Toxicity of glyphosate in feed for weanling piglets and the mechanism of glyphosate detoxification by the liver nuclear receptor CAR/PXR pathway

Huiyang Fu, Shengnan Qiu, Xinxin Yao, Feng Gao, Peng Tan, Teng Teng, Baoming Shi

PII: S0304-3894(19)31661-9

DOI: https://doi.org/10.1016/j.jhazmat.2019.121707

Reference: HAZMAT 121707

To appear in: *Journal of Hazardous Materials*

Received Date: 20 August 2019

Revised Date: 14 November 2019

Accepted Date: 15 November 2019

Please cite this article as: Fu H, Qiu S, Yao X, Gao F, Tan P, Teng T, Shi B, Toxicity of glyphosate in feed for weanling piglets and the mechanism of glyphosate detoxification by the liver nuclear receptor CAR/PXR pathway, *Journal of Hazardous Materials* (2019), doi: https://doi.org/10.1016/j.jhazmat.2019.121707

Toxicity of glyphosate in feed for weanling piglets and the mechanism of glyphosate detoxification by the liver nuclear receptor CAR/PXR pathway

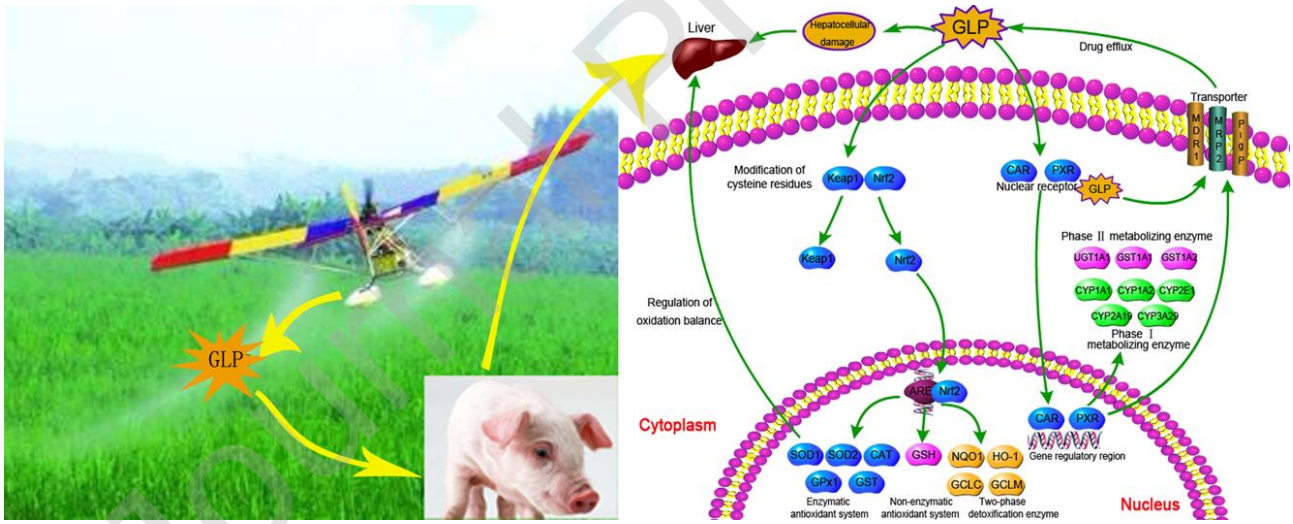
Huiyang Fu[a,#], Shengnan Qiu[a,#], Xinxin Yao[a], Feng Gao[a], Peng Tan[a], Teng Teng[a], Baoming Shi[a,*]

[a]Institute of Animal Nutrition, Northeast Agricultural University, Harbin, 150030, PR China

[#]These authors contributed equally to this work

[*]Corresponding author: Baoming Shi

Graphical Abstract



Highlights

- Glyphosate (GLP) is the herbicide with the largest dosage and yield in production.
- It is unprecedented to take sensitive weaned piglets as the research object of GLP test.
- The intake of GLP has no effect on the performance of weaned piglets

- GLP intake can cause changes in antioxidant enzymes activity.
- The toxicity can be alleviated by activating the nuclear receptor CAR/PXR signaling pathway.

Abstract: Glyphosate (GLP), the most widely used and productive pesticide worldwide, which safety and reliability gradually become a social concern. It is important to explore the toxic of GLP on the limitation level by governments on piglets and the potential role of hepatic CAR/PXR and Keap1-Nrf2 pathways in low levels of glyphosate detoxification. Compared with the control group, the production performance and organ index of GLP group showed no significant change. However, the liver GLP residue of 40 mg/kg group was significantly higher than the control group. We also found that the activity of ALP increased linearly and DBIL content increased quadratically. Furthermore, GLP could significantly increase SOD and GSH-Px and decrease T-AOC and CAT activities and significantly increase MDA and H_2O_2 contents (P<0.05); however, the genes expression of Keap1/Nrf2 pathway was not affected. Gene expression of CAR/PXR pathway showed that GLP could significantly stimulate the expression of CAR, but it could not affect the expression of phase Ⅰ (CYP1A1, CYP1A2, CYP2E1, CYP2A19, CYP3A29), phase Ⅱ (UGT1A6, GSTA1, GSTA2) detoxification enzymes and transporters (MDR1, MRP2, P-gp). Our study showed that although 10-40 mg/kg GLP would inevitably cause some liver damage and dysfunction, it can self-alleviating the toxic effect of GLP.

Keywords: glyphosate, weaned piglets, antioxidant, Keap1/Nrf2, CAR/PXR

1. Introduction

Glyphosate (GLP) is the active ingredient of the commercial, nonselective, broad-spectrum herbicide Roundup, which is the herbicide with the largest dosage and yield in production[1, 2]. Monsanto has

successfully developed the first commercialized GLP-resistant crop (Roundup-Ready Soybean)[3]. However, there has been more reckless use of GLP herbicides by farmers, leading to GLP pollution[4]. The consumption of GLP-containing crops that have undergone fog spraying and the consumption of water from the excessive irrigation runoff into rivers has triggered toxicity in animal tissues, which also depends on the level of GLP intake[5-7]. In vitro, exposure to GLP in porcine granulosa cells can significantly reduce cell proliferation, decrease cell viability and reduce steroid production[8]. Acute GLP supplementation at a certain dose or chronic exposure to GLP-containing environments for a long time alone leads to toxicity in the nervous system[9, 10].

In most cases, the metabolism of GLP is excreted directly with urine and feces[11]. The GLP excreted in vitro can be degraded by microorganisms as a nutrient and is an effective way to alleviate the environmental and health pressures of GLP[12, 13]. As the main antidotal organ, the liver can thoroughly decompose drugs or discharge them through low metabolism. This function is called "detoxification function". After long-term exposure to GLP, the liver is induced to produce a series of oxidative metabolites (ROS), such as superoxide anions, hydrogen peroxide, and oxygen free radicals[14]. As the main peroxidase, CAT can catalyze the rapid decomposition of H_2O_2[15], while GPx not only catalyzes the decomposition of hydrogen peroxide but also catalyzes reduced GSH to produce GSSG[16].

Nuclear receptors are transcription-related factors that regulate intracellular cascade reactions[17]. They can regulate the gene expression of phase Ⅰ detoxification enzymes and phase Ⅱ detoxification enzymes[18]. Studies have shown that PXR induces the upregulation of downstream CYP3A, MDR1 and MRP2 gene expression under the stimulation of exogenous drugs or endogenous products[19]. The upregulation of MDR1 and MRP2 expression can stimulate cells to transport harmful external toxins into cells and form decomposition products or drug complexes through the action of phase Ⅰ metabolic detoxification enzymes (CYPs) and phase Ⅱ metabolic detoxification enzymes (UGTs), which are

discharged with the circulation of urine or bile[20].

In this experience, GLP has important research significance, since it is an exogenous toxicant and may cause a toxic effect on the liver. The CAR/PXR and Keap1-Nrf2 signaling pathways may be involved in GLP detoxification, but it remains unclear which pathway plays a role. Therefore, we chose weanling piglets with high sensitivity to study whether different concentrations of GLP in the government limit level can cause toxic effects, and to explore the self-protection mechanism of piglets to GLP stimulation by detecting the expression of related genes and proteins in CAR/PXR and Keap1-Nrf2 signaling pathway, and achieve self-protection against foreign drugs. Additionally, previous studies have shown that GLP has toxic effects on animals, but they neither used sensitive weaned piglets as research models nor used the GLP of different concentrations on the basis of the Codex Alimentarius Commission (CODEX) for dietary supplementation (20 mg/kg), which is also the innovation point of this experimental study.

2. Materials and methods

2.1 Experimental design and animal feeding management

The GLP level in experimental diets was based on the Codex Alimentarius Commission (CODEX), which stipulates that the residue limit of GLP in dry soybean seeds is 20 mg/kg. In 2013, the United States relaxed the residue limit of GLP in soybean to 40 mg/kg. Therefore, the control group was fed a basic diet without GLP, while the treatment group was fed with 10, 20, or 40 mg/kg GLP supplemented with a basic diet. The basic diet is formulated according to National Research Council (NRC, 2012) standards, and all nutrient components meet NCR requirements. The ingredients and nutritional levels of the basic diet are shown in **Table 1**.

The twenty 28-day-old healthy weaned female crossbred piglets (Dorec × Landrace × Large white) were selected for the study. The piglets were subjected to seven days of pre-experimentation during the prefeeding period, in which the diets were supplemented with the basal diet without the addition of GLP, and the test diets were fed after the formal test over 35 days. Thereafter, all the piglets were divided into four groups according to their weight, with each group consisting of 5 replicates and one piglet per replicate. The weight range of all piglets after pre-experimentation was controlled at 12.20 ± 1.00 kg.

The experiment was carried out at Acheng Experimental Base of Northeast Agricultural University. All the piglets were fed separately from a single metabolic cage containing a water dispenser and feeding tank for free drinking and feeding. During the experiment, the pigsty temperature was kept between 23 and 25 ℃, meanwhile, the relative humidity was kept between 65 and 75%.

This study was performed in strict accordance with the recommendations of the National Research Council Guide (1996), and all of the animal experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang Province, China. The experimental proposals and procedures for the care and treatment of animals were approved by the Institutional Animal Care and Use Committee

of Northeast Agricultural University (NEAU-[2011]-9).

2.2 GLP and Chemicals

GLP was provided by the Roundup herbicide produced from Monsanto Company (St. Louis, Missouri, USA), in which the active ingredient is GLP with a purity of 30%. All the basic dietary raw materials came from Harbin Datang Minsheng Feed Co., Ltd. in Heilongjiang Province.

2.3 Preparation of GLP-treated diets

Before mixing the feed each time, 1.4 ml, 2.8 ml and 5.6 ml of Roundup were dissolved in water and sprayed evenly on the 2 kg basic diet. After a night in a cool and ventilated place, the mixture was evenly mixed with the 40 kg basic diet to form a treatment diet and was stored in a dry and ventilated room. The values of GLP in the treatment groups were 10, 20, and 40 mg/kg, while the control group contained no GLP.

2.4 Sample collection

After 35 days of feeding, pigs were fasted for 12 h before slaughtering, meanwhile, all the piglets were weighed on slaughter day. During this process, the organs of heart, liver, spleen, lung and kidney were quickly collected and weighed.

Blood samples (10 mL) were collected using heparin tubes from 20 piglets (each treatment group contained 5 pigs with similar BW) by venous puncture. The samples were then centrifuged at 3,000 g for 15 min at 4 °C. The plasma was separated and immediately stored at −80 °C until analysis. After blood sampling, the pigs were sacrificed using electricity (250 V, 0.5 A, for 5 to 6 s), with subsequent jugular exsanguination. The liver were obtained immediately, rinsed with physiological saline.

Next, 2 g of liver sample was placed in a liquid nitrogen tank and stored at -80 °C for RT-PCR analysis. Thereafter, ~1 cm^2 of liver tissue was cut and preserved in 10% formaldehyde solution for tissue section observation. Next, another 1 mm^2 of liver tissue was stored in electron microscopic solution at 4 °C for

later ultrastructural observation, and the remainder was stored at -20 °C for routine index analysis.

2.5 GLP residue in the liver

The pretreatment and determination methods for the liver were carried out in accordance with the "determination of GLP residues in food for import and export-HPLC-MA/MS method" (SN/T1923-2007).

2.6 Determination of the blood biochemical level

The hematological indexes were determined using an automatic biochemical analyzer (Roche, Cobus-Mira-Plus, Roche Diagnostic System Inc., Basel, Switzerland). The indicators of measurement included the levels of Total protein (TP), Albumin (ALB), Globulin (GLB), ALB/GLB (A/G), Alanine aminotransferase (ALT), Aspartate aminotransferase (ALT), AST/ALT, Gamma-glutamyl transferase (GGT), Alkaline phosphatase (ALP), Total bilirubin (TBIL), Direct bilirubin (DBIL), Triglyceride (TG), Cholesterol (CHOL), High-density lipoprotein (HDL), Low density lipoprotein (LDL), Lactic dehydrogenase (LDH), Creatine kinase (CK), Blood urea nitrogen (BUN), Creatinine (CREA), Uric acid (URIC), and Glucose (GLU).

Indicators for all measurements were assessed using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the method of Bradford according to the manufacturer's guidelines.

2.7 Antioxidant levels in the Liver

To determine the antioxidant indexes, a 10% liver/PBS (0.01 mol/L, pH=7.2-7.4) homogenate was first prepared. The liver SOD, GSH-Px, GST, CAT, GSH and MDA levels were determined using ELISA kits, and T-AOC and H_2O_2 levels were determined by colorimetry. All the kits were purchased from Shanghai Jinma Laboratory Equipment Co., Ltd. (Jinma, Shanghai, China), and each indicator was detected strictly according to the kit's specifications.

2.8 Expression of Keap1-Nrf2 and CAR/PXR pathway-related genes by RT-PCR

Total RNA was extracted with TRIZOL Reagent (Thermo Fisher Scientific Co., Ltd.). Next, 1000 ng of extracted RNA was reverse transcribed into cDNA using a gene amplification instrument (East Win Life Science, Inc., Beijing, China) and then was used for real-time polymerase chain reaction (RT-PCR). The concentration of RNA was determined using a nanophotometer (Implen GmbH, Munich, Germany), and an A_{260}/A_{280} ratio between 1.8 and 2.0 indicated a high purity of RNA. The reverse transcription kit was provided by Dalian Bao Biological Co., Ltd. (TaKaRa, Dalian, China). The total reaction volume was 20 μL and was divided into two steps. The first step is a volume of 10 μL, including 1μL of gDNA Eraser (1), 2 μL of 5× gDNA Eraser Buffer (2), 2 μL of RNase Free dH_2O (6) and 5 μL of total RNA reacting 42 °C for 2 min. Then 1 μL of PrimeScript RT Enzyme Mix Ⅰ (3), 4 μL of 5× PrimeScript Buffer 2 (for Real Time) (4), 1 μL of RT Primer Mix (5) and 4 μL of RNase Free dH_2O (6) were added on the basis of the first step reaction volume, following the reaction at 37 °C for 15 min, 85 °C for 5 s, and 4 °C for 30 min, so that the two steps totaled 20 μL. The specific steps of the reaction were strictly implemented according to the kit's instructions.

RT-PCR was determined using the ABI PRISM 7500 SDS thermal cycler apparatus (Applied Biosystems, Foster City, CA, USA), following the reaction procedure of one cycle at 95 °C for 30 s, 40 cycles at 95 °C for 5 s, and 61 °C for 34 s. The overall reaction system was 10 μL, including 1 μL of cDNA, 3.4 μL of 0.1% DEPC water, 5 μL of fluorescent dye TB-Green, 0.2 μL of correction solution ROX, and 0.2 μL each of upstream and downstream primers. The sequences of the primers used in the experiment are shown in **Table 2** and **Table 3**. All the primers were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Sangon Biotech, Shanghai, China), and TB-Green and ROX in the system were provided by Dalian Bao Biological Co., Ltd. (TaKaRa, Dalian, China). The Ct value was used as the output form of statistical analysis, and the gene expression was calculated as $2^{-\Delta\Delta Ct}$ calculated value[21].

2.9 Western blot analysis

Total proteins in liver were extracted with the RIPA Lysis Buffer and PMSF (Beyotime Biotechnology, Shanghai, China), and then the concentration of total proteins were determined by the Enhanced BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China). Under the action of 10% polyacrylamide gel electrophoresis (KeyGEN Biotech, Nanjing, China), according to the different moving rates of different molecular weight target proteins, the corresponding bands of gels were cut off and transferred to the Polyvinylidene Fluoride membranes (PVDF) (Beyotime Biotechnology, Shanghai, China) membrane, and incubated with β-actin, CAR, PXR, CYP1A2 and GSTA1 primary antibodies at 4 °C. After incubation overnight (12-15 h), the PVDF membrane was removed, washed three times with 1×TBST. Then the PVDF membrane was incubated in horseradish peroxidase labeled antibody at 35°C for 1 h, and then washed three times again with 1×TBST. Each protein emits fluorescence under the action of the fluorescence detection kit BeyoECL Star (Beyotime Biotechnology, Shanghai, China), and is exposed in the gel imaging and analysis system (UVItec, Cambridge, Britain). β-actin served as an internal control. The relative expressions of proteins were expressed as the ratio of band intensities of proteins to β-actin.

2.10 Statistical analyses

The data analysis software SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used to process and analyze the experimental data by one-way ANOVA, and orthogonal polynomials were used to analyze the linear and quadratic effects of GLP. Differences among groups were evaluated by Tukey test. $P < 0.05$ was considered to indicate statistical significance. All figures were made using Adobe Photoshop (Adobe Photoshop CS5, Inc., San Jose, California, USA) and GraphPad Prism (version 5.0, Graph Pad Software Inc., San Diego, CA, USA).

3 Results

3.1 Growth performance

The effects of different levels of GLP on the performance of weaned piglets are shown in **Table 4**. The IW, FW, ADG, ADFI and F/G levels were not affected by the GLP content in the diet.

3.2 Organ weight and organ index

In this study, organ index = organ weight/body weight.

As shown in **Table 5**, the weight of the heart, liver, spleen, lung, kidney and organ index of weaned piglets were not significantly affected by the GLP diets with different concentrations.

3.3 GLP residues in the liver

Table 6 shows that the residues were detected in the liver of the GLP groups, but no GLP residue was detected in the liver of the control group. Compared with the control group, the GLP content in the liver of the 40 mg/kg group was increased significantly ($P<0.05$).

3.4 Evaluation of pathological sections of the liver

The effect of supplementation with different GLP levels on liver slices in weaned piglets is shown in **Fig. 1**. There was no obvious damage in the control 0 mg/kg GLP group. The GLP group showed acute toxicity, which increased with the increase in the GLP content. Compared with the control group, the 10 mg/kg GLP treatment group of hepatocytes showed mild granular degeneration, hepatocyte swelling and evidence of fibrosis in the central vein; additionally, enlargement of the hepatic sinus was not obvious yet. In the 20 mg/kg GLP treatment group, blood cells appeared between the hepatic sinuses. The structure of the hepatic cord was not clear, but slight blood stasis could be observed. The wall of the hepatic central vein showed a high degree of fibrosis in the 40 mg/kg GLP treatment group. Additionally, more blood cells were detected in the hepatic sinusoids, as well as widening of the hepatic sinusoids and obvious necrosis of local hepatocytes, with the obvious phenomenon of blood stasis.

3.5 Ultrastructural assessment of the liver

The effects of different levels of GLP in the diet on the ultrastructure of hepatocytes in weaned piglets are shown in **Fig. 2**. Nonalcoholic fatty liver appeared in each treatment group, and the degree of disease increased gradually. In the 0 mg/kg GLP group, the mitochondrial ridges of hepatocytes were clearly visible, and the nuclear membrane was smooth and intact without damage. In the 10 mg/kg GLP treatment group, the hepatocyte nucleus decreased with abnormal chromatin aggregation, and the mitochondrial ridge was more obvious. An ambiguous chromatin structure in hepatocytes and fat granules began to appear in the 20 mg/kg GLP treatment group. Fibrosis began to appear in hepatocytes. In the 40 mg/kg GLP treatment group, the morphology of the hepatocyte nucleus was abnormal and the mitochondrial membrane was unclear. Endoplasmic reticulum ribosome exfoliation occurred, liver fibrosis was serious, and fat granules became larger.

3.6 Blood biochemical level

The serum collected after centrifugation was used to determine the hematological parameters, as shown in **Table 7**. With the increase in the GLP dose, the ALP level in the 10 mg/kg, 20 mg/kg and 40 mg/kg groups was linearly increased (P=0.018) and was significantly higher than that in the control group (P<0.05). The concentration of DBIL in the 10 mg/kg, 20 mg/kg and 40 mg/kg GLP treatment groups was quadratically increased (P=0.033) and the 20 mg/kg and 40 mg/kg GLP treatment groups were significantly higher than that in the control group (P<0.05), while adding GLP showed no linear and quadratic effects on other indexes.

3.7 Antioxidant capacity of the liver

The antioxidant index of the liver homogenate at 10% concentration is shown in **Table 8**. The table revealed that T-AOC in the liver was decreased linearly (P=0.023), and SOD activity increased linearly (P=0.002) and quadratically (P=0.003) in the 0 to 40 mg/kg GLP treated groups. The activity of GSH-Px

in the 40 mg/kg GLP treatment group was significantly higher than that in the control group (P<0.05). With the increase in the GLP concentration from 0 to 40 mg/kg, the CAT activity decreased linearly (P=0.002) and quadratically (P=0.001). As shown, the CAT activity in the 20 mg/kg and 40 mg/kg GLP treatment groups was significantly lower than that of the control group and 10 mg/kg treatment group (P<0.05) with no significant difference between the control group and 10 mg/kg treatment group. The MDA content increased quadratically (P=0.028) with the increase in the GLP concentration. The MDA content in the 40 mg/kg treatment group was significantly higher than that in the 20 mg/kg treatment group (P<0.05). In the 10 to 40 mg/kg GLP groups, the H_2O_2 concentration was increased linearly (P=0.006) and was significantly higher than that in the control group (P<0.05). The concentration of H_2O_2 in the 20 mg/kg and 40 mg/kg GLP treatment groups was also significantly higher than that in the 10 mg/kg treatment group (P<0.05). However, adding different levels of GLP showed no linear and quadratically effects on the GST activity and GSH concentration.

3.8 Expression of Keap1-Nrf2 antioxidant signaling pathway-related genes

Gene expression related to the Keap1-Nrf2 antioxidant signaling pathway in the liver is shown in **Fig. 3**. The graph shows that GLP addition had no significant effect on the mRNA levels of nuclear transcription-related factor (NFE2L2), enzymatic antioxidant system (SOD1, SOD2, CAT, GPX1, GST), nonenzymatic antioxidant system (GSH) and phase Ⅱ detoxifying enzyme (NQO1, HO-1, GCLC, GCLM) genes in the Keap1-Nrf2 antioxidant signaling pathway, indicating that GLP addition did not induce excessive oxidative damage to the liver.

3.9 Expression of nuclear receptor CAR/PXR signaling pathway-related genes

The expression of nuclear receptor CAR/PXR signaling pathway-related genes in the liver is shown in **Fig. 4**. The mRNA expression of constitutive androstane receptor (CAR) in piglets increased with the increase in the GLP concentration. In the detection of nuclear receptor gene (CAR, PXR) expression, we

compared the control group and found that the mRNA level of the CAR gene in the 40 mg/kg GLP group was increased significantly (P<0.05), but there was no significant difference between the 0, 10 and 20 mg/kg GLP groups. Regarding the determination of phase metabolic enzymes and the transporter protein gene expression, no significant difference was found in the levels of phase Ⅰ metabolic enzymes (CYP1A1, CYP1A2, CP2E1, CYP2A19, CYP4A29), phase Ⅱ metabolic enzymes (UGT1A6, GSTA1, GSTA2) and the transporter protein (MDR1 MRP2, P-gp) gene in each group, indicating that GLP did not increase the expression of phase Ⅰ metabolic enzymes, phase Ⅱ metabolic enzymes and transporter protein genes.

3.10 Analysis of the protein expression levels of nuclear receptor pathway-related genes

Analysis of the protein expression levels of nuclear receptor CAR/PXR signaling pathway-related genes in the liver is shown in **Fig. 5**. Compared with the control group, the protein level of nuclear receptor CAR in the 40 mg/kg GLP group was increased significantly (P<0.05), while the protein level of nuclear receptor CAR in 10 and 20 mg/kg GLP groups were not significantly different from that in the control group. It was found that different concentrations of GLP had no significant increase on the protein levels of nuclear receptor PXR, phase Ⅰ metabolic enzymes (CYP1A2) and phase Ⅱ metabolic enzymes (GSTA1).

4 Discussion

We established an oral exposure model combining food type with feeding pattern in weaned piglets for the experiments. In this study, we evaluated the hepatotoxicity of GLP supplementation in weaned piglets. The effects of low-dose GLP intake group on the growth performance, organ index and hematological parameters of piglets were also analyzed.

Numerous studies have shown that GLP intake has little effect on production performance. Studies have shown that drinking water containing 1% (w/v) GLP during pregnancy has no effect on the ADG of lactating offspring[14]. Furthermore, in the study of feed GLP granules for pregnant rats at 2 mg/kg BW/day and 200 mg/kg BW/day, no significant effect was noted on the average daily intake and body weight of postpregnant and weaning rats[22]. Feeding a GLP rat diet with 5 mg/kg body weight did not affect ADG, ADFI and organ index in rats[23]. In the study of piglets, it was also found that neither intravenous injection of 360 mg/mL glyphosate solution nor 40 wt% glyphosate isopropylamine solution could affect the performance of weaned piglets in a short time[24]. Obviously, these findings are basically in line with ours, glyphosate has no effect on production performance, which can be explained by GLP not reducing palatability.

Roundup is a commercial form of GLP. After adding 269.9 mg/kg of Roundup and 134.95 mg/kg of GLP to the diet of rats for one week and two weeks, the weights of the liver, spleen and kidney of rats were not affected[25]. Similarly, pigs were fed with non-GM (without glyphosate herbicide) and GM (with glyphosate herbicide) soybeans and maize respectively, which had no effect on their heart, liver, spleen, lung and kidney indices[26]. Because our trial dose is only exploring the toxic effects on weaned piglets at the level of the Ministry of agriculture, it is lower than previous studies, and our results are consistent with previous studies. Therefore, we consider that glyphosate at these dose are not enough to affect the organ weight and organ index of animals.

As the largest detoxification organ in the body, the liver must detoxify poisons and wastes produced in the body, as well as ingested poisons and drugs that are harmful to the liver[27]. Therefore, it is crucial to detect the deposition of GLP in the liver. In this study, the GLP residues in the liver of weaning piglets existed after the interruption of GLP intake, suggesting that GLP is also metabolized by the liver. Thus, as an exogenous poison, excessive deposition of GLP may lead to damage to liver tissue. We assessed the severity of liver injury by observing the histopathology and ultrastructure of the liver using Wister rats treated with GLP diets of 56 mg/kg and 560 mg/kg. The phagocytosis of rat phagocytes was accelerated after acute and long-term exposure to GLP, manifested as focal necrosis of the liver, leaching of monocytes and increased apoptotic cells[28]. This finding was similar to that observed with the pathological sections of the liver in our study. Other studies have shown that when tadpoles are raised in GLP (300 mg/L) aqueous solution, the number of liver melanomachrophagic cells (MMc) is increased, monocyte infiltration and blood stasis appear, and fat particles are deposited[29]. Moreover, long-term drinking of water containing glyphosate at low doses can also increase plasma triglyceride level and the content of most fatty acids (especially acylcarnitines) in liver[30]. There is a “multiple hits” hypothesis in the formation of nonalcoholic fatty liver. The “primary hits” is a metabolic disorder characterized by the accumulation of fatty acids in the liver, which makes the liver more sensitive to injury stimuli. This makes the liver more responsive to the “secondary hits”, such as oxidative stress, mitochondrial dysfunction and adipocytokines, resulting in lipid peroxidation, mitochondrial damage and hepatocyte damage, leading to non-alcoholic fatty liver disease and ultimately liver fibrosis[31]. Meanwhile, we observed the ultrastructure of the liver and showed fat particles in the liver cells, and also found that malondialdehyde increased significantly in the GLP group when the lipid peroxidation products of liver were measured. Thus, we believe that GLP can lead to lipid metabolism disorder and fat deposition in the liver, which may be a potential cause of non-alcoholic fatty liver disease.

The serum bilirubin level is an important indicator of hepatocyte function[32]. Pathological sections showed that 20 mg/kg and 40 mg/kg of GLP could cause blood stasis in the liver. After many red blood cells were destroyed, a large amount of hemoglobin was transformed into indirect bilirubin (IBIL) under the action of heme oxygenase and bilirubin reductase[33]. When IBIL enters the liver, it combines with glucuronic acid to produce excessive direct bilirubin (DBIL) by microsomal uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1)[32, 34]. Normally, DBIL enters the intestine from the liver and is converted by intestinal microorganisms to urinary bilirubin, which is excreted in vitro or reabsorbed via the portal vein[35]. Like DBIL, serum alkaline phosphatase (ALP) is another important index to evaluate the degree of liver injury[36] that needs to be excreted from the liver through the bile duct. However, in our study, the ultrastructure of the liver was changed, and the excretion inside and outside the liver was blocked, causing DBIL to not enter the biliary tract smoothly but only enter the blood circulation, and elevating the blood DBIL.

Animal infancy is the key period of vigorous growth and metabolism. It is very easy to produce reactive oxygen species (ROS) following external toxic stimulation[37]. The main source of ROS is ATP production by electron transfer through the mitochondrial respiratory chain complex. Because the liver is rich in mitochondria, oxidative stress induced by ROS is the common physiological basis of liver diseases and liver injury[38]. To resist oxidative stress induced by ROS, organisms have a complete network of antioxidant systems that interact to maintain the ROS level[39]. The antioxidant system consists of the nonenzymatic system and enzymatic system. Reduced glutathione (GSH) is a typical nonenzymatic antioxidant. It is not only a cofactor of many antioxidant enzymes but also participates in the redox process in vivo. It can combine with peroxides and free radicals and convert to oxidative glutathione (GSSG) under the action of glutathione reductase (GR) to resist the destruction of thiol by oxidants and protect cell membranes. Proteins containing sulfhydryl groups and enzymes containing sulfhydryl groups

are not destroyed, and they can resist the damage of free radicals to important organs[40]. No significant effect of the GSH content was observed in this study, suggesting that GLP may not destroy the balance of the transformation of nonenzymatic substances in weaned piglets. However, GLP can also promote ROS production and lipid peroxidation in the liver. The increase in malondialdehyde (MDA), a lipid peroxidation product, was observed in Albino rats treated with a sublethal dose of GLP or Roundup[25]. Another study showed that the content of MDA in human hepatocytes cultured with Roundup was increased significantly in vitro[41]. Under conditions of external stress, the dynamic balance of the production and removal of H_2O_2 is changed[42]. Like other ROS, H_2O_2 also participates in the mechanism of oxidative stress[43]. This is consistent with our results. Weaned piglets are stimulated by GLP to produce excessive MDA and H_2O_2. This oxidative damage alters membrane permeability and oxidative metabolism, leading to cell death[44]. To maintain the stability of ROS and oxidation levels, the body activates the antioxidant enzyme system.

The key antioxidant enzyme system comprises superoxide dismutase (SOD), glutathione peroxidase (GSH-Px/GPx), glutathione S-transferase (GST), and catalase (CAT), among others[39, 45-47]. Increased ROS levels stimulate the activity of these enzymes and affect the metabolism of nutrients such as protein and fat[48, 49]. SOD can catalyze not only the disproportionation of superoxide anions but also the decomposition of hydrogen peroxide. It has been proven that both GSH-Px and CAT can also catalyze the decomposition of hydrogen peroxide, which is the toxic intermediate in the metabolic reaction[50, 51]. As an exogenous oxidative stress inducer, GLP can produce a series of resistance responses. Studies have shown that oxidative stress occurs in multiple tissues exposed to GLP. Drinking water supplemented with 0.7 mg/L and 7 mg/L GLP significantly increased GSH-Px activity in the liver, kidney and small intestines of rats[52]. Another study showed that drinking water containing 1% GLP in pregnant rats induced lipid peroxidation in the liver of rats and their offspring but increased GPx activity in the

liver[14]. The oral concentration of 0.65 g/L and 1.3 g/L GLP water increased the activity of GPx in the brain of lactating rats but decreased the activity of CAT[53]. GLP not only affects the activity of antioxidant enzymes by feeding but also reduced the CAT activity in tortoises living in the GLP environment for a long time[39]. In our experiments, it can be understood that GPx is sufficient to maintain the stability of H_2O_2. Moreover, the total antioxidant capacity (T-AOC) of the liver of weaned piglets was also measured in our experiment, indicating that GLP showed oxidative damage to the liver. Consistent with previous studies, this damage can stimulate the activity of antioxidant enzymes to maintain the oxidative balance, which is an important mechanism of the body against oxidative damage.

The Keap1-Nrf2 signaling pathway is an effective way for the body to alleviate oxidative stress. Nrf2 (NF-E2-related factor 2) is a key factor in oxidative stress that is regulated by Kelch-like ECH-associated protein (Keap1) and regulates the expression of antioxidant protein and phase Ⅱ detoxifying enzyme by interacting with antioxidant response element (ARE), which plays an important role in resisting oxidative stress[54]. Normally, Nrf2 binds to its binding protein Keap1 (Kelch-like ECH-associated protein). Once Nrf2 is stimulated by oxidative stress from exogenous compounds or nucleophilic reagents, the cysteine residues in the structure of Keap1 protein will be modified. Next, Nrf2 and Keap1 protein will be separated and enters the nucleus to bind to ARE, triggering downstream gene expression[55, 56]. GLP is considered an exogenous compound. In our study, GLP enters the liver and affects the antioxidant system of the liver. Therefore, we also explored whether GLP could enhance the antioxidant protection ability by stimulating the gene expression of downstream antioxidant enzymes through the Keap1-Nrf2 signaling pathway. However, we found no significant differences in the gene expression levels of nuclear factor erythroid 2-related factor 2 (NFE2L2), the enzymatic antioxidant system (SOD1, SOD2, CAT, GPX1, GST), the nonenzymatic antioxidant system (GSH) and phase Ⅱ detoxification enzymes (NQO1, HO-1, GCLC, GCLM) in the Keap1-Nrf2 pathway between the groups. These results suggest that the GLP

dosage designed in this study does not activate the overexpression of genes related to the Keap1-Nrf2 signaling pathway in the liver but maintains the antioxidant homeostasis of the liver under GLP stress by stimulating enzyme activity. Because we set the experimental concentration gradient according to the maximum limit of soybean products set rely on Ministry of Agriculture Limits on the basis of the Codex Alimentarius Commission (CODEX) for dietary supplementation (20 mg/kg), we infer that this phenomenon is the result of low dosage, and it can be proven that diets containing 10 mg/kg, 20 mg/kg and 40 mg/kg GLP have no effect on weaned piglets at the molecular level.

The nuclear receptor superfamily (NRs) comprises ligand-activated transcription factors that regulate cell growth and differentiation by establishing connections between signaling molecules and transcriptional responses. Nuclear receptor CAR/PXR is thought to play an important role in regulating the transcription of phase Ⅰ metabolizing enzyme, phase Ⅱ metabolizing enzyme and transporters. The metabolism of drugs in vivo mainly undergoes two stages. The first step is a phase Ⅰ reaction, during which drugs are oxidized, reduced or hydrolyzed, catalyzing the key metabolic enzymes in the one-phase reaction, namely, P450 enzyme; the second step is a phase Ⅱ reaction, including glucuronidase, glutathione-S-transferase and N-acetyltransferase, which are all important enzymes in drug-binding reactions[57]. Its function is to catalyze the binding of drugs or metabolites with endogenous small molecules of glucuronic acid, glutathione and acetyl to form polar compounds that are excreted from urine and bile. The expression of CYP3A, CYP2B and CYP1A is regulated by nuclear receptor CAR or PXR[18]. In untreated animals, the expression of these genes is at a low level and increased when stimulated by exogenous chemicals[58]. At the same time, by increasing the gene expression of drug transporter (MDR1, MRP2, P-gp), the drug can be fully transported into cells for complete metabolism. The chemical structures of various drugs are different, while their binding relationships with CAR or PXR receptors show abundant overlapping relationships[59]. Our aim was to investigate whether GLP can metabolize

exogenous toxic GLP by activating nuclear receptors CAR and PXR, phase Ⅰ metabolizing enzymes and phase Ⅱ metabolizing enzymes to alleviate oxidative damage. Additionally, we observed that the gene expression and protein expression levels of constitutive androstane receptor (CAR) is dose dependent with the addition of GLP. Therefore, we speculate that GLP, similar to other drugs, can induce the gene expression of the nuclear receptor CAR to realize the detoxification of GLP by the liver itself. However, the gene expression of detoxifying enzyme and transporters remained unchanged, likely because the concentration of GLP in our experiment did not reach the range to activate its overexpression of downstream gene. Thus, the nuclear receptor CAR/PXR signaling pathway plays an important role in resisting GLP toxicity.

In conclusion, dietary GLP supplementation had no effect on the performance and organ index of weaned piglets, but it can lead to liver dysfunction and tissue damage. We showed that the liver alleviates the oxidative imbalance caused by GLP by enhancing its antioxidant capacity rather than by activating the expression of detoxifying enzymes downstream of the Keap1/Nrf2 signaling pathway. Furthermore, our results showed that moderate GLP supplementation could antagonize GLP-induced toxicity by activating the gene expression and protein expression levels of nuclear receptor CAR, which was the self-detoxifying function of liver (**Fig. 6**). However, the 10 mg/kg to 40 mg/kg concentration did not reach the range needed to activate the overexpression of other genes. This further confirms that the concentration of GLP added according to the Ministry of Agriculture limit was not sufficient to cause serious toxicity to weaned piglets, providing a strong guarantee for production safety.

Author Contributions

Huiyang Fu and Baoming Shi designed and conceived the experiments. Huiyang Fu, Shengnan Qiu, Xinxin Yao and Feng Gao conducted the main experiments assay. Huiyang Fu wrote the main manuscript text. Peng Tan and Teng Teng supervised the work and

revised the final version of the manuscript. All of the authors have read and approved the final version of the manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

GLP, Glyphosate; **IW**, Initial weight; **FW**, Final weight; **ADG**, The average daily gain; **ADFI**, The average daily feed intake; **F/G**, Feed conversion ratio or feed-gain ratio; **TP**, Total protein; **ALB**, Albumin; **GLB**, Globulin; **A/G**, ALB/GLB; **ALT**, Alanine aminotransferase; **AST**, Aspartate aminotransferase; **GGT**, Gamma-glutamyl transferase; **ALP**, Alkaline phosphatase; **TBIL**, Total bilirubin; **DBIL**, Direct bilirubin; **TG**, Triglyceride; **CHOL**, Cholesterol; **HDL**, High-density lipoprotein; **LDL**, Low density lipoprotein; **LDH**, Lactic dehydrogenase; **CK**, Creatine kinase; **BUN**, Blood urea nitrogen; **CREA**, Creatinine; **URIC**, Uric acid; **GLU**, Glucose; **T-AOC**, Total antioxidant capacity; **SOD**, Superoxide dismutase; **GSH-Px/GPx**, Glutathione peroxidase; **GST**, Glutathione S-transferase; **CAT**, Catalase; **GSH**, Reductive glutathione; **MDA**, Methane dicarboxylic aldehyde; **H_2O_2** , Hydrogen peroxide; **Keap1**, Kelch-like ECH-associated protein; **NFE2L2**, Nuclear factor erythroid 2-related factor 2; **SOD1**, Superoxide dismutase, soluble; **SOD2**, Superoxide dismutase, mitochondrial; **GPX1**, Glutathione peroxidase cytoplasm; **NQO1**, NAD(P)H quinone oxidoreductase 1; **HO-1**, Heme oxygenase 1; **GCLC**, Glutamate cysteine ligase catalyzes subunits; **GCLM**, Glutamic acid cysteine ligase modified subunit; **CAR**, Constitutive androstane receptor; **PXR**, Pregnenolone X receptor; **CYP1A1**, Cytochrome P450 1A1; **CYP1A2**, Cytochrome P450 1A2; **CYP2E1**, Cytochrome P450 2E1; **CYP2A19**, Cytochrome P450 2A19; **CYP3A29**, Cytochrome P450 3A29; **UGT1A6**, Uridine diphosphate glucuronosyltransferase 1A6; **GSTA1**, Glutathione S-transferase A1; **GSTA2**, Glutathione S-transferase A1; **MDR1**, multidrug resistance-1; **MRP2**, Multidrug resistance

associated protein 2; **P-gp**, Permeability glycoprotein.

Acknowledgements

This work was supported by grants from National Key R&D Program (2018YFD0501101) and the Provincial Science Fund for Distinguished Young Scholars (JQ2019C002).

References

[1] Q. Mao, F. Manservisi, S. Panzacchi, D. Mandrioli, I. Menghetti, A. Vornoli, L. Bua, L. Falcioni, C. Lesseur, J. Chen, F. Belpoggi, J. Hu, The Ramazzini Institute 13-week pilot study on glyphosate and Roundup administered at human-equivalent dose to Sprague Dawley rats: effects on the microbiome, Environ Health, 17 (2018) 50-61.

[2] N. Yu, Y. Tong, D. Zhang, S. Zhao, X. Fan, L. Wu, H. Ji, Circular RNA expression profiles in hippocampus from mice with perinatal glyphosate exposure, Biochem Biophys Res Commun, 501 (2018) 838-845.

[3] T. Bohn, M. Cuhra, T. Traavik, M. Sanden, J. Fagan, R. Primicerio, Compositional differences in soybeans on the market: glyphosate accumulates in Roundup Ready GM soybeans, Food Chem, 153 (2014) 207-215.

[4] A.V. Sviridov, T.V. Shushkova, I.T. Ermakova, E.V. Ivanova, D.O. Epiktetov, A.A. Leontievsky, Microbial degradation of glyphosate herbicides (Review), Applied Biochemistry and Microbiology, 51 (2015) 188-195.

[5] J.C. Brodeur, S. Malpel, A.B. Anglesio, D. Cristos, M.F. D'Andrea, M.B. Poliserpi, Toxicities of glyphosate- and cypermethrin-based pesticides are antagonic in the tenspotted livebearer fish (Cnesterodon decemmaculatus), Chemosphere, 155 (2016) 429-435.

[6] D. Bridi, S. Altenhofen, J.B. Gonzalez, G.K. Reolon, C.D. Bonan, Glyphosate and Roundup((R)) alter morphology and behavior in zebrafish, Toxicology, 392 (2017) 32-39.

[7] G.A. Altamirano, M.B. Delconte, A.L. Gomez, P.I. Ingaramo, V.L. Bosquiazzo, E.H. Luque, M. Munoz-de-Toro, L. Kass, Postnatal exposure to a glyphosate-based herbicide modifies mammary gland growth and development in Wistar male rats, Food Chem Toxicol, 118 (2018) 111-118.

[8] P. Gigante, M. Berni, S. Bussolati, F. Grasselli, S. Grolli, R. Ramoni, G. Basini, Glyphosate affects swine ovarian and adipose stromal cell functions, Anim Reprod Sci, 195 (2018) 185-196.

[9] D. Cattani, V.L. de Liz Oliveira Cavalli, C.E. Heinz Rieg, J.T. Domingues, T. Dal-Cim, C.I. Tasca, F.R. Mena Barreto Silva, A. Zamoner, Mechanisms underlying the neurotoxicity induced by glyphosate-based herbicide in immature rat hippocampus: involvement of glutamate excitotoxicity, Toxicology, 320 (2014) 34-45.

[10] G. Wang, X.N. Fan, Y.Y. Tan, Q. Cheng, S.D. Chen, Parkinsonism after chronic occupational exposure to glyphosate, Parkinsonism Relat Disord, 17 (2011) 486-487.

[11] L. Niemann, C. Sieke, R. Pfeil, R. Solecki, A critical review of glyphosate findings in human urine samples and comparison with the exposure of operators and consumers, Journal of Consumer Protection and Food Safety, 10 (2015) 3-12.

[12] H. Zhan, Y. Feng, X. Fan, S. Chen, Recent advances in glyphosate biodegradation, Appl Microbiol Biotechnol, 102 (2018) 5033-5043.

[13] A. Obojska, N.G. Ternan, B. Lejczak, P. Kafarski, G. McMullan, Organophosphonate Utilization by the Thermophile Geobacillus caldoxylosilyticus T20, Applied and Environmental Microbiology, 68 (2002) 2081-2084.

[14] C.J. Beuret, F. Zirulnik, M.S. Gimenez, Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses, Reprod Toxicol, 19 (2005) 501-504.

[15] X. Liao, C. Huang, D. Zhang, J. Wang, J. Li, H. Jin, C. Huang, Mitochondrial catalase induces cells transformation through nucleolin-dependent Cox-2 mRNA stabilization, Free Radic Biol Med, 113 (2017) 478-486.

[16] L.E. Rikans, K.R. Hornbrook, Lipid peroxidation, antioxidant protection and aging, Biochimica et Biophysica Acta, 1326 (1997) 116-127.

[17] B. Shi, Y. Su, S. Chang, Y. Sun, X. Meng, A. Shan, Vitamin C protects piglet liver against zearalenone-induced oxidative stress by modulating expression of nuclear receptors PXR and CAR and their target genes, Food Funct, 8 (2017) 3675-3687.

[18] Yi-Zhong Gu, John B. Hogenesch, C.A. Bradfiel, THE PAS SUPERFAMILY: Sensors of Environmental

and Developmental Signals, Annu. Rev. Pharmacol. Toxicol., 40 (2000) 519-561.
[19] S. Teng, M. Piquette-Miller, The involvement of the pregnane X receptor in hepatic gene regulation during inflammation in mice, J Pharmacol Exp Ther, 312 (2005) 841-848.
[20] E.S. Tien, M. Negishi, Nuclear receptors CAR and PXR in the regulation of hepatic metabolism, Xenobiotica, 36 (2006) 1152-1163.
[21] G. Lin, D. Luo, J. Liu, X. Wu, J. Chen, Q. Huang, L. Su, L. Zeng, H. Wang, Z. Su, Hepatoprotective Effect of Polysaccharides Isolated from Dendrobium officinale against Acetaminophen-Induced Liver Injury in Mice via Regulation of the Nrf2-Keap1 Signaling Pathway, Oxid Med Cell Longev, 2018 (2018) 6962439.
[22] M.M. Milesi, V. Lorenz, G. Pacini, M.R. Repetti, L.D. Demonte, J. Varayoud, E.H. Luque, Perinatal exposure to a glyphosate-based herbicide impairs female reproductive outcomes and induces second-generation adverse effects in Wistar rats, Archives of Toxicology, 92 (2018) 2629-2643.
[23] J. Tang, P. Hu, Y. Li, T.T. Win-Shwe, C. Li, Ion Imbalance Is Involved in the Mechanisms of Liver Oxidative Damage in Rats Exposed to Glyphosate, Front Physiol, 8 (2017) 1-12.
[24] H.L. Lee, C.D. Kan, C.L. Tsai, M.J. Liou, H.R. Guo, Comparative effects of the formulation of glyphosate-surfactant herbicides on hemodynamics in swine, Clinical toxicology, 47 (2009) 651-658.
[25] N.S. El-Shenawy, Oxidative stress responses of rats exposed to Roundup and its active ingredient glyphosate, Environ Toxicol Pharmacol, 28 (2009) 379-385.
[26] H.R.V. Judy A. Carman, Larry J. Ver Steeg, Verlyn E. Sneller, Garth W. Robinson, Catherine A. Clinch-Jones1, Julie I. Haynes, John W. Edwards, A long-term toxicology study on pigs fed a combined genetically modified (GM) soy and GM maize diet, Journal of Organic Systems, 8 (2013) 38-54.
[27] Hartmut Jaeschke, Gregory J. Gores, Arthur I. Cederbaum, J.A. Hinson, Dominique Pessayre, J.J. Lemasters, Mechanisms of Hepatotoxicity, TOXICOLOGICAL SCIENCES, 65 (2002) 166-176.
[28] S. Caglar, D. Kolankaya, The effect of sub-acute and sub-chronic exposure of rats to the glyphosate-based herbicide Roundup, Environ Toxicol Pharmacol, 25 (2008) 57-62.
[29] N.C. Bach, D.J.G. Marino, G.S. Natale, G.M. Somoza, Effects of glyphosate and its commercial formulation, Roundup((R)) Ultramax, on liver histology of tadpoles of the neotropical frog, Leptodactylus latrans (amphibia: Anura), Chemosphere, 202 (2018) 289-297.
[30] R. Mesnage, G. Renney, G.E. Seralini, M. Ward, M.N. Antoniou, Multiomics reveal non-alcoholic fatty liver disease in rats following chronic exposure to an ultra-low dose of Roundup herbicide, Sci Rep, 7 (2017) 39328.
[31] D. Gnocchi, C. Custodero, C. Sabba, A. Mazzocca, Circadian rhythms: a possible new player in non-alcoholic fatty liver disease pathophysiology, Journal of molecular medicine, 97 (2019) 741-759.
[32] X. Wang, J.R. Chowdhury, N.R. Chowdhury, Bilirubin metabolism: Applied physiology, Current Paediatrics, 16 (2006) 70-74.
[33] X. Wang, L. Zheng, J. Wu, B. Tang, M. Zhang, D. Zhu, X. Lin, Constitutive androstane receptor activation promotes bilirubin clearance in a murine model of alcoholic liver disease, Mol Med Rep, 15 (2017) 3459-3466.
[34] L. Weaver, A.R. Hamoud, D.E. Stec, T.D. Hinds, Jr., Biliverdin reductase and bilirubin in hepatic disease, Am J Physiol Gastrointest Liver Physiol, 314 (2018) G668-G676.
[35] A.R. Hamoud, L. Weaver, D.E. Stec, T.D. Hinds, Jr., Bilirubin in the Liver-Gut Signaling Axis, Trends Endocrinol Metab, 29 (2018) 140-150.
[36] F. Geyikoglu, H. Turkez, T.O. Bakir, M. Cicek, The genotoxic, hepatotoxic, nephrotoxic, haematotoxic and histopathological effects in rats after aluminium chronic intoxication, Toxicol Ind Health, 29 (2013) 780-791.
[37] P. Monaghan, N.B. Metcalfe, R. Torres, Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation, Ecol Lett, 12 (2009) 75-92.
[38] D.R. LIVINGSTONE, C.H. Plymouth Marine Laboratory, Plymouth,Devon PLI 2PB,UK, Contaminant-stimulated Reactive Oxygen Species Production and Oxidative Damage in Aquatic Organisms, Marine Pollution Bulletin, 48 (2001) 656-666.

[39] P.M. Burella, L.M. Odetti, M.F. Simoniello, G.L. Poletta, Oxidative damage and antioxidant defense in Caiman latirostris (Broad-snouted caiman) exposed in ovo to pesticide formulations, Ecotoxicol Environ Saf, 161 (2018) 437-443.
[40] F. Regoli, M.E. Giuliani, Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms, Mar Environ Res, 93 (2014) 106-117.
[41] L. Luo, F. Wang, Y. Zhang, M. Zeng, C. Zhong, F. Xiao, In vitro cytotoxicity assessment of roundup (glyphosate) in L-02 hepatocytes, J Environ Sci Health B, 52 (2017) 410-417.
[42] L.A.d. Rı´o1, F. Javier Corpas, Luisa M. Sandalio, Jose´ M. Palma, M. Go´mez, J.B. Barroso, Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes, Journal of Experimental Botany, 53 (2002) 1255-1272.
[43] R. Mittler, S. Vanderauwera, N. Suzuki, G. Miller, V.B. Tognetti, K. Vandepoele, M. Gollery, V. Shulaev, F. Van Breusegem, ROS signaling: the new wave?, Trends Plant Sci, 16 (2011) 300-309.
[44] C.R. Murussi, M.D. Costa, J.W. Leitemperger, L. Guerra, C.C. Rodrigues, C.C. Menezes, E.S. Severo, F. Flores-Lopes, J. Salbego, V.L. Loro, Exposure to different glyphosate formulations on the oxidative and histological status of Rhamdia quelen, Fish Physiol Biochem, 42 (2016) 445-455.
[45] S. Yang, K. He, T. Yan, H. Wu, J. Zhou, L. Zhao, Y. Wang, Q. Gong, Effect of starvation and refeeding on oxidative stress and antioxidant defenses in Yangtze sturgeon (Acipenser dabryanus), Fish Physiol Biochem, 4 (2019) 1-9.
[46] N. Singh, M.A. Savanur, S. Srivastava, P. D'Silva, G. Mugesh, A manganese oxide nanozyme prevents the oxidative damage of biomolecules without affecting the endogenous antioxidant system, Nanoscale, 11 (2019) 3855-3863.
[47] X.Y. Yuan, G.Z. Jiang, C.C. Wang, K.P. Abasubong, Q. Zou, Y.Y. Zhou, W.B. Liu, Effects of partial replacement of fish meal by yeast hydrolysate on antioxidant capability, intestinal morphology, and inflammation-related gene expression of juvenile Jian carp (Cyprinus carpio var. Jian), Fish Physiol Biochem, 45 (2019) 187-197.
[48] G. Chaufan, A. Juarez, S. Basack, E. Ithuralde, S.E. Sabatini, G. Genovese, M.L. Oneto, E. Kesten, C. Rios de Molina Mdel, Toxicity of hexachlorobenzene and its transference from microalgae (Chlorella kessleri) to crabs (Chasmagnathus granulatus), Toxicology, 227 (2006) 262-270.
[49] S.E. Sabatini, G. Chaufan, A.B. Juarez, I. Coalova, L. Bianchi, M.R. Eppis, C. Rios de Molina Mdel, Dietary copper effects in the estuarine crab, Neohelice (Chasmagnathus) granulata, maintained at two different salinities, Comp Biochem Physiol C Toxicol Pharmacol, 150 (2009) 521-527.
[50] R.S. Araújo, K.R.S. Sousa, F.C.B. Sousa, A.C. Oliveira, L.R.B. Dourado, S.E.F. Guimarães, W. Silva, D. Biagiotti, G.F.V. Bayão, A.C. Araujo, Effect of glycerin supplementation on the expression of antioxidant and mitochondrial genes in broilers, Animal Production Science, 59 (2019) 408-415.
[51] C.C.D. Ribeiro, R.M. Silva, V. Campanholo, D.A. Ribeiro, A.P. Ribeiro Paiotti, N.M. Forones, Effects of Grape Juice in Superoxide Dismutase and Catalase in Colorectal Cancer Carcinogenesis Induced by Azoxymethane, Asian Pac J Cancer Prev, 19 (2018) 2839-2844.
[52] K. Larsen, R. Najle, A. Lifschitz, G. Virkel, Effects of sub-lethal exposure of rats to the herbicide glyphosate in drinking water: glutathione transferase enzyme activities, levels of reduced glutathione and lipid peroxidation in liver, kidneys and small intestine, Environ Toxicol Pharmacol, 34 (2012) 811-818.
[53] C.E. Gallegos, C.J. Baier, M. Bartos, C. Bras, S. Dominguez, N. Monaco, F. Gumilar, M.S. Gimenez, A. Minetti, Perinatal Glyphosate-Based Herbicide Exposure in Rats Alters Brain Antioxidant Status, Glutamate and Acetylcholine Metabolism and Affects Recognition Memory, Neurotox Res, 34 (2018) 363-374.
[54] L. Sang, X.M. Wang, D.Y. Xu, L.X. Sang, Y. Han, L.Y. Jiang, Morin enhances hepatic Nrf2 expression in a liver fibrosis rat model, World J Gastroenterol, 23 (2017) 8334-8344.
[55] Y.H. Ni, L.J. Huo, T.T. Li, Antioxidant axis Nrf2-keap1-ARE in inhibition of alcoholic liver fibrosis by IL-22,

World J Gastroenterol, 23 (2017) 2002-2011.
[56] R.S. Wible, Q.T. Tran, S. Fathima, C.H. Sutter, T.W. Kensler, T.R. Sutter, Pharmacogenomics of Chemically Distinct Classes of Keap1-Nrf2 Activators Identify Common and Unique Gene, Protein, and Pathway Responses In Vivo, Mol Pharmacol, 93 (2018) 297-308.
[57] S. Chang, Y. Su, Y. Sun, X. Meng, B. Shi, A. Shan, Response of the nuclear receptors PXR and CAR and their target gene mRNA expression in female piglets exposed to zearalenone, Toxicon, 151 (2018) 111-118.
[58] R.D. Finn, C.J. Henderson, C.L. Scott, C.R. Wolf, Unsaturated fatty acid regulation of cytochrome P450 expression via a CAR-dependent pathway, Biochem J, 417 (2009) 43-54.
[59] JODI M. MAGLICH, CATHERINE M. STOLTZ, BRYAN GOODWIN, DIANE HAWKINS-BROWN, JOHN T. MOORE, S.A. KLIEWER, Nuclear Pregnane X Receptor and Constitutive Androstane Receptor Regulate Overlapping but Distinct Sets of Genes Involved in Xenobiotic Detoxification, MOLECULAR PHARMACOLOGY, 62 (2002) 638-646.

Table1 Basic diet ingredients and nutrient levels

Basic diet ingredients	Content (%)
Corn	62.00
Peeling soybean meal (46.2% CP)	16.20
Corn gluten meal (62.7% CP)	2.00
Full-fat soybean (35.5% CP)	9.00
Whey Powder	3.00
Fish meal (64% CP)	3.00
Soybean oil	1.50
Lysine (98%)	0.17
Calcium hydrogen phosphate	0.84
Stone powder	0.89
NaCl	0.40
Premix[a]	1.00
Total	100.00
Nutrient levels[b]	
Digestible energy (Mcal/kg)	3.19
Crude protein	19.01
Crude fat	5.97
Lysine	1.15
Methionine	0.34
Threonine	0.75
Tryptophan	0.22
Calcium	0.73
Total phosphorus	0.56
Available phosphorus	0.36
Sodium	0.25
Chlorine	0.25

[a] Provided the following per kilogram of diet: Fe, 190 mg; Cu, 190 mg; Mn, 45 mg; Zn, 140 mg; Se, 0.4 mg; I, 0.5 mg; vitamin A, 45,000,000 IU; vitamin D_3, 8,500,000 IU; vitamin E, 80,000 mg; vitamin K_3, 5,000 mg; vitamin B_1, 8,000 mg; vitamin B_2, 20,000 mg; vitamin B_6, 8,000 mg; vitamin B_{12}, 100 mg; niacin, 100,000 mg; D-pantothenic acid, 45,000 mg; D-biotin, 500 mg; and folate, 4,000 mg.

[b] Treatments consisted of a basal diet supplemented with glyphosate at 0, 10, 20, or 40 mg kg^{-1}.

Table 2 Primer design and synthesis used for RT-PCR of the Keap1-Nrf2 Pathway in piglets

Gene (Gene Bank)	Sequences (5'→3')	Fragments sizes	Gen Bank No.
NFE2L2	F: CCAATTCAGCCAGCACAACACATC	149 bp	XM_013984303.2
	R: GACTGAGCCTGGTTAGGAGCAATG	149 bp	
SOD1	F: CCAGTGCAGGTCCTCACTTCAATC	172 bp	NM_001190422.1
	R: CGGCCAATGATGGAATGGTCTCC	172 bp	
SOD2	F: TGTATCCGTCGGCGTCCAAGG	93 bp	NM_214127.2
	R: TCCTGGTTAGAACAAGCGGCAATC	93 bp	
CAT	F: AGCCTACGTCCTGAGTCTCTGC	90 bp	NM_214301.2
	R: TCCATATCCGTTCATGTGCCTGTG	90 bp	
GST	F: GGACTCTCCTGGTCCTGAATGCC	115 bp	NM_214050.2
	R: AGAACTGGCACCAGACCTGAGG	115 bp	
GPX1	F: GCGTCGCTCTGAGGCACAAC	167 bp	NM_214201.1
	R: GGTCGGACGTACTTGAGGCAATTC	167 bp	
GSH	F: TGCACGAATTCTCAGCCAAGGAC	80 bp	L12743.1
	R: GGTGACGATGCACACGTAGCC	80 bp	
NQO1	F: AGTATCCTGCCGAGACTGCTCTG	95 bp	NM_001159613.1
	R: CACAAGGTCTGCGGCTTCCAC	95 bp	
HO-1	F: GTTTGAGGAGGTGCAGGAGC	184 bp	NM_001004027.1
	R: GAGTGTCAGGACCCATCGGA	184 bp	
GCLC	F: GCATGTGGCTCACCTCTTCATCAG	135 bp	XM_021098556.1
	R: GGAGGCTTGAATCTCATCGTCTGC	135 bp	
GCLM	F: CACAGCGAGGAGCTTCGAGAC	119 bp	XM_001926378.4
	R: ACTGCGTGAGACACAGTACATTCC	119 bp	

Table 3 Primer design and synthesis used for RT-PCR of the CAR/PXR Pathway in piglets

Gene (Gene Bank)	Sequences (5'→3')	Fragments sizes	Gen Bank No.
β-actin	F:ATGCTTCTAGGCGGACTGT	211 bp	AY550069
	R:CCATCCAACCGACTGCT	211 bp	
CAR	F:TCTTCAGGCGAACAGTCAAC	133 bp	AB214979.1
	R: GCATCTAGGCACTTCTGCAA	133 bp	
PXR	F:CCTTTGACACCACCTTCACA	121 bp	NM_001038005.1
	R:CTCCACTTGGCAGCTTCTTC	121 bp	
CYP1A1	F:CCTTCACCATCCCTCACAGT	122 bp	NM_214412.1
	R:GTCATCCCACAGCTTCTGGT	122 bp	
CYP1A2	F:CAAAGGCCTGAAGAGTCCAC	124 bp	NM_001159614.1
	R:TTGCAGCACATCTCCATAGC	124 bp	
CYP2E1	F:TGCCGTGGTACATGAGATTC	118 bp	NM_214421.1
	R:AATTACCACTGTGCCCTTGG	118 bp	
CYP2A19	F:GGATGAGAACGGGCAGTTTA	126 bp	NM_214417.1
	R:TTCTGCAGGATGTTGGTGAG	126 bp	
CYP3A29	F:TTGCTGGCTACGAGACCACTAG	78 bp	NM_214423.1
	R:CTGCTGGACATCAGGGTGAGT	78 bp	
UGT1A6	F:ATCTGCTTGGTCACCCAAAG	122 bp	NM_001278750.1
	R:ATCCATCTGATCCCCAAACA	122 bp	
GSTA1	F:TCAACTACATCGCCACCAAG	120 bp	NM_214389.2
	R:CACAGTGGCAACAGCAAGAT	120 bp	
GSTA2	F:TCAATTACATCGCCACCAAG	121 bp	NM_213850.1
	R:CACAGTGGCAACAGCAAGAT	121 bp	
MDR1	F:CAATAGCTCGTGCCCTTGTCAGA	146 bp	NM_001308246.1
	R:CGGTGAGCGATCACGATGCAG	146 bp	
MRP2	F:CCAACAACAGCAGCAAGACC	120 bp	XM_003481109.4
	R:GAGCGGTGCAATAAGCTGTG	120 bp	
PGP	F:CCAACAACAGCAGCAAGACC	120 bp	XM_003481109.4
	R:GAGCGGTGCAATAAGCTGTG	120 bp	

Table 4 Performance of piglets fed different levels of glyphosate

Items/GLP		Control	GLP (mg/kg diet)			SEM[a]	*P*-value	
		0	10	20	40		Liner	Quadratic
IW (kg)		12.32	12.16	12.22	12.06	0.138	0.946	0.744
FW (kg)		35.49	34.98	34.14	35.84	0.480	0.457	0.532
ADG (kg/d)		0.66	0.65	0.63	0.69	0.012	0.343	0.437
ADFI (kg/d)		1.32	1.36	1.29	1.37	0.023	0.453	0.240
F/G		1.99	2.09	2.06	2.02	0.024	0.333	0.550

The values are the means from 5 individual pigs.

[a] SEM: pooled standard error of the means.

Table 5 Organ weight and organ index of piglets fed different levels of glyphosate

Items/GLP		Control	GLP (mg/kg diet)			SEM[a]	*P*-value	
		0	10	20	40		Liner	Quadratic
Heart (g)		161.20	183.00	162.60	166.20	4.513	0.216	0.128
Relative heart (%)		0.45	0.53	0.48	0.46	0.134	0.151	0.186
Liver (g)		773.20	792.80	750.25	791.80	9.700	0.269	0.123
Relative liver (%)		2.18	2.27	2.19	2.21	0.027	0.462	0.325
Spleen (g)		73.40	69.60	73.80	74.80	2.396	0.798	0.632
Relative spleen (%)		0.21	0.20	0.22	0.21	0.006	0.720	0.426
Lung (g)		374.20	385.60	370.00	399.60	6.242	0.445	0.247
Relative lung (%)		1.06	111	1.05	1.11	0.023	0.673	0.382
Kidney (g)		161.40	165.20	144.40	151.80	4.427	0.414	0.196
Relative kidney (%)		0.45	0.47	0.42	0.42	0.012	0.498	0.271

The values are the means from 5 individual pigs. Relative organ=organ weight / FW.

[a] SEM: pooled standard error of the means.

Table 6 Glyphosate residues in the liver of piglets fed different levels of glyphosate

Items/GLP		Control	GLP (mg/kg diet)			SEM[c]	*P*-value	
		0	10	20	40		Liner	Quadratic
Liver residues (ng/g)		0[b]	6.29[ab]	4.51[b]	15.97[a]	2.222	0.121	0.071

The values are the means from 5 individual pigs.

[a,b] In the same row, the values with different small superscript letters indicate a significant difference ($P < 0.05$).

[c] SEM: pooled standard error of the means.

Table 7 Effects of different glyphosate supplementation levels on the blood biochemical level in piglets

Items/GLP		Control	GLP (mg/kg diet)			SEM[d]	*P*-value	
		0	10	20	40		Liner	Quadratic
TP (g/L)		67.94	66.70	67.26	65.04	0.787	0.787	0.538
ALB (g/L)		40.20	39.96	42.43	40.18	0.555	0.352	0.195
GLB (g/L)		27.74	26.74	23.54	24.86	0.835	0520	0.369
A/G		1.48	1.53	1.90	1.64	0.072	0.167	0.132
ALT (IU/L)		49.67	49.40	59.00	54.00	2.305	0.517	0.283
AST (IU/L)		70.33	89.00	88.67	76.00	3.388	0.061	0.795
AST/ALT		1.49	1.71	1.46	1.28	0.083	0.382	0.472
GGT (IU/L)		37.60	39.00	42.00	37.50	1.546	0.594	0.547
ALP (IU/L)		125.00[c]	168.67[b]	207.33[a]	198.33[ab]	10.211	0.018	0.243
TBIL (IU/L)		9.37	8.30	10.63	11.30	0.620	0.523	0.373
DBIL (IU/L)		1.46[b]	2.53[b]	6.08[a]	5.58[a]	0.639	0.054	0.033
TG (mmol/L)		0.57	0.60	0.54	0.67	0.039	0.595	0.439
CHOL (mmol/L)		2.74	2.60	2.61	2.85	0.051	0.174	0.868
HDL (mmol/L)		0.99	0.98	0.99	1.06	0.023	0.715	0.866
LDL (mmol/L)		1.53	1.38	1.35	1.53	0.040	0.126	0.819
LDH (IU/L)		886.00	933.00	925.33	860.00	17.271	0.318	0.985
CK (IU/L)		3010.80	3250.20	3700.60	2824.00	170.100	0.176	0.316
BUN (mmol/L)		5.33	4.38	3.80	5.10	0.263	0.084	0.493
CREA (μmol/L)		92.77	96.50	100.20	98.60	1.267	0.460	0.611
URIC (μmol/L)		4.84	5.16	4.96	4.82	0.120	0.589	0.615
GLU (mmol/L)		5.50	5.38	5.80	4.98	0.311	0.730	0556

The values are the means from 5 individual pigs.

[a,b,c] In the same row, the values with different small superscript letters indicate a significant difference ($P < 0.05$).

[d] SEM: pooled standard error of the means.

Table 8 Antioxidant indexes in the liver of piglets fed different levels of glyphosate.

Items/GLP		Control	GLP (mg/kg diet)			SEM[d]	*P*-value	
		0	10	20	40		Liner	Quadratic
T-AOC (U/mL)		8.72[a]	7.77[b]	7.22[b]	7.42[b]	0.187	0.023	0.651
SOD (U/L)		1302.40[b]	1568.20[a]	1468.60[a]	1580.20[a]	31.989	0.002	0.003
GSH-Px (IU/L)		139.20[b]	146.37[ab]	149.55[ab]	154.65[a]	1.965	0.782	0.590
GST (mIU/L)		17.04	16.59	16.32	16.99	0.182	0.363	0.675
CAT (U/L)		1281.30[a]	1229.70[a]	934.98[b]	939.65[b]	43.440	0.002	0.001
GSH (ng/L)		887.71	843.85	808.83	885.03	13.440	0.086	0.383
MDA (nmol/L)		7.67[ab]	7.92[ab]	7.61[b]	8.13[a]	0.077	0.058	0.028
H_2O_2 (pg/mL)		27.08[c]	32.43[b]	34.88[a]	36.05[a]	0.833	0.006	0.531

The values are the means from 5 individual pigs.

[a,b,c] In the same row, values with different small superscript letters mean significant difference ($P < 0.05$).

[d] SEM: pooled standard error of the means.

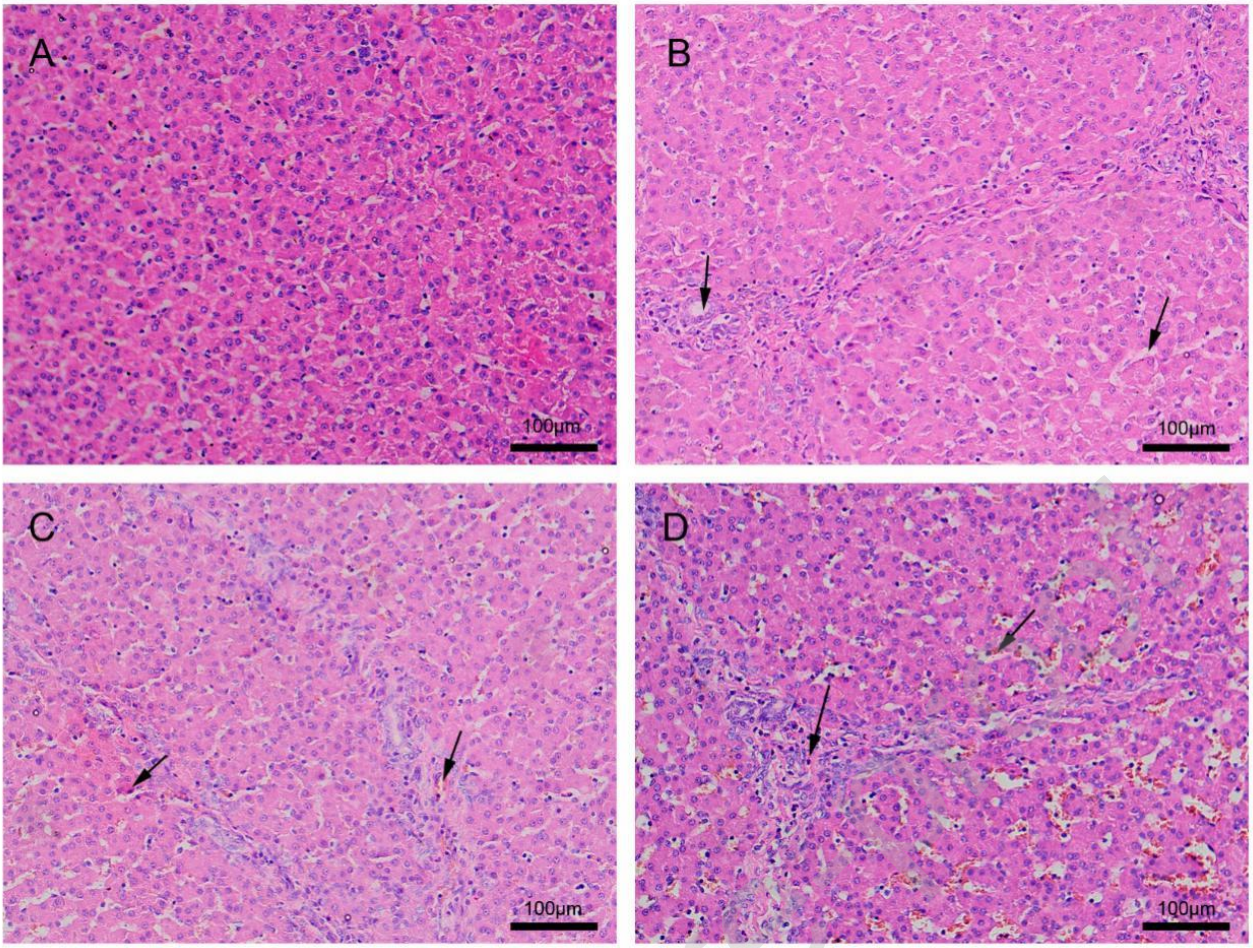


Fig. 1 Effects of different GLP diet levels on liver slices of piglets. A: No obvious damage was noted in the control group. B: The 10 mg/kg GLP treatment group showed slight granular degeneration of hepatocytes and hepatocyte swelling. Enlargement of the hepatic sinus was not obvious. C: In the 20 mg/kg GLP treatment group, blood cells appeared between the hepatic sinuses. The structure of the hepatic cord is unclear, slight blood stasis is observed. D: The 40 mg/kg GLP treatment group showed a thicker wall in the central vein. More blood cells were observed in the hepatic sinusoids, as well as widening of the hepatic sinusoids and obvious necrosis of local hepatocytes. The phenomenon of blood stasis was obvious.

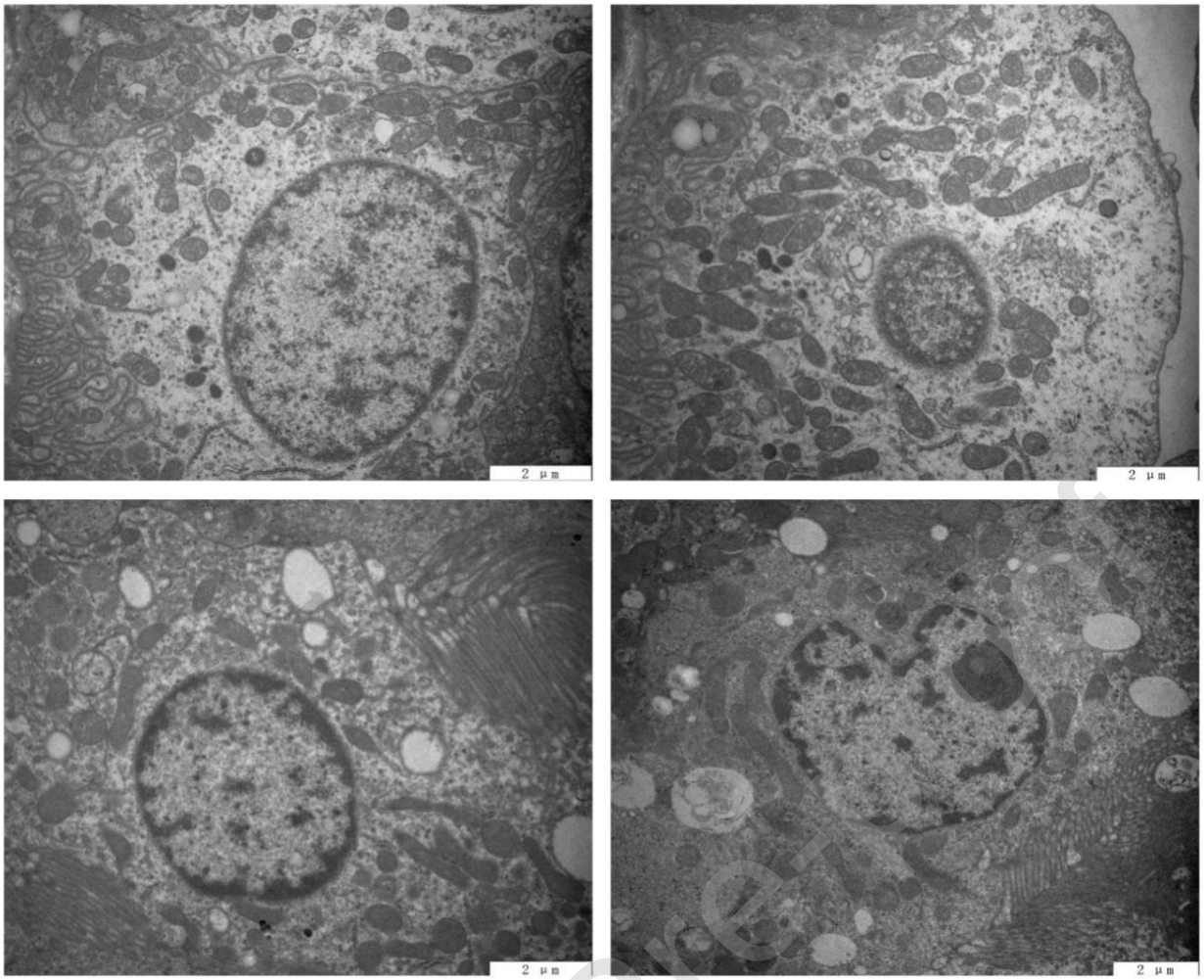


Fig. 2 Effects of different GLP diet levels on the ultrastructure of liver in piglets. A: In the control group, the nuclear membrane of hepatocytes was smooth and the mitochondrial ridge was obvious. B: In the 10 mg/kg GLP treatment group, the nucleus of liver cells was smaller and the nuclear membrane was blurred, while the mitochondrial ridge was more obvious. C: In the 20 mg/kg GLP treatment group, the chromatin of hepatocytes was dispersed and fat granules began to appear. Fibrosis began to appear in hepatocytes. D: In the 40 mg/kg GLP treatment group, the morphology of the hepatocyte nucleus was abnormal and the mitochondrial membrane was unclear. Endoplasmic reticulum ribosome exfoliation and liver fibrosis were serious, and fat granules became larger.

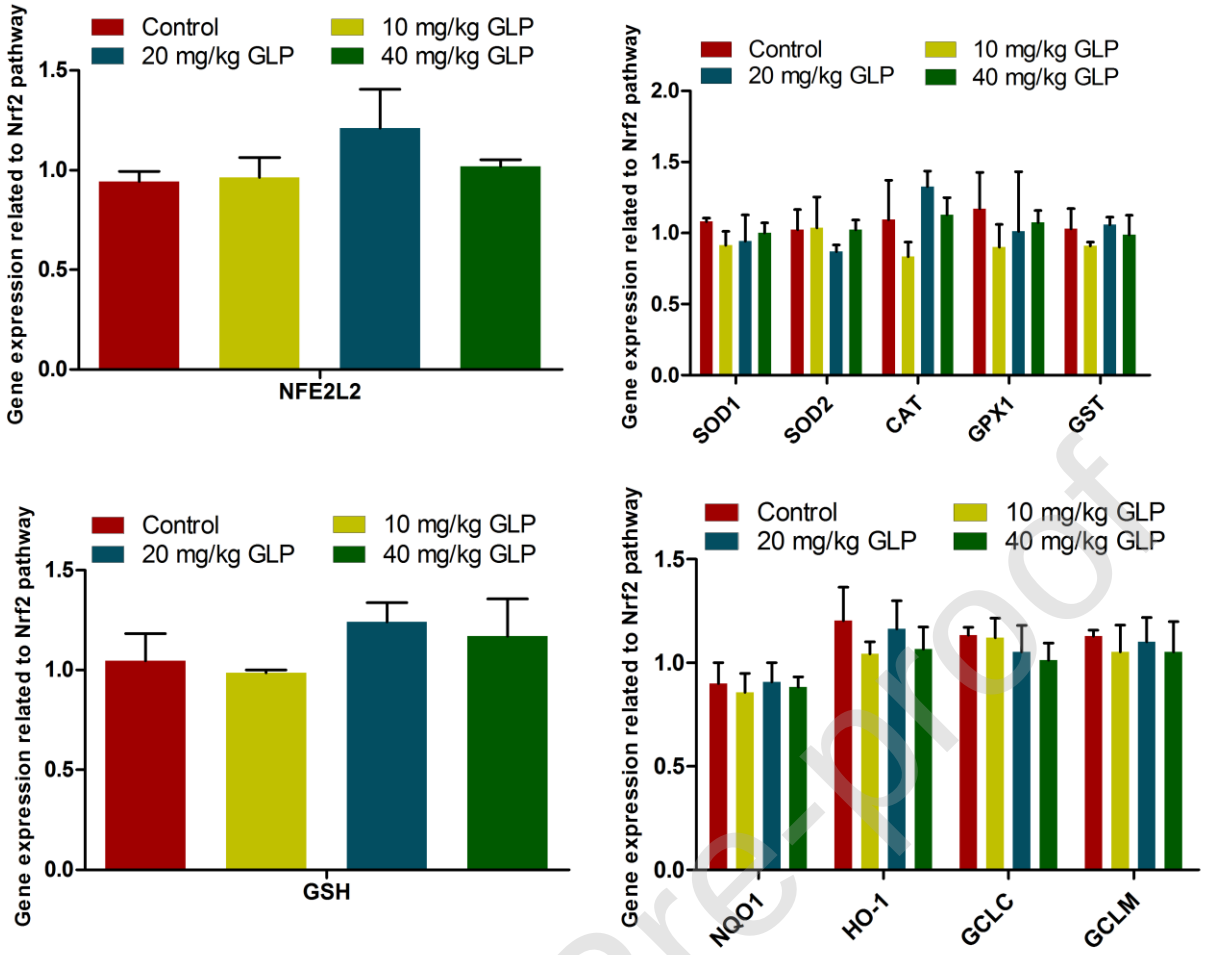


Fig. 3 Gene expression related to the Keap1-Nrf2 antioxidant signaling pathway. Four images were used to determine the gene expression levels of nuclear transcription factor (NFE2L2), the enzymatic antioxidant system (SOD1, SOD2, CAT, GPX1, GST), the nonenzymatic antioxidant system (GSH) and phase Ⅱ detoxification enzymes (NQO1, HO-1, GCLC, GCLM) in the Keap1-Nrf2 pathway. Bars not sharing the same letters are significantly different ($P < 0.05$) from each other. The data are expressed as the mean ± SD (n = 5).

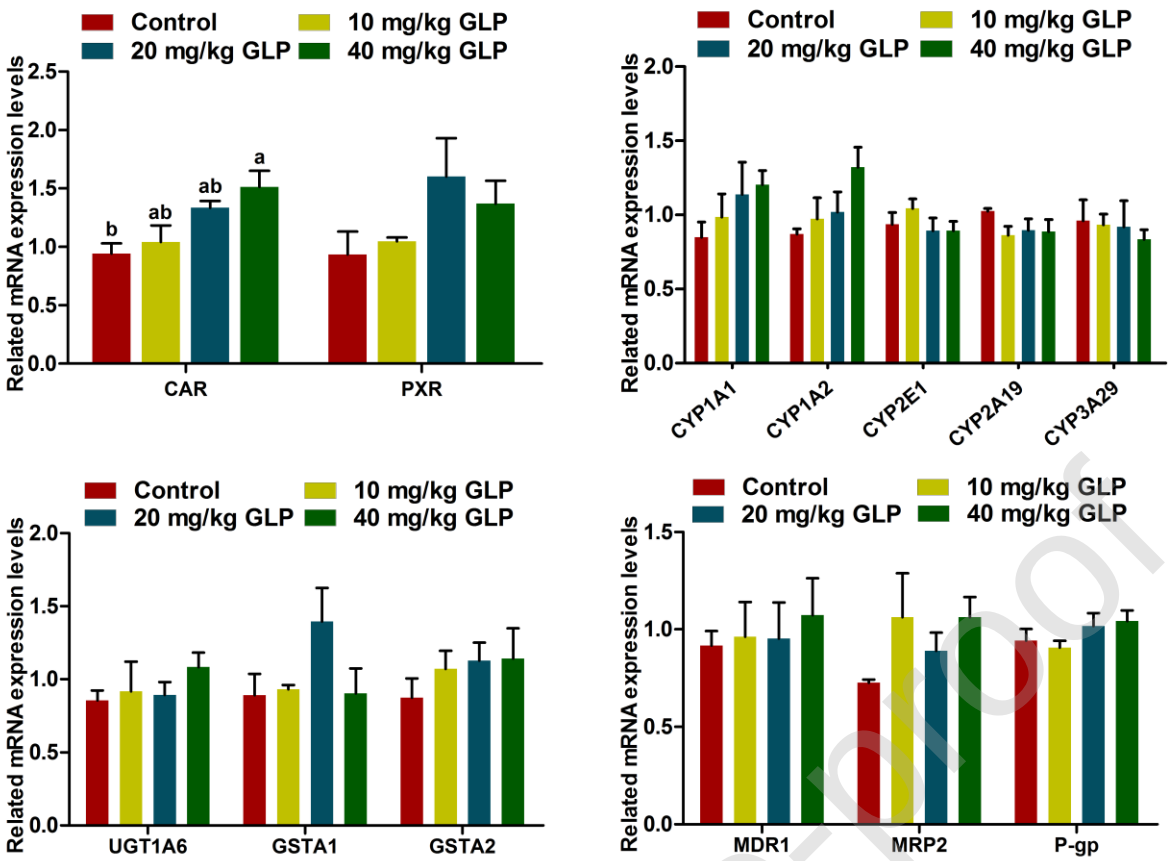


Fig. 4 Gene expression related to the nuclear receptor CAR/PXR signaling pathway. Four pictures were taken to determine the gene expression of liver nuclear receptor (CAR, PAR), phase Ⅰ metabolizing enzyme (CYP1A1, CYP1A2, CYP2E1, CYP2A19, CYP3A29), phase Ⅱ metabolizing enzyme (UGT1A6, GSTA1, GSTA2) and transporter (MDR1, MRP2, P-gp) respectively. Bars not sharing the same letters are significantly different ($P < 0.05$) from each other. The data are expressed as the mean ± SD (n = 5).

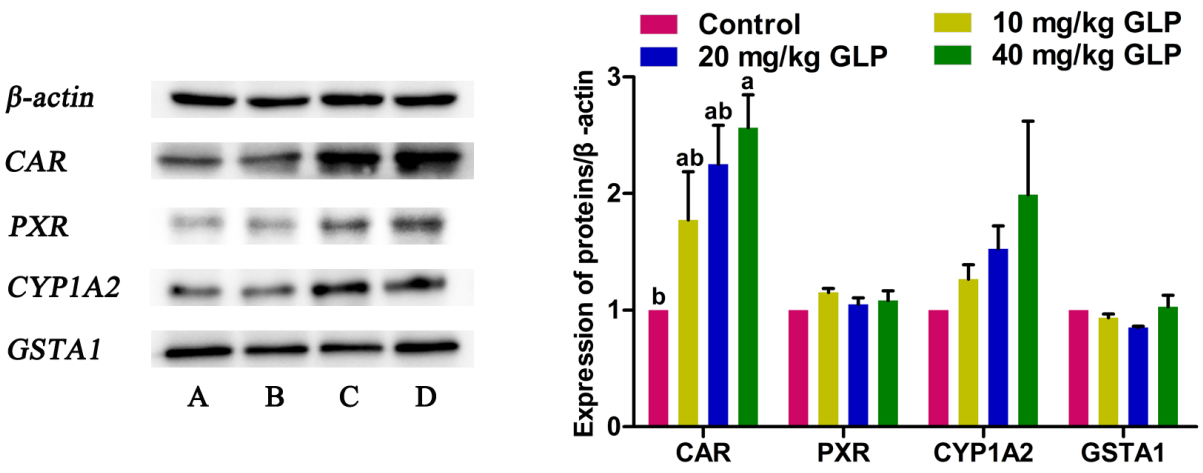


Fig. 5 Analysis of the protein expression levels of nuclear receptor pathway-related genes under different levels of GLP. The relative expression levels of enzymes were expressed as the ratio of band intensities of the enzymes to β-actin. A: The control group; B: The 10 mg/kg GLP treatment group; C: The 20 mg/kg GLP treatment group; D: The 40 mg/kg GLP treatment group. [a,b] Mean values with different superscripts for each enzyme among all four groups differ significantly ($P < 0.05$).

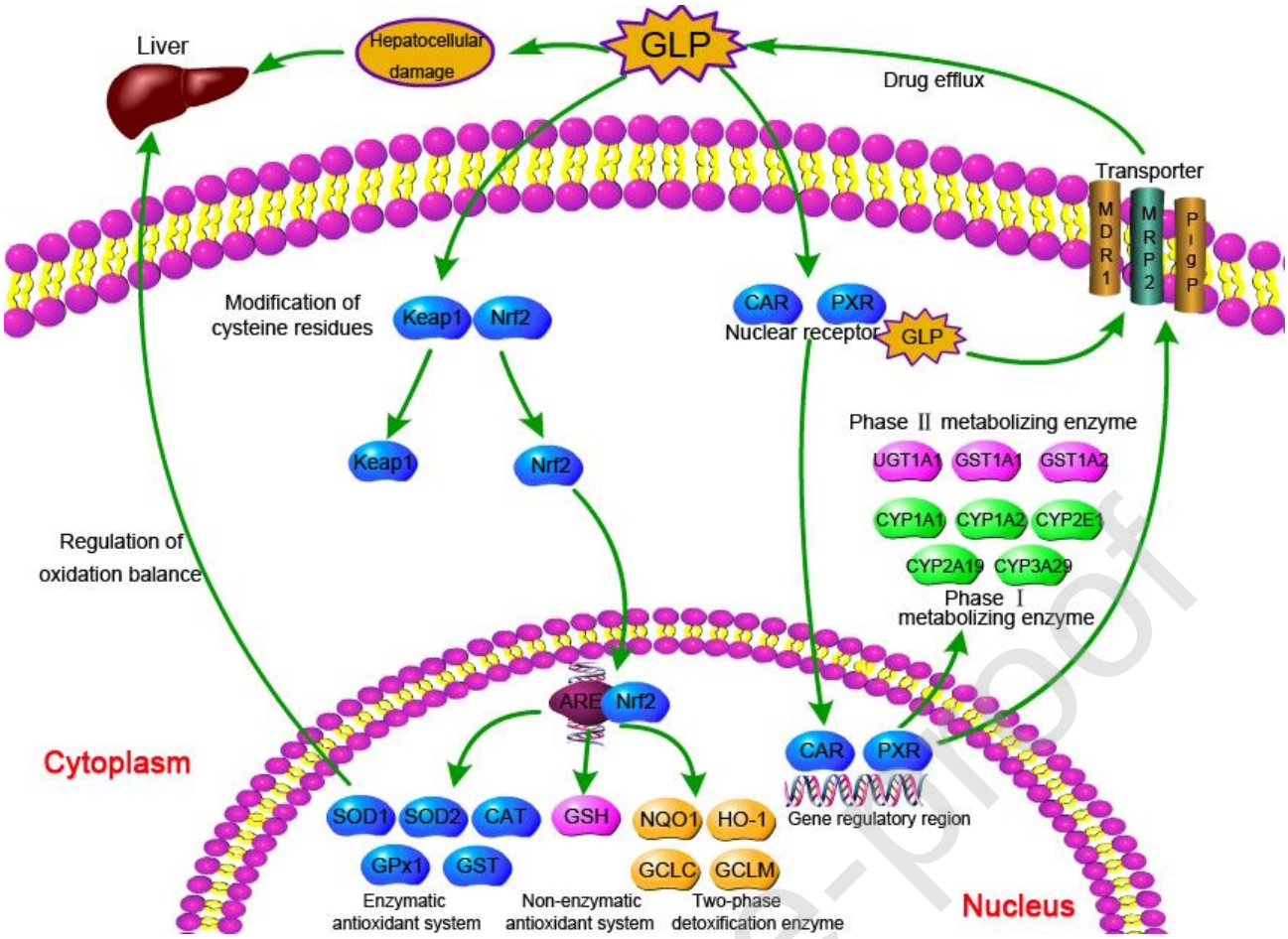


Fig. 6 The picture shows the self-detoxification mechanism of GLP through the CAR/PXR signaling pathway to regulate the expression of downstream phase Ⅰ metabolizing enzymes (CYP1A1, CYP1A2, CYP2E1, CYP2A19, CYP3A29), phase Ⅱ metabolizing enzymes (UGT1A6, GSTA1, GSTA2) and transporters (MDR1, MRP2, P-gp), as well as the antioxidant damage mechanism of downstream antioxidant enzymes in the Keap1/Nrf2 pathway.