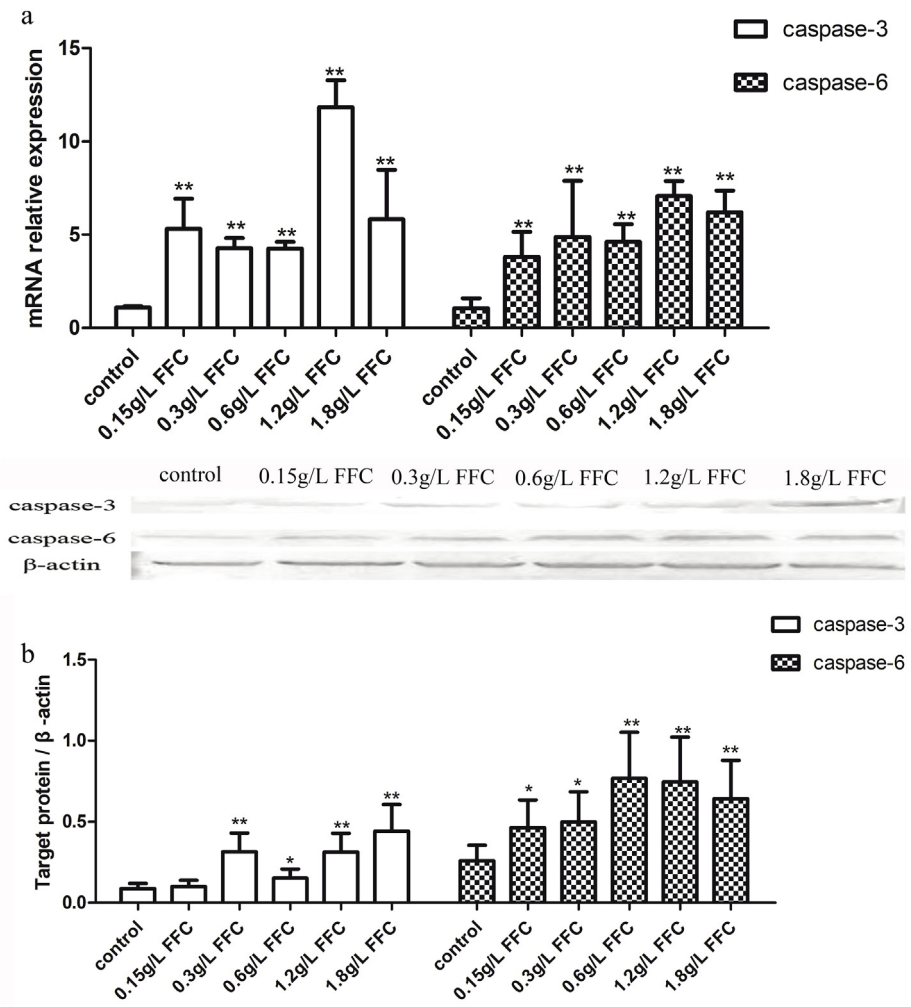

Fig. 4. Caspase-3 and caspase-6 mRNA transcriptional levels (a) and protein expression (b) in liver tissues of chicks in each group. **P < 0.01, Compared with the control group; *P < 0.05, compared with the control group.

NOQ-1 and HO-1, as downstream proteins of Nrf2-ARE pathway, play a synergistic role in antioxidant activity in vivo. At the same time, it is regulated by Nrf2 protein (Yang et al., 2012). Theoretically, the expression of NOQ-1 and HO-1 increases when oxidative stress occurs in the body, thereby reducing oxidative stress (Gonzalez-Burgos, 2012). Previous research have demonstrated FFC increased MDA contents and caused cellular oxidative damage to the aquatic organisms (Zhang et al., 2019). Our results also showed that FFC significantly inhibited the transcription of NQO-1 and HO-1 genes and protein expression compared with the control group. It is concluded that FFC can cause oxidative stress in broiler liver by inhibiting the expression of antioxidant proteins Nrf2, HO-1 and NQO-1, thereby inhibiting the expression of antioxidant factors GSH, SOD and CAT in liver, strengthening the content of ROS in liver tissue and causing oxidative stress and injury in liver tissue.

Direct hepatocyte damage to the drug itself or to metabolites, Antibiotics, aflatoxins and heavy metals are the most common causes of liver injury in broiler chickens. Most of their metabolites have hepatotoxicity. There are two steps for the drug in the liver, For example, phase I and phase II reactions. Phase I reaction: hydrolysis, oxidation and reduction of drugs to produce metabolites. The main metabolic enzymes are cytochrome P450 (CYP), CYP1A1 and CYP2H1, which are key oxidases related to drug-induced liver injury (Dohnal et al., 2014). Therefore, the content of hepatic drug enzymes in hepatocytes is directly related to the detoxification ability of the liver. Our results showed that the levels of CYP1A1 and CYP2H1 were significantly decreased in the liver of chickens fed with FFC. It suggested that FFC could increase the metabolic time of FFC in chickens and the residue of FFC in vivo by inhibiting the activity of liver pharmacological enzymes.

Hepatocyte apoptosis can occur in acute liver injury caused by many factors, and is the main cause of liver failure (Jaeschke et al., 2004; Togo et al., 2004). In order to detect the effect of FFC on hepatocyte apoptosis in chickens, we tested the apoptotic rate of hepatocytes in each group by TUNEL test. The results showed that FFC could significantly increase the number of apoptotic cells in chicken liver tissue. In addition, caspase family is an important participant in the process of apoptosis. caspase-3 and caspase-6 are the executors of apoptosis. Activated executors, caspase family, cause programmed cell death by hydrolyzing caspase target proteins (Thornberry and Lazebnik, 1998). The expression of caspase-3 and caspase-6 was detected by fluorescence quantitative PCR and Western Bolting. It was found that FFC could significantly increase the expression of caspase-3 and caspase-6 in liver tissue. These results suggest that FFC can accelerate the apoptosis of hepatocytes and lead to liver injury. Although FFC has not been reported to induce apoptosis of hepatocytes before, many studies have proved that FFC can induce apoptosis of immune organs (Hu et al., 2016; Yang et al., 2016). Those results showed that FFC has toxic effect on multiple organs.

Authors' contributions

WS and YB conceived and designed the study, and critically revised

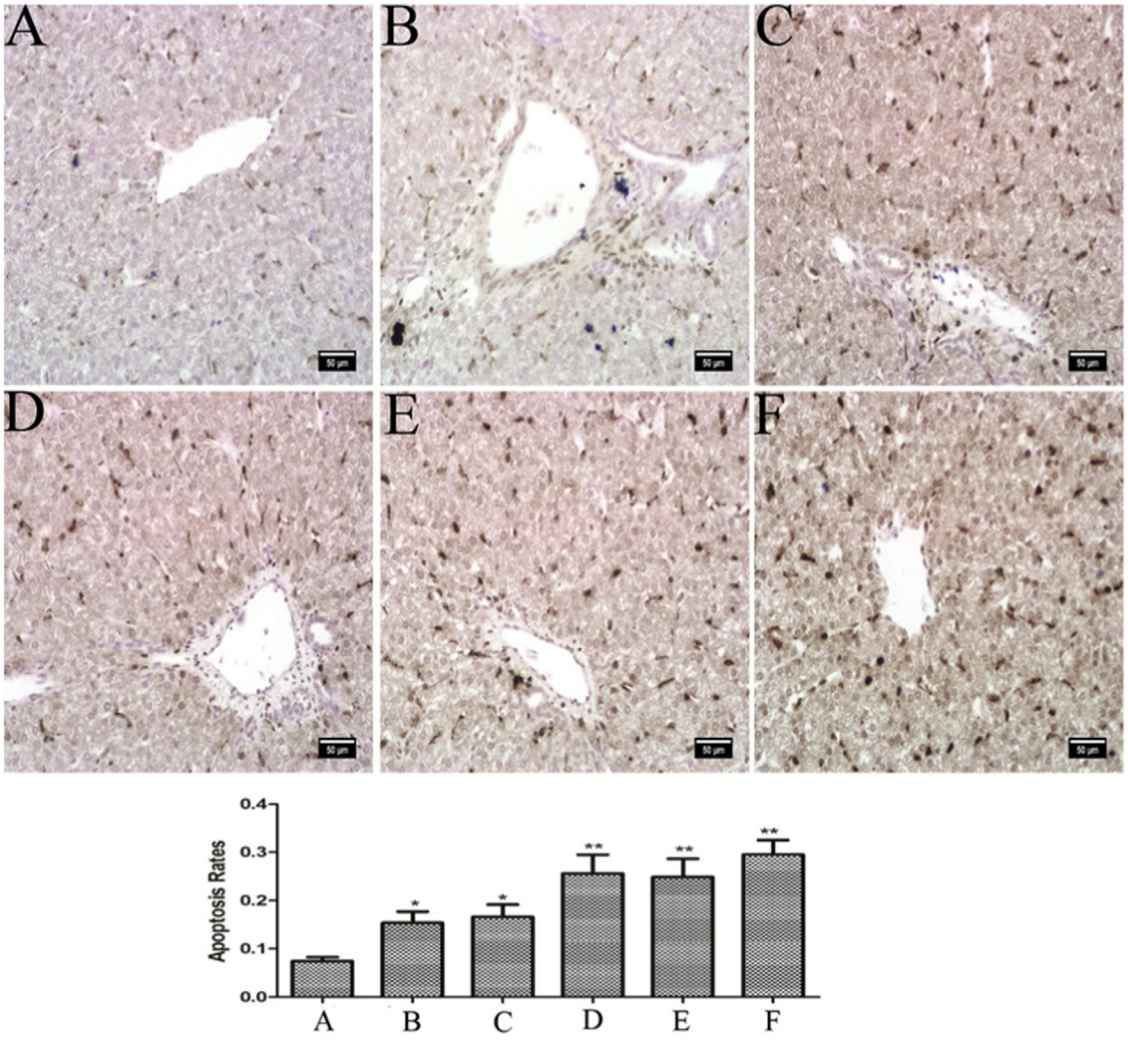

Fig. 5. Apoptosis in liver tissues of chicks in each group. Group A: control group (0 mg/L FFC); Group B:0.15 g/L FFC group; Group C: 0.3 g/L FFC group; Group D: 0.6 g/LFFC group; Group E: 1.2 g/L FFC group; Group F: 1.8 g/L FFC group. $^{**}P < 0.01$, Compared with the group A; $^{*}P < 0.05$, compared with the group A.

the manuscript. CH performed most of the experiments and analyzed the results, CH and YW drafted the manuscript. YB assisted in experimental design, data interpretation and manuscript preparation. YG and YW contributed to samples collection, performed qPCR experiment and helped with the data analysis. YC and CH participated in HE, TUNEL and Western blot experiments. All authors read and approved the final manuscript.

Declaration of competing interest

The authors have no conflicts of interest.

Acknowledgments

This study was financially supported by Subsidized Project for Cultivating Innovative Ability of Postgraduates of Hebei province (no.CXZZBS2019096).

References

Ben Abdennebi, H., Zaoualí, M., Alfany-Fernandez, I., et al., 2011. How to protect liver graft with nitric oxide. World J. Gastroenterol. 17, 2879–2889.
Bjornsson, E.S., 2017. Drug-induced liver injury due to antibiotics. Scand. J. Gastroenterol. 52, 617–623.
Bretzlaff, K.N., Neff-Davis, C.A., Ott, R.S., et al., 1987. Florfenicol in non-lactating dairy cows: pharmacokinetics, binding to plasma proteins, and effects on phagocytosis by blood neutrophils. J. Vet. Pharmacol. Therapeut. 10, 233–240.
Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–310.
Dohnal, V., Wu, Q., Kuča, K., 2014. Metabolism of aflatoxins: key enzymes and interindividual as well as interspecies differences. Arch. Toxicol. 88, 1635–1644.
Donoghue, D.J., 2003. Antibiotic residues in poultry tissues and eggs: human health concerns? Poultry Sci. 82, 618–621.
Doumas, B.T., Biggs, H.G., Arends, R.L., et al., 1972. Determination of ssrum albumin 1. Stand Method ClinChem 7, 175–188.
Fraunfelder, F.T., Bagby, G.C., Kelly, D.J., et al., 1982. Fatal aplastic anemia following topical administration of ophthalmic chloramphenicol. Am. J. Ophthalmol. 93, 356–360.
Gonzalez-Burgos, E., 2012. Diterpenoids isolated from Sideritis species protect astrocytes against oxidative stress via Nrf2. J. Nat. Prod. 75, 1750–1758.
Goth, L., 1991. A simple method for determination of serum catalase activity and revision of reference range. Clin. Chim. Acta 196, 143–151.
Hanekamp, J.C., Bast, A., 2015. Antibiotics exposure and health risks: Chloramphenicol. Environ. Toxicol. Pharmacol. 39, 213–220.
Hassanin, O., Abdallah, F., Awad, A., 2014. Effects of florfenicol on the immune responses and the interferon-inducible genes in broiler chickens under the impact of E. coli infection. Vet. Res. Commun. 38, 51–58.
Hu, D.F., Han, Z., Li, C., et al., 2016. Florfenicol induces more severe hemotoxicity and immunotoxicity than equal doses of chloramphenicol and thiamphenicol in Kunming mice. Immunopharmacol. Immunotoxicol. 38, 472–485.
Jaeschke, H., Gujral, J.S., Bajt, M.L., 2004. Apoptosis and necrosis in liver disease. Liver Int. 24, 85–89.
Khalifeh, M.S., Amawi, M.M., Abu-Basha, E.A., et al., 2009. Assessment of humoral and cellular-mediated immune response in chickens treated with tilmicosin, florfenicol, or enrofloxacin at the time of Newcastle disease vaccination. Poultry Sci. 88, 2118–2124.
Khor, T.O., Huang, Y., Wu, T.Y., et al., 2011. Pharmacodynamics of curcumin as DNA hypomethylation agent in restoring the expression of Nrf2 via promoter CpGs

demethylation. Biochem. Pharmacol. 82, 1073–1078.
Kohen, R., Nyska, A., 2002. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol. Pathol. 30, 620–650.
Li, B., Zhang, Y., Wei, J., et al., 2015. Characterization of a novel small plasmid carrying the florfenicol resistance gene floR in Haemophilus parasuis. J. AntimicrobChemoth 70, 3159–3161.
Li, W., Li, Y., Jiang, X., et al., 2018. Compound ammonium glycyrrhizin protects hepatocytes from injury induced by Lipopolysaccharide/Florfenicol through a mitochondrial pathway. Molecules 23, E2378.
Motohashi, H., Yamamoto, M., 2004. Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends Mol. Med. 10, 549–557.
Nishikimi, M., Appaji, N., Yagi, K., 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem. Biophys. Res. Commun. 46, 849–854.
Osman, K.M., Badr, J., Orabi, A., et al., 2019. Poultry as a vector for emerging multidrug resistant Enterococcus spp.: first report of vancomycin (van) and the chloramphenicol–florfenicol (cat-fex-cfr) resistance genes from pigeon and duck faeces. Microb. Pathog. 128, 195–205.
Paige, J.C., Tollefson, L., Miller, M., 1997. Public health impact on drug residues in animal tissues. Vet. Hum. Toxicol. 39, 162–169.
Pentecost, R.L., Niehaus, A.J., Werle, N.A., et al., 2013. Pharmacokinetics of florfenicol after intravenous and intramuscular dosing in llamas. Res. Vet. Sci. 95, 594–599.
Picco, E.J., Díaz, D.C., Valtorta, S.E., et al., 2001. Chronotoxicology of florfenicol. Chronobiol. Int. 18, 567–572.
Pozniak, B., Pawłowski, P., Pasławska, U., et al., 2017. The influence of rapid growth in broilers on florfenicol pharmacokinetics - allometric modelling of the pharmacokinetic and haemodynamic parameters. Br. Poultry Sci. 58, 184–191.
Reitman, S., Frankel, S., 1957. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am. J. Clin. Pathol. 28, 56–63.
Shah, J.M., Qureshi, T.A., Shah, T., et al., 2016. Impact of therapeutic and high doses of florfenicol on kidney and liver functional indicators in goat. Vet. World 9, 1135–1140.
Shin, S.J., Kang, S.G., Nabin, R., et al., 2005. Evaluation of the antimicrobial activity of florfenicol against bacteria isolated from bovine and porcine respiratory disease. Vet. Microbiol. 106, 73–77.
Shuang, G., Yu, S., Guo, W.X., et al., 2011. Immunosuppressive activity of florfenicol on the immune responses in mice. Immunol. Invest. 40, 356–366.
Sidhu, P., Rassouli, A., Illambas, J., et al., 2014. Pharmacokinetic-pharmacodynamic integration and modelling of florfenicol in calves. J. Vet. Pharmacol. Therapeut. 37, 231–242.
Skolimowski, I.M., Knight, R.C., Edwards, D.I., 1983. Molecular basis of chloramphenicol and thiamphenicol toxicity to DNA in vitro. J. Antimicrob. Chemother. 12, 535–542.
Surh, Y.J., Na, H.-K., 2008. NF-κB and Nrf2 as prime molecular targets for chemoprevention and cytoprotection with anti-inflammatory and antioxidant phytochemicals. Genes; Nutr. 2, 313–317.
Syriopoulou, V.P., Harding, A.L., Goldmann, D.A., et al., 1981. In vitro antibacterial activity of fluorinated analogs of chloramphenicol and thiamphenicol. Antimicrob. Agents Chemother. 19, 294–297.
Thornberry, N.A., Lazebnik, Y., 1998. Caspases: enemies within. Science 281, 1312–1316.
Togo, S., Makino, H., Kobayashi, T., et al., 2004. Mechanism of liver regeneration after partial hepatectomy using mouse cDNA microarray. J. Hepatol. 40, 464–471.
Verner-Jeffreys, D.W., Brazier, T., Perez, R.Y., et al., 2017. Detection of the florfenicol resistance gene floR in Chryseobacterium isolates from rainbow trout. Exception to the general rule? FEMS Microbiol. Ecol. 93, fix015.
Wisselink, H.J., Veldman, K.T., Eede, C.V.D., et al., 2006. Quantitative susceptibility of Streptococcus suis strains isolated from diseased pigs in seven European countries to antimicrobial agents licenced in veterinary medicine. Vet. Microbiol. 113, 73–82.
Yang, C.M., Huang, S.M., Liu, C.L., et al., 2012. Apo-8′-lycopenalInduces expression of HO-1and NQO-1 via the ERK/p38-Nrf2-ARE pathway in human HepG2 cells. J. Agric. Food Chem. 60, 1576–1585.
Yang, G., Tian, X.L., Dong, S.L., et al., 2016. Effects of dietary rhubarb,bacillus cereus, yeast polysaccharide, and florfenicol supplementation on growth, intestinal morphology, and immune responses of sea cucumber (apostichopus japonicus). Aquacult. Int. 24, 675–690.
Yatzidis, H., 1977. New colorimetric method for quantitative determination of protein in urine. Clin. Chem. 23, 811–812.
Zhang, Y.X., Guo, P.X., Wu, Y.M., et al., 2019. Evaluation of the subtle effects and oxidative stress response of chloramphenicol, thiamphenicol, and florfenicol in Daphnia magna. Environ. Toxicol. Chem. 38, 575–584.
Zhang, Z.F., Cho, J.H., Kim, I.H., 2013. Effects of Bacillus subtilis UBT-MO2 on growth performance, relative immune organ weight, gas concentration in excreta, and intestinal microbial shedding in broiler chickens. Livest. Sci. 155, 343–347.