Original Research Article

Cytochrome P450 enzymes mediated by DNA methylation is involved in deoxynivalenol-induced hepatoxicity in piglets

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ABSTRACT

Deoxynivalenol (DON) is an inevitable contaminant in animal feed and can lead to liver damage, then decreasing appetite and causing growth retardation in piglets. Although many molecular mechanisms are related to hepatoxicity caused by DON, few studies have been done on cytochrome P450 (CYP450) enzymes and DNA methylation. To explore the role of CYP450 enzymes and DNA methylation in DON-induced liver injury, male piglets were fed a control diet, or diet containing 1.0 or 3.0 mg/kg DON for 4 weeks. DON significantly raised the activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glutamyl transpeptidase (GGT) (P < 0.01), leading to liver injury. In vivo study found that DON exposure increased the expression of CYP450 enzymes (such as CYP1A1, CYP2E1, CYP3A29) (P < 0.05), and disturbed the expression of nicotinamide N-methyltransferase (NNMT), galanin-like peptide (GALP) and insulin-like growth factor 1 (IGF-1) (P < 0.05), in which DNA methylation affected the expression of these genes. In vitro study (human normal hepatocytes L02) further proved that DON elevated the expression of CYP1A1, CYP2E1 and CYP3A4 (P < 0.05), and inhibited cell growth in a dose-dependent manner, resulting in cell necrosis. More importantly, knockdown of CYP1A1 or CYP2E1 could alleviate DON-induced growth inhibition by promoting IGF-1 expression. Taken together, increased CYP450 enzymes expression was one of the mechanisms of hepatoxicity and growth inhibition induced by DON, suggesting that the decrease of CYP450 enzymes can antagonize the hepatoxicity in animals, which provides some value for animal feed safety.

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1. Introduction

Deoxynivalenol (DON) is the most frequent mycotoxin produced by several pathogenic fungi, such as Fusarium graminearum (Zhou et al., 2020). DON is mainly present in wheat, barley, oats, corn and animal feeds (Wang et al., 2019), bread, biscuits (Suman et al., 2019), animal milk, eggs (Molina et al., 2019) and fruit (Pallares et al., 2019). DON contamination levels in grains and cereals range from 0.250 to 50.289 mg/kg (Streit et al., 2012). It is reported that the concentration of DON in the primary effluent samples of a Swiss waste water treatment plant is 32 to 118 ng/L (Wettstein and Bucheli, 2010). Among all organs, the liver is the most important target organ for DON-induced toxicity. Therefore, in view of the universality and severity of DON contamination, its hepatotoxicity should be paid great attention.

Cytochrome P450 (CYP450) enzymes in the liver do not directly metabolise DON (Peng et al., 2017), but these enzymes may be related to hepatic damage. Previous studies have found many
mechanisms of liver injury caused by DON, such as lipid peroxidation, oxidative stress (Yu et al., 2019), apoptosis (Zhou et al., 2020), inflammatory response (Reddy et al., 2018) and autophagy (Peng et al., 2019b). CYP450 enzymes are the most important enzyme for drug metabolism. However, it has been discovered in recent years that even though CYP450 does not metabolize drugs, it may also be an important target of drug-induced liver toxicity (Balakina et al., 2017; Bou-Fakhredin et al., 2021). Moreover, the literature showed that a diet containing 8 mg DON/kg feed fed to pigs for 4 weeks resulted in an increased expression of CYP1A26 in the liver (Reddy et al., 2018). Therefore, we speculate that CYP450 may also be closely related to liver damage caused by DON. However, it is unclear whether these enzymes mediated DON-induced hepatotoxicity and growth inhibition.

In addition, DNA methylation is closely associated with toxin-induced liver damage and growth inhibition (Liu et al., 2019a). However, to the best of our knowledge, the potential role of DNA methylation in DON-induced CYP450 enzymes expression has been unclear. The expression of growth hormone, growth hormone receptor and insulin-like growth factor 1 (IGF-1) were influenced by DNA methylation (Ouni et al., 2015; Strobl et al., 1986). A study showed that 8 mg/kg of DON in the diet was responsible for significant decreases in the mRNA expression of nicotinamide N-methyltransferase (NNMT), galanin-like peptide (GALP) and IGF-1, but significantly promoted the expression of insulin-like growth factor-binding protein 2 (IGFBP2) in pig liver, resulting in growth stunting of pigs (Reddy et al., 2018). However, whether DNA methylation affects the expression of these genes in DON-exposed liver is unclear.

Thus, the present study was designed to investigate whether CYP450 enzymes and DNA methylation were involved in DON-induced hepatotoxicity and growth inhibition, which used piglets as an in vivo model and human normal hepatocytes L02 as an in vitro model to study. The dose used in this study was selected on the basis of previous research reports on DON in piglets (Gerez et al., 2015; Wang et al., 2018; Van Le Thanh et al., 2016; Zhang et al., 2020b), and piglets were exposed to toxic levels of 1 and 3 mg/kg for 4 weeks of treatment, which resulted in weight gain and feed intake of piglets (Liu et al., 2020a, b; Zhang et al., 2020b). The effect of DON on the liver function was determined by analysing the changes of liver function indices in serum. The expression of CYP450 enzymes, growth- and DNA methylation-related genes was tested by quantitative real-time PCR (qPCR) and Western blot. Furthermore, the level of 5-methyl cytosine (5-mC) in the whole genome was detected by colorimetry. The methylation level of CYP450 enzyme gene promoter and growth-related gene promoter in the liver was detected by methylation-specific PCR (MSP) and bisulphite sequencing (BSP), respectively. The effect of DON on CYP450 enzymes expression, cell growth and pathological changes were further studied in vitro. In L02 cells, after siRNA interference of CYP1A1 and CYP2E1, the role of CYP450 enzymes in DON-induced growth inhibition was further verified.

2. Materials and methods

2.1. Experimental animals and design

The animal protocol for this study was approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University, China. In total, 21 male castrated crossbred ((Large white × Landrace) × Large white) weanling piglets (3 weeks of age) were randomly allocated to 3 treatment groups; each group was assigned to 7 pens of 1 piglet/pen. Piglets were allowed free access to water and fed twice a day (08:00 and 17:00) with a commercial soybean based diet (control; Table S1) formulated to meet the NRC nutritional requirements (National Research Council (NRC), 2012), or the control diet spiked with 1.0 or 3.0 mg/kg DON. The experiment lasted 28 d, and during this period piglets were immunised with the OVA (0.5 mg + Quil A adjuvant) pseudorabies (PS) vaccine, swine fever (SF) vaccine and porcine circoviruses (PC) vaccine at 1 and 2 weeks into the feeding trial. The use dose of these vaccines (Huapai Bio-engineering Group Co., Ltd. Sichuan, China) was 1/2 recommended dose. On 28 d of experiment, all pigs were humanely sacrificed to harvest blood and liver samples. The blood was centrifuged to obtain serum and stored at −80 °C for subsequent biochemical detection. The tissues were washed with ice-cold saline, then divided into aliquots that were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. All animal work was in compliance with NIH Publication, The Development of Science Based Guidelines for Laboratory Animal Care.

2.2. Cell culture and cell viability assay

DON (CAS NO. 514810–8) was dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich, St. Louis, MO, USA) and then was diluted in RPMI-1640 (Gibco) medium. The L02 cell line was purchased from Suer Biotechnology Co., Ltd of Shanghai, China. L02 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS, Gibco), 1% glutamine, 1% penicillin and 1% streptomycin, at 37 °C in a 5% CO2 incubator. The cells were sub-cultured 3 to 4 times and then used in subsequent experiments. To determine the optimal incubation concentration and time for the toxicity of DON to impact L02 cells, the cells (1 × 105) were seeded in 96-well plates and treated with different concentrations of DON for 12, 24 and 36 h, respectively. After incubation, the cells were treated with 10 μl/well with CCK-8 reagent for 2 h at 37 °C. The optical density (OD) was measured using a micro-quant plate reader (Bio-Tek Instruments) (TECAN A-5082, Magellan, Austria). Cells were cultured in cell plates and incubated with DON (2.5, 5, 10 μmol/L) for 24 h for follow-up experiments. Every experiment was carried out thrice on three separate occasions.

2.3. Measurement of hepatic biochemical indices

The 21 blood samples from piglets were analyzed biochemically. The activity of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glutamyl transpeptidase (GGT) was determined by an autoanalyzer (Hitachi Model 7600 analyzer; Hitachi, Tokyo, Japan) using AST, ALT and GGT assay kits purchased from Mindray (Shenzhen, China) as described previously (Peng et al., 2019a; Santana et al., 2018; Wang et al., 2018a).

2.4. qPCR examination

Total RNA Extraction Reagent was used to obtain RNA from 21 piglets’ liver and L02 cells. The quality of RNA was determined by micro ultraviolet spectrophotometer. Then the RNA was reverse-transcribed into cDNA using HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) for qPCR. The qPCR primers are shown in Table S2 and Table S3. The qPCR reaction system was based on work previously described (Liu et al., 2019a). In this study, the housekeeping gene β-actin was used as an internal calibrator reference gene for expression profiling of genes. Data were analyzed and quantified using the 2−ΔΔCt methods: ΔΔCt = (Ctexperimental group target gene − Ctexperimental group β-actin) − (Ctcontrol target gene − Ctcontrol β-actin).
2.5. Protein extraction and Western blot assay

The protein from 21 piglets’ liver tissue and L02 cells were collected with RIPA Lysis Buffer (Servicebio, Wuhan, China) containing protease inhibitor cocktail and phosphatase inhibitors (Servicebio, Wuhan, China). The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). Blots were blocked with 5% bovine serum albumin (BSA) for 1 h and incubated with primary antibodies (species specificity: Human, Mouse, Rat) (ABclonal Technology, Wuhan, China) for NNMT Rabbit pAb (A18033) (1:500), CYP1A1 Rabbit pAb (A2159) (1:1,000), CYP2E1 Rabbit pAb (A2160) (1:1,000), CYP3A4 Rabbit pAb (1:1,000), IGF1 Rabbit pAb (A11985) (1:500), GALP Rabbit pAb (A17445) (1:1,000) and GAPDH Rabbit pAb (A2159) (1:1,000), CYP2E1 Rabbit pAb (A2160) (1:1,000), CYP3A4 Rabbit pAb (1:1,000), IGF1 Rabbit pAb (A11985) (1:500), GALP Rabbit pAb (A17445) (1:1,000) and GAPDH Rabbit pAb (1:1,000) at room temperature for 1 h and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were subsequently incubated with secondary antibodies HRP Goat Anti-Rabbit IgG (H + L) (AS014) (ABclonal Technology, Wuhan, China) (1:5,000) at room temperature for 1 h before developing the membranes with a LAS-4000 luminescent image analyzer (Fujifilm, Tokyo, Japan). Finally, we applied Quantity One software to analyze the protein gray value.

2.6. Genome-wide DNA methylation and MSP assays

In order to detect the effect of DON on the methylation of genomic DNA, a coloredimetric method was used to detect the level of 5-mC in all nucleotides in genomic DNA of the liver tissue. The specific steps of this method were based on work previously described (Liu et al., 2019a).

To determine the 5-mC level of gene promoter regions related to the metabolism and growth in liver tissue, the EZ DNA Methylation-Gold kit was used for bisulfite conversion of guanine-cytosine-rich DNA. Methylated and unmethylated primers for CYP450 enzymes and NNMT, GALP, IGF-1 and IGFBP2 genes are shown in Table S4. MSP analysis was conducted according to a previous study (Liu et al., 2019a).

2.7. BSP analysis

DNA of piglet liver was modified with bisulfite to detect cytosine-phosphate-guanine (CpG) dinucleotide methylation in the promoter regions of NNMT, GALP, IGF-1 and IGFBP2. These gene promoter regions were amplified from the bisulfite-modified sequence by BSP primers in Table S5. For the follow-up steps, please refer to previous studies (Liu et al., 2019a, 2019b). In short, the PCR products were recovered and purified, then connected with the vector, cloned and sequenced by selecting white single colonies.

2.8. Hematoxylin and eosin (H&E) and 5-ethyl-2’-deoxyuridine (EdU) staining of L02 cells

L02 cells (1 × 10^4) were inoculated in 12-well plates covered with glass coverslips and grown to 70% to 80% confluency. DON (2.5, 5, 10 μmol/L) was added to L02 cells. Then, after 24 h, the cells were fixed and stained with H&E and EdU kit (Guangzhou RiboBio Co., Ltd.). Cell damage, including whether the cell edge was clear, the change of nuclear morphology, and whether the cell had necrosis was observed under an inverted microscope (Olympus BX 41, Japan).

2.9. CYP1A1 and CYP2E1 gene silencing by siRNA and cell proliferation assay

CYP1A1 and CYP2E1 were transiently knocked out by siRNA transfection under the guidance of the manufacturer. Briefly, L02 cells were cultured to 60% to 70% confluency. The negative control (siNC), siCYP1A1 (sense: GUGAGCACUUCCAAAUUGCAUdT, anti-sense: UGAUAUUUGGAAGUCUCdCdTdT) and siCYP2E1 (UGUGGUGAUUGCAAGCCGUU) were mixed with Lipofectamine 2,000 reagent (Invitrogen, USA) and added to the culture medium. After 6 h of transfection, the culture medium was changed to fresh medium for a further 18 h. The interference efficiency of CYP1A1 and CYP2E1 was tested by qPCR and Western blot assays.

L02 cells (1 × 10^5) were seeded in 24-well plates covered with glass coverslips and grown to 60% to 70% confluency. SiRNA was transfected into L02 cells, and then L02 cells were cultured for 24 h, followed by DON (5 μmol/L) incubating for 24 h. The cells were stained with EdU kit and the cell proliferation was analyzed by fluorescence microscope (Olympus, Tokyo, Japan).

3. Results

3.1. DON affected serum liver biochemical indices in piglets

To observe the effect of DON on piglet liver function, serum chemical parameters including AST, ALT, and GGT were measured. According to the data presented in Fig. 1A, serum ALT and AST activity in the DON group significantly increased compared to 0 mg/kg group (P < 0.01). It should be noted that the ratio of AST to ALT (AST/ALT >1) in the DON-treated groups exhibited higher levels than that of the control group (P < 0.01), suggesting that the liver was damaged to a certain extent. Additionally, a sharp increase was observed in GGT activity following DON exposure when compared to the control group (P < 0.01).

3.2. DON exposure caused changes in CYP450 enzymes expression by DNA methylation in piglet liver

To check whether DON exposure caused abnormal expression of CYP450 enzymes, the mRNA level was examined. As shown in Fig. 1B, the mRNA expression levels of CYP450 enzymes, including CYP1A1, CYP1A2, CYP450 2B22 (CYP2B22), CYP450 2C33 (CYP2C33), CYP450 3A22 (CYP3A22), CYP450 2D25 (CYP2D25), CYP2E1 and CYP450 3A29 (CYP3A29), were significantly increased after DON administration at 3 mg/kg (P < 0.05). However, the expression of CYP2D25 and CYP2E1 in the DON group at 1 mg/kg exhibited a significant decrease compared to the control group (P < 0.05). The trend of protein expression and mRNA expression of CYP1A1, CYP2E1 and CYP3A29 was consistent in Fig. 1E. These results showed that DON exposure changed the expression of CYP450 enzymes.
In DNA methylation analysis, we focused primarily on the level of 5-mC, which is regulated by DNA methyltransferases (DNMT1, DNMT3A, DNMT3B). As shown in Fig. 1C, the mRNA expression levels of DNMT1 and DNMT3B were significantly increased after DON exposure compared to the control group (\( P < 0.01 \)). However, the DNMT3A expression of DON groups showed no significant difference compared to the control group. We tested the genomic 5-mC levels in pig liver, where our results uncovered that DON (1 or 3 mg/kg) significantly increased the total level of 5-mC compared with the control group (\( P < 0.01 \)) (Fig. 1D).

The MSP was used to detect the methylation levels of CpG sites in the CYP450 enzymes gene promoter region. As shown in Fig. 2A and B, in one DON exposure group (3 mg/kg), the methylation levels of CYP1A1 (\( P < 0.05 \)), CYP2D25 (\( P < 0.05 \)), CYP2E1 (\( P < 0.01 \)), and CYP3A29 (\( P < 0.05 \)) promoters were significantly decreased compared to the control, thus promoting the expression of these genes. Additionally, the methylation level of CYP1A2 promoter in DON-exposure groups was lower than the control group (\( P < 0.01 \)), which possibly promoted the transcription of the CYP1A2 gene. Although the methylation level of CYP2B22 promoter was increased (\( P < 0.05 \)), the expression of CYP2B22 gene was raised.

3.3. DON exposure caused changes of growth-regulating gene expression by DNA methylation in piglet liver

To investigate the effect of DON exposure on piglet growth, we also examined the expression of growth-related genes in the liver (Fig. 3A, and B). The results demonstrated that the expression levels of NNMT were markedly decreased after DON administration (1 or 3 mg/kg; \( P < 0.01 \)) compared to the control group. DON exposure at 3 mg/kg sharply elevated the expression of GALP (\( P < 0.01 \)), IGF-1 (\( P < 0.05 \)) and IGFBP2 (\( P < 0.01 \)) when compared to the control group (\( P < 0.05 \)), which might be the compensatory effect of DON-induced growth suppression toxicity.
To further determine whether DNA methylation was involved in the expression of NNMT, GALP, IGF-1 and IGFBP2 after exposure to DON in pig liver, MSP and BSP were applied (Fig. 3C, and D). For these genes, the CpG sites of MSP assay were included in the sequence of BSP analysis. Although only methylated primers amplified the bands of all groups, the methylated bands of the DON treatment group were stronger, suggesting an increased methylation level of NNMT to some extent. BSP results of NNMT gene also revealed that some CpG sites in DON-exposed groups were completely methylated compared to the control group, thus promoting NNMT transcription. The methylation level of GALP in one DON group (3 mg/kg) was lower than the control group and induced GALP expression. DON treatment could decrease the promoter methylation level of IGF-1, promoting IGF-1 expression. However, the IGFBP2 methylation level in DON groups exhibited no significant difference compared to the control group.

3.4. DON exposure increased the expression of CYP450 enzymes and decreased the expression of growth-related genes in L02 cells

The results of cell viability assay displayed a dose- and time-dependent decrease in cell viability induced by DON in L02 cells (Fig. S1). Considering the median inhibitory concentration (IC50) and the state of L02 cells, DON concentration (2.5, 5, 10 μmol/L) and 24 h of exposure time were selected for the subsequent studies. As shown in Fig. 4A, DON (5, 10 μmol/L) significantly \( P < 0.01 \) increased the mRNA expression of CYP1A1, CYP2E1 and CYP3A4 genes, which is consistent with the results of our in vivo study. In addition, the expression of growth-related genes (IGF-1, IGFBP2 and NNMT) was significantly \( P < 0.01 \) downregulated after DON exposure. The protein level of CYP450 enzymes and growth-related genes was consistent with the mRNA expression trend (Fig. 4B, and C).

3.5. DON led to cell necrosis and inhibited cell growth in a dose-dependent manner

In the control group, the nucleus was oval and chromatin was clearly distributed in the nucleus. Compared with the control group, DON exposure resulted in chromatin marginalization, nuclear pyknosis and nuclear karyorrhexis, which is a typical feature of cell necrosis. Importantly, DON treatment inhibited cell growth in a dose-dependent manner (Fig. 4E).

3.6. Silencing CYP1A1 increased the expression level of IGF-1, promoting cell growth

To prove whether CYP1A1 mediates growth inhibition induced by DON, L02 cells were transfected with CYP1A1-specific siRNA. The knockdown efficiency was detected by qPCR and Western blot analysis. The results illustrated that the expression level of CYP1A1 was significantly reduced \( P < 0.01 \) in CYP1A1-siRNA-transfected cells compared with the siNC group (Fig. 5A, and B). To detect the
The effect of CYP1A1 inhibition on the expression of cell growth-related genes, the mRNA and protein expression of IGF-1 was analysed at 24 h after transfection. Knockout of CYP1A1 gene significantly increased IGF-1 expression ($P < 0.01$) (Fig. 5A, and B). Correspondingly, compared with the siNC group, CYP1A1 knockout promoted cell growth ($P < 0.01$) (Fig. 5C, and D). Interestingly, decreased CYP1A1 alleviated growth inhibition induced by DON to some extent ($P < 0.01$) (Fig. 5C, and D).

3.7. Silencing CYP2E1 promoted the expression level of IGF-1, promoting cell growth

To prove whether CYP2E1 also mediates the growth inhibition induced by DON, L02 cells were transfected with CYP2E1-specific siRNA or siNC. The knockdown efficiency was detected by qPCR and Western Blot analysis. The results illustrated that the expression level of CYP2E1 was significantly decreased in CYP2E1-siRNA-transfected cells compared with the siNC group ($P < 0.01$) (Fig. 6A, and B). In addition, CYP2E1 silencing promoted IGF-1 expression ($P < 0.01$) (Fig. 6A, and B). Correspondingly, compared with the siNC group, knockdown of CYP2E1 induced cell growth ($P < 0.01$) (Fig. 6C). Noticeably, low expression of CYP1A1 also alleviated the growth inhibition induced by DON ($P < 0.01$) (Fig. 6C, and D).

4. Discussion

Due to high toxicity levels and widespread occurrence in animal feed and food, DON is considered an unavoidable contaminant in nature, which leads to hepatotoxicity and even growth retardation of animals (Wang et al., 2012). The liver is an important organ for metabolism and detoxification, and its damage is closely related to stunting growth under DON exposure. Our previous study found that 1.0 or 3.0 mg/kg DON reduced the body weight of piglets (Liu et al., 2020a; Zhang et al., 2020b), however, the role of DON-induced liver injury and the mechanism in growth retardation is unclear. Additionally, although there are increasing studies on DON-induced hepatotoxicity, the role of CYP450 enzymes and DNA methylation is still unclear. The present study showed DNA demethylation elevated the expression of CYP450 enzymes in DON-treated piglet livers, with an increase of AST and ALT. DON exposure reduced the expression of NNMT and GALP through DNA methylation, with the decrease in feed intake and weight of piglets (Liu et al., 2020a; Zhang et al., 2020b). In vitro studies found that different concentrations of DON inhibited cell growth and led to cell necrosis. In addition, it was found for the first time that CYP450 enzymes such as CYP1A1 and CYP2E1 could mediate DON-induced cell growth inhibition, although they do not metabolize DON.
Fig. 4. The effects of different concentrations of DON on the expression of CYP450 enzymes and growth-related genes, on cell damage and cell growth were tested in human normal hepatocytes L02 cells. (A) The mRNA levels of CYP1A1, CYP2E1, CYP3A4, NNMT, IGF-1 and IGFBP2 were detected by qPCR. (B and C) The protein levels of CYP1A1, CYP2E1, CYP3A4, and IGF-1 were detected by Western blot. (D) The pathological changes of L02 cells under DON exposure were detected by H&E staining. (E) L02 cell proliferation after DON treatment was detected by the EdU reagent (100× magnification, scale bar = 50 μm). *P < 0.05, **P < 0.01 versus the control group. EdU = 5-ethynyl-2′-deoxyuridine.
Feed levels of 1 and 3 mg/kg of DON are possible daily doses for piglets. In Portugal, by using high-pressure liquid chromatography (HPLC) to analyse 307 samples of plant crops, the highest level of DON concentration was found to be 17.9 mg/kg (Marques et al., 2008). It was reported that the average content of 23,980 samples contaminated by DON was as follows: wheat, 9.9 mg/kg; corn, 4.772 mg/kg; rice, 0.183 mg/kg; barley, 6.349 mg/kg; oats, 0.537 mg/kg; and rye, 0.190 mg/kg (Tardivel et al., 2015). DON was also detected in pasta with the highest level in the European Union of 3.2 mg/kg (Marin et al., 2013). Therefore, the dose used in this study was within the exposure dose range of animals to DON.

Although CYP450 enzymes do not participate in the direct metabolism of DON, abnormal upregulation in CYP450 enzymes were the mechanisms for DON-induced hepatoxicity and then growth inhibition. Recent literature suggested that CYP450 enzymes are involved in oxidative stress, apoptosis and inflammatory response (Cong et al., 2019; Xie et al., 2018). One study reported that DON (4.6 mg/kg feed) had no effect on the mRNA expression of CYP1A4 and CYP3A37 in broiler chickens’ liver (Antonissen et al., 2017). However, the present study stated clearly that DON (3 mg/kg feed) could significantly increase the expression level of CYP1A1, CYP2E1 and CYP3A4 in piglet liver. The differences in the result may be related to the DON dose, and the sensitivity of piglets to DON.

IGF-1 is an endocrine hormone produced in the liver and a sensitive index reflecting the function of growth hormone. DON (3 mg/kg) increased the expression of IGF-1 and IGFBP2 in piglet liver, but their expression was reduced under DON exposure in vitro. This may be related to the different self-repair environments in which in vivo and in vitro cells are located. The adverse consequences of DON in the body of piglet growth retardation may stimulate the production of IGF-1 and IGFBP2 to protect the body. However, the cell environment in vitro is simple and the compensatory effect is poor (Paravidino et al., 2021). Therefore, when DON inhibited growth, the expression of IGF-1 decreased.

Additionally, in vitro studies have found that forced reduction of CYP1A1 and CYP2E1 could raise the expression of IGF-1 and alleviate DON-caused growth inhibition. However, in vivo studies have shown that DON promoted the expression of CYP450, and the expression of IGF-1 also increased. Danicke et al. (2005) found that when 300 μg pure DON/kg was fed to 34 female pigs for 2 weeks after weaning under standardized conditions, serum IGF-1 levels were increased, but were decreased in 1,200 μg pure DON/kg groups at 8 weeks compared to the control group (Danicke et al., 2005). Therefore, the effect of DON on the level of IGF-1 in pigs may be related to the dose of DON, animal sensitivity and growth environment. In addition, DON inhibits the synthesis of growth hormone and affects the growth hormone/IGF axis (Guo et al., 2021; Liu et al., 2020b), thereby stimulating the expression of IGF-1 to resist the adverse effects of DON. At the same time, the highly expressed IGF-1 may also activate the PI3 kinase (PI3K)/serine kinase (AKT) pathway, thereby reducing the degree of cell growth inhibition caused by DON (Li et al., 2020).
Piglets are one of the most sensitive species to DON contaminated feed (Amuzie and Pestka, 2010; Pestka, 2010). The liver plays a key role in the metabolism and detoxification of DON (Wu et al., 2013). However, various investigations into DON in piglet liver have generated inconsistent results, with some showing hepatotoxicity of DON, and others not. For example, DON at 3.1 mg/kg feed for 37 d had no impact on pig liver (Stanek et al., 2012). However, growing pigs given DON-contaminated feed (6 mg/kg for 21 d) increased the values of ALT and AST in serum (Wu et al., 2013). A diet containing 8 mg/kg DON fed for 4 weeks disrupted the immune-related processes in the liver of piglets (Reddy et al., 2018). In the present study we selected the administration dose of DON of 1 and 3 mg/kg feed and fed piglets for 4 weeks. Herein, compared with the control group, DON elevated ALT, AST and GGT activity, and the ratio of AST to ALT was greater than 1, demonstrating that DON caused severe liver damage (Wang et al., 2020; Zhang et al., 2020a).

DNA methylation could be used as a sensitive molecular indicator of DON-induced liver injury. Regarding DNA methylation, DNMT1, DNMT3A and DNMT3B, maintain synergistically the stability of DNA methylation (Liu et al., 2019a). Mycotoxins can lead to changes in gene DNA methylation, thereby changing gene expression (Liu et al., 2019a, 2019b). It was found that 10 mM DON increased the percentage of 5-mC in DNA from 4.5% to 9% in Caco-2 cells (Kouadio et al., 2007). In the current study, DON exposure raised the genomic 5-mC level in piglet livers. We also observed that with the higher concentration of DON the effect was also greater. This might indicate that the dynamic changes of 5-mC level may be closely related to the degree of liver injury induced by DON (Ye et al., 2016). Additionally, DNA methylation is a molecular switch that regulates CYP450 enzymes expression of DON-exposed piglet liver. In the present study, it was found that DON at 3 mg/kg could decrease the methylation level of the promoter region of enzymes, including CYP1A1, CYP1A2, CYP2D25, CYP2E1 and CYP3A29, and thus increase their mRNA expression levels. Strangely, the results found that DON at 3 mg/kg significantly increased the methylation level of the CYP2B22 gene promoter, but the expression of CYP2B22 was significantly increased, which suggested that DON also affected CYP2B22 expression through other transcriptional factors (Gray and Squires, 2013; Messina et al., 2009). There were differences in the regulation of DNA methylation on the expression of different CYP450 enzymes, which may be related to the sequence of gene promoter, specific transcription factors and gene polymorphisms of different individuals (Tang and Chen, 2015).

DON also affected the expression of genes related to animal feeding and growth. NNMT is closely related to ALT release and liver inflammation (Sternak et al., 2010). In our study, DON significantly reduced the expression of NNMT, which may be an important factor in the weight loss of piglets. GALP is involved in regulating appetite and inflammation and energy metabolism (Dungan Lemko et al., 2008; Kageyama et al., 2005; Man and Lawrence, 2008). A high dose of DON (3 mg/kg) significantly increased the expression of GALP in the liver, which may be due to the rapid weight loss, malnutrition and increased leptin secretion of piglets fed with long-term high dose of DON (Lawrence and Fraley, 2011). Meanwhile, DON at 3 mg/kg could demethylate CpG sites in the promoter region of the GALP gene, leading to a sharp increase in the expression of GALP, which is the first time this has been discovered.

![Fig. 6. Effects of CYP2E1 silencing on cell proliferation. After L02 cells were transfected with the siNC and siCYP2E1 for 24 h, and then added DON (5 μmol/L) for 24 h. (A and B) The expression levels of CYP2E1 and IGF-1 were analyzed by qPCR and Western blot methods. (C) L02 cell proliferation was detected by the EdU reagent (100 × magnification, scale bar = 50 μm). (D) The level of cell proliferation in Fig. 6C was quantified. *P < 0.05, **P < 0.01 versus the siNC group; #P < 0.05, ##P < 0.01 versus the siNC + DON group, and the data were expressed as SD (n = 3).](image-url)
5. Conclusion
DON significantly raised the levels of AST, ALT and GGT, probably reflecting liver injury. In vivo research also found that DON increased the expression level of CYP450 enzymes and disturbed the expression of growth-related genes NNMT, GALP, IGF-1 and IGFBP2, in which DNA methylation meditated the expression of these genes. In vitro experiments showed that DON exposure increased the expression of metabolic enzymes CYP3A4, CYP1A1 and CYP2E1, and inhibited cell growth in a dose-dependent manner and led to cell necrosis. The silence CYP1A1 or CYP2E1 could slow down DON-caused cell growth inhibition by elevating IGF-1 expression. These results suggest that CYP450 enzymes may be therapeutic targets to alleviate hepatotoxicity and growth delay under DON exposure. In the future, this needs to be further confirmed in different models.

Author contributions
Xu Wang and Lv-hui Sun: financial support, experimental guidance and manuscript editing. Aimei Li: concept and design, performed research, analysed data, manuscript writing. Yaqin Yang, Jingchao Guo, Yan Gao, Qinghua Wu and Ling Zhao: participate in experiments, animal breeding and statistical data.

Declaration of competing interest
We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Supplementary Tables
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jani.2021.11.009.

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