Article

Mitigation of Aflatoxin B_1 Hepatoxicity by Dietary *Hedyotis diffusa* Is Associated with Activation of NRF2/ARE Signaling in Chicks

Ling Zhao [1], Jiang Deng [1], Zi-Jian Xu [1], Wan-Po Zhang [2], Mahmoud Mohamed Khalil [3], Niel Alexander Karrow [4] and Lv-Hui Sun [1,*]

[1] Department of Animal Nutrition and Feed Science, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan 430070, China; zling@mail.hzau.edu.cn (L.Z.); JiangDeng@webmail.hzau.edu.cn (J.D.); zijianxu@webmail.hzau.edu.cn (Z.-J.X.)

[2] Department of Veterinary Pathology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China; zwp@mail.hzau.edu.cn

[3] Animal Production Department, Faculty of Agriculture, Benha University, Cairo 13736, Egypt; mahmoud.khalil@fagr.bu.edu.eg

[4] Department of Animal Biosciences, University of Guelph, Guelph, ON N1G2W1, Canada; nkarrow@uoguelph.ca

* Correspondence: lvhuisun@mail.hzau.edu.cn; Tel./Fax :+86-027-87282091

Abstract: The objective of this study was to explore the mechanism of *Hedyotis diffusa* (HD) in mediating the detoxification of aflatoxin B_1 (AFB$_1$)-induced hepatic injury in chicks. A total of 144 one-day-old male broilers (Cobb 500) were randomly assigned to four treatment groups (n = 6 cages/diet, 6 chicks/cage). After three days of acclimation, the broilers were fed either a control diet (Control), Control plus 0.5 mg/kg of AFB$_1$, or Control plus 0.5 mg/kg AFB$_1$ with 500 or 1000 mg/kg HD for two weeks. Both serum and liver were collected at the end of the feeding trial for biochemistry, histology, and NF-E2-related nuclear factor 2 (NRF2)/antioxidant response element (ARE) signaling analysis. Compared with Control, the AFB$_1$ treatment caused liver injury and decreased ($p < 0.05$) body weight gain, feed intake, feed conversion ratio, and serum albumin and total protein by 6.2–20.7%. AFB$_1$ also induced swelling, necrosis, and severe vacuolar degeneration in chicks' livers. Notably, HD supplementation at 500 and 1000 mg/kg mitigated ($p < 0.05$) the alterations induced by AFB$_1$. HD supplementation alleviated ($p < 0.05$) AFB$_1$-induced impairment in hepatic glutathione peroxidase activity, protein carbonyl, and exo-AFB$_1$-8,9-epoxide (AFBO)–DNA concentrations by 57.7–100% and increased ($p < 0.05$) the activities of superoxide dismutase and catalase by 23.1–40.9% more than those of AFB$_1$ treatment alone. Furthermore, HD supplementation at the two doses upregulated ($p < 0.05$) NRF2, NAD(P)H: quinone oxidoreductase-1, heme oxygenase-1, glutathione cysteine ligase catalytic subunit, and glutathione-S transferase A2 and A3 in livers relative to the AFB$_1$ group by 0.99–3.4-fold. Overall, dietary supplementation of HD at a high dose displayed better protection effects against aflatoxicosis. In conclusion, a dietary HD supplementation at 500 and 1000 mg/kg protected broilers from AFB$_1$-induced hepatotoxicity, potentially due to the activation of NRF2/ARE signaling in the chicks.

Keywords: *Hedyotis diffusa*; aflatoxin B_1; hepatotoxicity; NRF2/ARE signaling; broilers

1. Introduction

Aflatoxins (AFs) are secondary metabolites of the fungi *Aspergillus flavus* and *Aspergillus parasiticus* that can be detected in various agricultural commodities [1,2]. Aflatoxin B_1 (AFB$_1$) is considered the most toxic variant among AFs and their metabolites. It exhibits harmful teratogenic, mutagenic, and hepatotoxic effects on both humans and livestock [3–5]. Moreover, AFB$_1$ is also classified as a Group I carcinogen [6]. The biotransformation pathways of AFB$_1$ represent the main reason for its toxicity. The bioactivation

of AFB_1 in the liver by cytochrome P450 (CYP450), a member of the phase I metabolizing enzymes, into the highly reactive exo-AFB_1-8,9-epoxide (AFBO) [5,7] forms adducts with DNA and proteins, resulting into cytotoxicity, mutations, and DNA lesions [5,8]. On the contrary, detoxification of AFBO can occur in conjunction with glutathione (GSH), forming a non-toxic adduct that can be catalyzed by glutathione-S transferases (GST), the phase II detoxification enzymes [8]. Moreover, AFB_1 also can induce the generation of reactive oxygen species (ROS), leading to oxidative stress and oxidation of DNA, proteins, and lipids [6,9,10]. Several strategies have been proposed to detoxify and deactivate these toxins in order to decrease their harmful effects on animals. Lately, the use of natural active biological compounds derived from plants has been attracting more attention. Some metabolites of herbs have been proven to be an effective alternative that can ameliorate the adverse effects of AFB_1 in broiler diets [11–13].

Hedyotis diffusa (HD), a natural medicinal plant, is composed of flavonoids, iridoids, triterpenes, anthraquinones, alkaloids, lignans, coumarins, cerebrosides, and sterols [11]. These chemical compounds provide HD with multiple biological properties including antioxidant, anti-inflammatory, antitumor chemo-preventive, proapoptotic, and anti-angiogenic effects [14,15]. Since it possesses these properties, HD has been widely applied for the treatment of inflammation-related diseases, bronchitis, urethritis, and appendicitis and has been proposed as a potential therapy for liver, breast, lung, colon, and pancreatic cancers [14–19].

Interestingly, HD has shown the ability to protect against AFB_1-induced mutation and carcinoma by inhibiting CYP450-mediated bioactivation of AFB_1 to toxic AFBO [20,21]. Numerous natural compounds protect against toxicity of xenobiotics through regulating NF-E2-related nuclear factor 2 (NRF2)/antioxidant response element (ARE) pathway [22]. NRF2 binds to ARE, which promotes the transcriptional expression of phase II-detoxifying enzymes and antioxidant genes, such as GST, superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutathione cysteine ligase catalytic subunit (GCLC), heme oxygenase-1 (HO1), and NAD(P)H: quinone oxidoreductase-1 (NQO1) [23–25]. To our knowledge, no information is available about HD's potential to influence NRF2/ARE signaling in the context of AFB_1-induced hepatoxicity. Therefore, the present study was conducted to investigate whether the dietary supplementation of HD has any effect in mitigating AFB_1-induced hepatotoxic effects through the regulation of the NRF2/ARE signaling pathway in chicks.

2. Materials and Methods

2.1. Birds, Dietary Treatments, and Sample Collections

The animal trial was conducted following the protocol approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University, China. A total of 144 one-day-old male broiler chickens (Cobb 500) were randomly assigned to 4 experimental groups, with 6 replicates of 6 chicks/cage. Chicks were fed a corn/soybean-based diet (BD) formulated to meet the nutritional requirements of broilers (Supplemental Table S1) [10] with unlimited access to water. After 3 days of adaptation, chicks in the four experimental groups were fed either the BD diet (Control), Control plus 0.5 mg AFB_1/kg (AFB_1), Control plus 0.5 mg AFB_1/kg and 500 mg HD/kg (AFB_1 + 500HD), or Control plus 0.5 mg AFB_1/kg and 1000 mg HD/kg (AFB_1 + 1000HD). The HD was added as the form of the extract of HD plant, which was prepared as previously described [26]. Briefly, the dried whole plant (1000 g) of HD was extracted with 70% ethanol at room temperature for 2 days and repeated three times. Then, it was vacuum dried and given 81.5 g of HD extract. The AFB_1 was bought from Sigma Chemical Co. (St. Louis, MO, USA). The experimental duration was chosen on the basis of previous studies that reported that dietary consumption of >100 µg AFB_1/kg $\geq$ 2 weeks induced hepatotoxicity in broilers [10,27]. The experiment lasted for 2 weeks, and body weights and feed intake were measured weekly. At the end of the feeding trial, 1 broiler from each replicate (thus, 6 broilers from each treatment group) were slaughtered for blood and liver sample collection for serologic and liver histologic

examination, as previously described [10]. The liver samples were washed with ice-cold isotonic saline, divided into aliquots, snap-frozen in liquid nitrogen, and stored at $-80\,^\circ$C until use [28].

2.2. Serum Biochemical and Histologic Analysis

The serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and the concentrations of total bilirubin (TBIL), total protein (TP), and albumin (ALB) were measured by an automatic biochemistry analyzer (Beckman Synchron CX4 PRO). The liver tissues were examined microscopically after being fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin [29].

2.3. Hepatic Antioxidant Parameters and AFBO–DNA Adduct Concentrations Analysis

The activities of SOD, GPX, and CAT, along with concentrations of malondialdehyde (MDA) and protein carbonyl (PC), were measured by specific assay kits (A001, A005, A007–1, A003, and A087–1–2) purchased from the Nanjing Jiancheng Bioengineering Institute of China. Protein concentrations were measured by the bicinchoninic acid assay. The hepatic AFBO–DNA adduct concentrations were analyzed as previously described [10]. Briefly, hepatic genomic DNA was extracted by a DNA isolation kit (Tiangen Biotech Co., Ltd., Beijing, China). Then, approximately 15 μg of the genomic DNA was used to determine the AFBO–DNA adduct concentration using an AFBO–DNA adduct competitive ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA).

2.4. Real-Time qPCR and Western Blot Analyses

Real-time q-PCR analyses of the pertaining samples were conducted as previously described [10]. Primers (Supplemental Table S2) for the NRF2/ARE signaling-related genes and the reference gene β-actin were designed using Primer Express 3.0 (Applied Biosystems). The $2^{-\text{ddCt}}$ method was used for the quantification with β-actin as a reference gene, and the relative abundance was normalized to the Control group. Western blot analyses of the pertaining samples were performed as previously described [30]. The primary antibodies used for each gene product are presented in Supplementary Table S3. Protein concentrations were measured by the bicinchoninic acid assay.

2.5. Statistical Analysis

The data were analyzed by one-way ANOVA using SPSS statistics software (version 19, IBM) with the significance level declared at $p < 0.05$, and the Tukey–Kramer method was used for multiple mean comparisons. The data are presented as means $\pm$ standard errors (SE).

3. Results

3.1. Growth Performance, Serum Biochemistry, and Liver Histology

No differences ($p \geq 0.05$) were observed for the initial body weight of broilers among the four groups (Table 1). After two weeks of experimental treatments, AFB$_1$ decreased ($p < 0.05$) the final body weight, body weight gain, feed intake, and gain/feed efficiency of chicks by 6.2–12.3% (Table 1). These changes induced by AFB$_1$ were significantly mitigated ($p < 0.05$) by HD supplementation at 500 and 1000 mg/kg. Furthermore, although AFB$_1$ did not alter ($p \geq 0.05$) serum ALT and AST activities and TBIL concentration, it decreased ($p < 0.05$) ALB and TP concentrations by 16.6–20.7% relative to the Control (Table 2). Interestingly, dietary supplementation with HD at 500 and 1000 mg/kg alleviated ($p < 0.05$) AFB$_1$-induced changes on these biochemistry variables. Moreover, the histological results indicated that AFB$_1$ induced liver damage including swelling, necrosis, severe vacuolar degeneration, and bile duct hyperplasia (Figure 1). However, the hepatic injury induced by AFB$_1$ was alleviated by dietary HD supplementation in a dose-dependent manner.

Table 1. Effects of dietary aflatoxin B_1 and HD on growth performances of broilers [1].

	Control	AFB_1	AFB_1 + 500HD	AFB_1 + 1000HD
Initial Body Weight, g	69.4 ± 0.1	69.2 ± 0.1	69.3 ± 0.1	69.4 ± 0.2
Final Body Weight, g	647 ± 8.3 [c]	575 ± 12.5 [a]	592 ± 17.4 [ab]	619 ± 8.8 [b]
Body Weight Gain, g	577 ± 8.3 [c]	506 ± 12.5 [a]	523 ± 17.4 [ab]	550 ± 8.8 [b]
Feed Intake, g	676 ± 9.8 [b]	632 ± 12.9 [a]	636 ± 16.7 [ab]	662 ± 8.8 [ab]
Gain/Feed, g/kg	854 ± 9 [b]	801 ± 24 [a]	822 ± 17 [ab]	831 ± 5 [a]

[1] Values are means ± SEs, *n* = 6. Means in a row not sharing a common letter are significantly different, where $p < 0.05$. HD, *Hedyotis diffusa*; Control, base diet; AFB_1, base diet supplement with 0.5 mg/kg AFB_1; AFB_1 + 500HD, base diet supplement with 0.5 mg/kg AFB_1 plus 500 mg/kg HD; AFB_1 + 1000 HD, base diet supplement with 0.5 mg/kg AFB_1 plus 1000 mg/kg HD.

Table 2. Effects of dietary aflatoxin B1 and HD on serum biochemistry of broilers [1].

	Control	AFB_1	AFB_1 + 500HD	AFB_1 + 1000HD
ALT, U/L	3.40 ± 0.43	2.73 ± 0.55	3.79 ± 0.56	3.23 ± 0.72
AST, U/L	268 ± 18.7	270 ± 27.4	230 ± 5.8	243 ± 21.7
TBIL, μmol/L	3.64 ± 0.06	3.65 ± 0.08	3.70 ± 0.15	3.75 ± 0.2
ALB, g/L	16.3 ± 0.5 [b]	14.1 ± 0.9 [a]	17.6 ± 1.7 [ab]	16.6 ± 0.5 [b]
TP, g/L	26.1 ± 1.2 [b]	20.7 ± 3.0 [a]	23.8 ± 2.0 [ab]	37.5 ± 5.0 [c]

[1] Values are means ± SEs, *n* = 6. Means in a row not sharing a common letter are significantly different, where $p < 0.05$. ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HD, *Hedyotis diffusa*; TBIL, total bilirubin; TP, total protein; Control, base diet; AFB1, base diet supplement with 0.5 mg/kg AFB1; AFB1+500HD, base diet supplement with 0.5 mg/kg AFB1 plus 500 mg/kg HD; AFB1+1000HD, base diet supplement with 0.5 mg/kg AFB1 plus 1000 mg/kg HD.
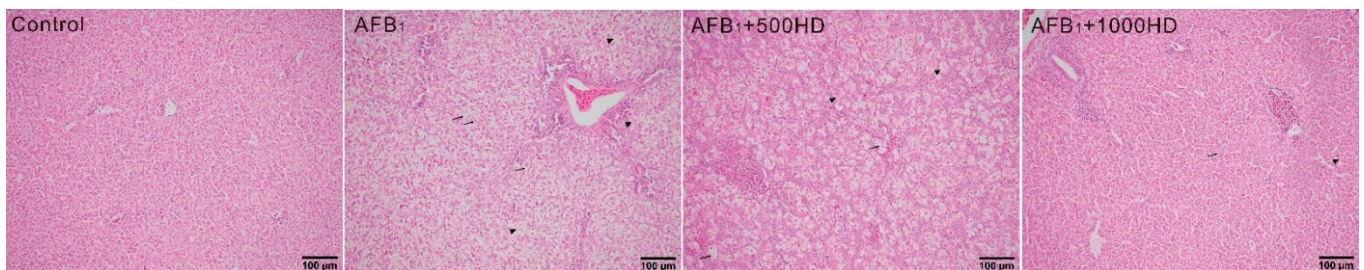

Figure 1. Influence of dietary AFB_1 and HD on the histopathology of livers of chicks. The liver sections were stained with hematoxylin and eosin; Photo-micrographs are shown at 200× magnification. Here, arrows indicate swelling and necrosis; arrowheads indicate vacuolar degeneration. Control, base diet; AFB_1, base diet supplement with 0.5 mg/kg AFB_1; AFB_1 + 500HD, base diet supplement with 0.5 mg/kg AFB_1 plus 500 mg/kg HD; AFB_1 + 1000HD, base diet supplement with 0.5 mg/kg AFB_1 plus 1000 mg/kg HD.

3.2. Hepatic Antioxidant Variables and AFBO–DNA Adduct Concentration

Birds exposed to AFB_1 treatment for two weeks showed significant alterations in the hepatic antioxidant variables, as presented in Table 3. Compared to the Control, AFB_1 decreased ($p < 0.05$) the activity of GPX (19.8%) but increased the concentration of PC (33.4%) in the liver of broilers. Notably, these changes induced by AFB_1 were significantly mitigated ($p < 0.05$) by the dietary supplementation of HD at 500 and 1000 mg/kg. Interestingly, the activities of SOD and CAT in the liver were increased ($p < 0.05$) by 23.1–40.9% in the AFB_1 + 1000HD group compared to the AFB_1 group. However, the concentration of MDA in the liver was not affected ($p \geq 0.05$) by AFB_1 or HD. Additionally, AFB_1 increased ($p < 0.05$) the concentration of AFBO–DNA adducts by 11-fold in the liver compared with the Control (Figure 2). Notably, dietary supplementation with HD at 500 and 1000 mg/kg reduced ($p < 0.05$) the concentration of AFBO–DNA adducts by 51.0–62.7% in comparison to the AFB_1-supplemented group.

Table 3. Effects of dietary aflatoxin B_1 and HD on hepatic redox status of broilers [1].

	Control	AFB$_1$	AFB$_1$ + 500HD	AFB$_1$ + 1000HD
SOD, U/mg Protein	88.7 ± 12.1 [ab]	83.7 ± 7.1 [a]	94.5 ± 12.4 [ab]	103 ± 17.5 [b]
GPX, U/mg Protein	26.2 ± 4.8 [b]	21.0 ± 2.1 [a]	24.0 ± 5.3 [ab]	30.6 ± 7.8 [b]
CAT, U/mg Protein	28.4 ± 5.1 [ab]	25.9 ± 3.0 [a]	27.5 ± 5.4 [ab]	36.5 ± 9.9 [b]
MDA, nmol/mg Protein	1.15 ± 0.26	1.01 ± 0.24	1.18 ± 0.22	1.25 ± 0.32
PC, nmol/mg Protein	6.19 ± 1.04 [a]	8.26 ± 2.09 [b]	6.08 ± 0.92 [a]	5.58 ± 0.18 [a]

[1] Values are means ± SEs, n = 6. Labeled means in a row without a common letter differ, where $p < 0.05$. HD, *Hedyotis diffusa*; SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde; PC, protein carbonyl; Control, base diet; AFB1, base diet supplement with 0.5 mg/kg AFB1; AFB1+500 HD, base diet supplement with 0.5 mg/kg AFB1 plus 500 mg/kg HD; AFB1+1000HD, base diet supplement with 0.5 mg/kg AFB1 plus 1000 mg/kg HD.
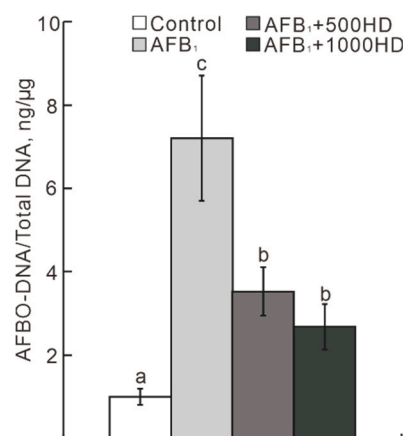

Figure 2. Effects of dietary AFB$_1$ and HD on the concentrations of AFBO–DNA adducts in the liver of chicks. Values are means ± SEs, n = 6. Means for a given gene not sharing a common letter are significantly different, where $p < 0.05$. AFBO, exo-AFB1-8,9-epoxide; Control, base diet; AFB$_1$, base diet supplement with 0.5 mg/kg AFB$_1$; AFB$_1$ + 500HD, base diet supplement with 0.5 mg/kg AFB$_1$ plus 500 mg/kg HD; AFB$_1$ + 1000 HD, base diet supplement with 0.5 mg/kg AFB$_1$ plus 1000 mg/kg HD.

3.3. Expression of the NRF2/ARE Signaling-Related Genes

Among the six NRF2/ARE signaling-related genes examined in the liver of chicks, three genes were influenced by AFB$_1$ and HD treatment (Figure 3). Specifically, AFB$_1$ upregulated ($p < 0.05$) the mRNA levels of HO1 and downregulated ($p < 0.05$) the mRNA level of *GSTA2*. Interestingly, dietary supplementation of HD at 500 and 1000 mg/kg increased ($p < 0.05$) the mRNA levels of HO1, GSTA1, and GSTA3 compared to the AFB$_1$ treatment group. Moreover, Western blot results showed that the hepatic protein levels of NRF2, NQO1, HO1, GCLC, GSTA2, and GSTA3 were affected by AFB$_1$ and HD treatment (Figure 4). Specifically, AFB$_1$ downregulated ($p < 0.05$) the protein levels of NRF2, NQO1, HO1, GSTA2, and GSTA3 in the liver of broilers. Notably, these changes induced by AFB$_1$ were significantly mitigated ($p < 0.05$) by the dietary supplementation of HD at 500 and 1000 mg/kg. Strikingly, the protein levels of GSTA3 and GCLC were much higher ($p < 0.05$) in the AFB$_1$ + 500HD and AFB$_1$ + 1000HD groups compared to the Control and AFB$_1$ groups.
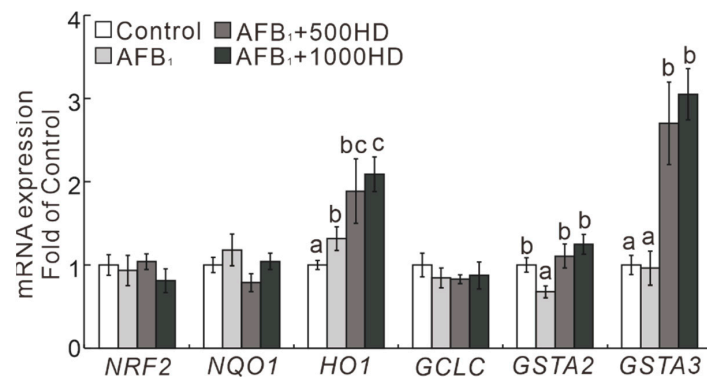
Figure 3. Effects of dietary AFB_1 and HP on the relative mRNA abundances of NRF2–ARE signaling in the livers of chicks. Values are means $\pm$ SEs, $n = 6$. Means for a given gene not sharing a common letter are significantly different, where $p < 0.05$. *NRF2*, NF-E2-related nuclear factor 2; *NQO1*, NAD(P) H: quinone oxidoreductase-1; *HO1*, heme oxygenase-1; *GCLC*, glutathione cysteine ligase catalytic subunit; *GST*, glutathione-S transferase. Control, base diet; AFB_1, base diet supplement with 0.5 mg/kg AFB_1; AFB_1 + 500HD, base diet supplement with 0.5 mg/kg AFB_1 plus 500 mg/kg HD; AFB_1 + 1000HD, base diet supplement with 0.5 mg/kg AFB_1 plus 1000 mg/kg HD.
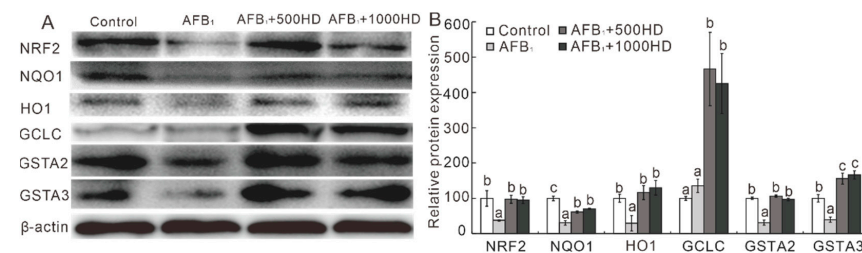

Figure 4. Effects of dietary AFB_1 and HP on the relative protein abundances of NRF2–ARE signaling in the livers of chicks. For a given protein, the band was a representative image of 3–5 independent analyses (**A**), and the value shows the relative density of protein bands (mean $\pm$ SEs, $n = 3$–5; (**B**)). Means for a given protein not sharing a common letter are significantly different, where $p < 0.05$. NRF2, NF-E2-related nuclear factor 2; NQO1, NAD(P) H: quinone oxidoreductase-1; HO1, heme oxygenase-1; GCLC, glutathione cysteine ligase catalytic subunit; GST, glutathione-S transferase; Control, base diet; AFB_1, base diet supplement with 0.5 mg/kg AFB_1; AFB_1 + 500HD, base diet supplement with 0.5 mg/kg AFB_1 plus 500 mg/kg HD; AFB_1 + 1000HD, base diet supplement with 0.5 mg/kg AFB_1 plus 1000 mg/kg HD.

4. Discussion

The current study showed that HD was able to mitigate hepatotoxicity induced by AFB_1 in broilers. Birds that consumed AFB_1 diets exhibited a reduction in final body weight, body weight gain, feed intake, and gain/feed of broilers, all of which remain in agreement with previous studies [13,31,32]. This poor growth performance could be related to anorexia and inhibition of lipogenesis and protein synthesis [32,33]. Meanwhile, AFB_1 also induced the typical clinical and pathological signs of hepatic injury, including decreased concentrations of ALB and TP in serum as well as swelling, necrosis, severe vacuolar degeneration, and bile duct hyperplasia in the liver of broilers and Japanese quail [29,31,34]. Intriguingly, dietary supplementation of HD at 500 and 1000 mg/kg alleviated AFB_1-induced toxic effects on performance, serum biochemistry, and hepatic histopathology. Notably, dietary supplementation with HD at the 1000 mg/kg displayed better mitigation based on these parameters than 500 mg/kg. These findings are similar to previous studies that showed that dietary supplementation of HD can mitigate AFB_1-induced growth retardation and hepatic damage [20,21].

AFB_1 dysregulation of the response of hepatic redox parameters (GPX and PC) indicated that the chicks exposed to AFB_1 suffered from oxidative stress in the present study.

Specifically, AFB$_1$ reduced the antioxidant enzyme GPX activity and induced the protein oxidation maker PC [10,27,32]. These outcomes are similar to previous studies [10,27,32], which explained that AFB$_1$ can induce oxidative stress and results in proteins, lipids and DNA oxidation, thereby causing cytotoxicity and genotoxicity [9,10,32]. Interestingly, dietary supplementation with HD at 500 mg/kg mitigated AFB$_1$-induced changes in these redox parameters in the liver. Moreover, dietary supplementation of HD at 1000 mg/kg even increased the hepatic antioxidant enzymes activities of SOD, GPX, and CAT compared to levels in the AFB$_1$ groups. Taken together, these outcomes indicate that HD could alleviate AFB$_1$-induced oxidative stress in the liver of chicks. These findings are similar to previous studies, which have reported that HD could detoxify other xenobiotic-induced oxidative stress both in vivo and in vitro [16,35,36].

A novel finding from the current study is that the alleviation of AFB$_1$-induced hepatotoxicity by HD was associated with the activation of NRF2/ARE signaling in broilers. Although the mRNA of NRF2 was not affected by AFB$_1$ treatment, the NRF2 protein was upregulated by the HD treatment in the liver of broilers. As the NRF2-targeted antioxidative genes, NQO1 and HO1 played important roles in the protection against oxidative stress [25,37,38]. AFB$_1$ sharply downregulated approximately 70% abundances of these two proteins, which might promote AFB$_1$-induced oxidative injury in the liver of broilers. These findings are similar to previous reports [39,40]. Intriguingly, dietary supplementation of HD at 500 and 1000 mg/kg mitigated AFB$_1$-induced downregulation of NQO1 and HO1 proteins, which may be attributed to reduced oxidative injury in livers induced by AFB$_1$. Moreover, the phase II metabolizing enzymes GSTA2, GSTA3, and GCLC, which play roles in the detoxification of AFB$_1$ by catalyzing the conjugation of toxic AFBO with GSH [8,10,27,41], can be transcriptional activated by NRF2 [23–25]. Similar to previous studies, AFB$_1$ decreased the mRNA and protein levels of GSTA2 and GSTA3 in the liver, which may be contributed to its induced hepatic injury [8,42–44]. Notably, dietary supplementation with HD at 500 and 1000 mg/kg inhibited the AFB$_1$-induced reduction of GSTA2 and GSTA3 proteins and increased the GCLC protein, which could help to ameliorate the AFB$_1$-induced hepatotoxicity of chicks. Indeed, as the primary toxic adduct of AFBO [10,45], AFBO–DNA was sharply decreased in the liver of broilers exposed to AFB$_1$ by the HD dietary supplementation. These results indicate that the protective effects of HD against AFB$_1$ toxicity are potentially mediated by activating the pivotal GSTs isozymes that promote the detoxification of the highly toxic AFBO.

Paradoxically, the abundance of hepatic proteins NRF2, NQO1, HO1, and GCLC did not correlate well with their mRNA abundance. Additionally, as the target genes of NRF2, the mRNA levels of NQO1, HO1, and GCLC also did not change as well as expected. These divergences may be attributed to complex feedback or posttranscriptional mechanisms regulating the synthesis of these proteins [28,32].

5. Conclusions

In summary, the present study has illustrated that dietary HD supplementation could mitigate poor growth performance and hepatic injury induced by AFB$_1$ in broilers, as illustrated by the amelioration of the changes in serum biochemistry and histopathologic lesions. The protective mechanism of HD against AFB$_1$-induced hepatoxicity is associated with the activation of NRF2/ARE signaling in broiler chicks. Specifically, (1) HD could mitigate AFB$_1$-induced oxidative stress by increasing the expression of the proteins with antioxidant capacities and (2) HD could effectively promote the expression of phase II detoxification enzymes, which play crucial roles in the detoxification of AFB$_1$ by catalyzing the conjugation of highly toxic AFBO with GSH. These findings support the use of HD to prevent aflatoxicosis in chicks.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10 .3390/antiox10060878/s1: Table S1: Basal diet formulation and nutritional values; Table S2: List of primers used for Q-PCR analysis; Table S3: Name, type, dilution, and source of primary antibodies.

References

1. Koehler, P.E.; Hanlin, R.T.; Beraha, L. Production of aflatoxins B_1 and G_1 by *Aspergillus flavus* and *Aspergillus parasiticus* isolated from market pecans. *Appl. Microbiol.* **1975**, *30*, 581–583. [CrossRef] [PubMed]
2. Sun, L.H.; Lei, M.Y.; Zhang, N.Y.; Zhao, L.; Krumm, C.S.; Qi, D.S. Hepatotoxic effects of mycotoxin combinations in mice. *Food Chem. Toxicol.* **2014**, *74*, 289–293. [CrossRef]
3. International Agency for Research on Cancer (IARC). *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans; Overall Evaluation of Carcinogenicity: An Updating of IARC Monographs*; IARC: Lyon, France, 1987.
4. Guengerich, F.P.; Johnson, W.W.; Shimada, T.; Ueng, Y.F.; Yamazaki, H.; Langouët, S. Activation and detoxication of aflatoxin B_1. *Mutat. Res.* **1998**, *402*, 121–128. [CrossRef]
5. Yunus, A.W.; Razzazi-Fazeli, E.; Bohm, J. Aflatoxin B_1 in affecting broiler's performance, immunity, and gastrointestinal tract: A review of history and contemporary issues. *Toxins* **2011**, *3*, 566–590. [CrossRef] [PubMed]
6. Sun, L.H.; Lei, M.Y.; Zhang, N.Y.; Gao, X.; Li, C.; Krumm, C.S.; Qi, D.S. Individual and combined cytotoxic effects of aflatoxin B1, zearalenone, deoxynivalenol and fumonisin B_1 on BRL 3A rat liver cells. *Toxicon* **2015**, *95*, 6–12. [CrossRef] [PubMed]
7. Hussein, H.S.; Brasel, J.M. Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology* **2001**, *167*, 101–134. [CrossRef]
8. Deng, J.; Zhao, L.; Zhang, N.Y.; Karrow, N.A.; Krumm, C.S.; Qi, D.S.; Sun, L.H. Aflatoxin B_1 metabolism: Regulation by phase I and II metabolizing enzymes and chemoprotective agents. *Mutat. Res.* **2018**, *778*, 79–89. [CrossRef] [PubMed]
9. Mary, V.S.; Theumer, M.G.; Arias, S.L.; Rubinstein, H.R. Reactive oxygen species sources and biomolecular oxidative damage induced by aflatoxin B_1 and fumonisin B_1 in rat spleen mononuclear cells. *Toxicology* **2012**, *302*, 299–307. [CrossRef]
10. Sun, L.H.; Zhang, N.Y.; Zhu, M.K.; Zhao, L.; Zhou, J.C.; Qi, D.S. Prevention of Aflatoxin B1 Hepatoxicity by Dietary Selenium Is Associated with Inhibition of Cytochrome P450 Isozymes and Up-Regulation of 6 Selenoprotein Genes in Chick Liver. *J. Nutr.* **2016**, *146*, 655–661. [CrossRef] [PubMed]
11. Abidin, Z.; Khatoon, A.; Numan, M. Mycotoxins in broilers: Pathological alterations induced by aflatoxins and ochratoxins, diagnosis and determination, treatment and control of mycotoxicosis. *Worlds Poult. Sci. J.* **2011**, *67*, 485–496. [CrossRef]
12. Manafi, M.; Heydari, M.; Yari, M. Aflatoxicosis and herbal detoxification: The effectiveness of thyme essence on performance parameters and antibody titers of commercial broilers fed aflatoxin B_1. *Res. Zool.* **2014**, *4*, 43–50.
13. Nazarizadeh, H.; Hosseini, S.M.; Pourreza, J. Effect of plant extracts derived from thyme and chamomile on the growth performance, gut morphology and immune system of broilers fed aflatoxin B_1 and ochratoxin A contaminated diets. *Ital. J. Anim. Sci.* **2019**, *18*, 1073–1081. [CrossRef]
14. Wang, C.; Zhou, X.; Wang, Y.; Wei, D.; Deng, C.; Xu, X.; Xin, P.; Sun, S. The Antitumor Constituents from Hedyotis Diffusa Willd. *Molecules* **2017**, *22*, 2101. [CrossRef]
15. Gupta, S.; Zhang, D.; Yi, J.; Shao, J. Anticancer activities of Oldenlandia diffusa. *J. Herb. Pharmacother.* **2004**, *4*, 21–33. [CrossRef]
16. Ahmad, R.; Ali, A.M.; Israf, D.A.; Ismail, N.H.; Shaari, K.; Lajis, N.H. Antioxidant, radical-scavenging, anti-inflammatory, cytotoxic and antibacterial activities of methanolic extracts of some Hedyotis species. *Life Sci.* **2005**, *76*, 1953–1964. [CrossRef]
17. Lin, C.C.; Ng, L.T.; Yang, J.J.; Hsu, Y.F. Anti-inflammatory and hepatoprotective activity of peh-hue-juwa-chi-cao in male rats. *Am. J. Chin. Med.* **2002**, *30*, 225–234. [CrossRef]
18. Ren, F.; Liu, G.; Zhang, L.; Niu, G. Studies on chemical constituents of Hedyotis diffusa Willd. *Chin. Pharm. J.* **2005**, *40*, 502–504.
19. Ahmad, R.; Shaari, K.; Lajis, N.H.; Hamzah, A.S.; Ismail, N.H.; Kitajima, M. Anthraquinones from Hedyotis capitellata. *Phytochemistry* **2005**, *66*, 1141–1147. [CrossRef] [PubMed]
20. Wong, B.; Lau, B.; Yamasaki, T.; Teel, R. Inhibition of dexamethasone-induced cytochrome P450-mediated mutagenicity and metabolism of aflatoxin B_1 by Chinese medicinal herbs. *Eur. J. Cancer Prev.* **1993**, *2*, 351–356. [CrossRef]

21. Wong, B.Y.; Lau, B.H.; Tadi, P.P.; Teel, R.W. Chinese medicinal herbs modulate mutagenesis, DNA binding and metabolism of aflatoxin B$_1$. *Mutat. Res.* **1992**, *279*, 209–216. [CrossRef]

22. Yarmohammadi, F.; Rezaee, R.; Karimi, G. Natural compounds against doxorubicin-induced cardiotoxicity: A review on the involvement of Nrf2/ARE signaling pathway. *Phytother. Res.* **2021**, *35*, 1163–1175. [CrossRef]

23. Guo, Y.; Yu, S.; Zhang, C.; Kong, A.T. Epigenetic regulation of Keap1-Nrf2 signaling. *Free Radic. Biol. Med.* **2015**, *88*, 337–349. [CrossRef]

24. Lee, J.M.; Johnson, J.A. An important role of Nrf2-ARE pathway in the cellular defense mechanism. *J. Biochem. Mol. Biol.* **2004**, *37*, 139–143. [CrossRef] [PubMed]

25. Shaw, P.; Chattopadhyay, A. Nrf2-ARE signaling in cellular protection: Mechanism of action and the regulatory mechanisms. *J. Cell Physiol.* **2020**, *235*, 3119–3130. [CrossRef] [PubMed]

26. Gu, G.W.; Barone, I.; Gelsomino, L.; Giordano, C.; Bonofiglio, D.; Statti, G.; Menichini, F.; Catalano, S.; Andò, S. Oldenlandia diffusa extracts exert antiproliferative and apoptotic effects on human breast cancer cells through ERα/Sp1-mediated p53 activation. *J. Cell Physiol.* **2012**, *227*, 3363–3372. [CrossRef]

27. Zhao, L.; Deng, J.; Ma, L.B.; Zhang, W.P.; Khalil, M.M.; Karrow, N.A.; Qi, D.S.; Sun, L.H. Dietary Se deficiency dysregulates metabolic and cell death signaling in aggravating the AFB1 hepatotoxicity of chicks. *Food Chem. Toxicol.* **2021**, *149*, 111938. [CrossRef] [PubMed]

28. Zhao, L.; Sun, L.H.; Huang, J.Q.; Briens, M.; Qi, D.S.; Xu, S.W.; Lei, X.G. A novel organic selenium compound exerts unique regulation of selenium speciation, selenogenome, and selenoproteins in broiler chicks. *J. Nutr.* **2017**, *147*, 789–797. [CrossRef] [PubMed]

29. Sun, L.H.; Zhang, N.Y.; Sun, R.R.; Gao, X.; Gu, C.; Krumm, C.S.; Qi, D.S. A novel strain of Cellulosimicrobium funkei can biologically detoxify aflatoxin B$_1$ in ducklings. *Microb. Biotechnol.* **2015**, *8*, 490–498. [CrossRef]

30. Huang, J.Q.; Ren, F.Z.; Jiang, Y.Y.; Xiao, C.; Lei, X.G. Selenoproteins protect against avian nutritional muscular dystrophy by metabolizing peroxides and regulating redox/apoptotic signaling. *Free Radic. Biol. Med.* **2015**, *83*, 129–138. [CrossRef]

31. Liu, J.; Song, W.J.; Zhang, N.Y.; Tan, J.; Krumm, C.S.; Sun, L.H.; Qi, D.S. Biodetoxification of aflatoxin B$_1$ in cottonseed meal by fermentation of *Cellulosimicrobium funkei* in duckling diet. *Poult. Sci.* **2017**, *96*, 923–930. [CrossRef]

32. Zhao, L.; Feng, Y.; Deng, J.; Zhang, N.Y.; Zhang, W.P.; Liu, X.L.; Rajput, S.A.; Qi, D.S.; Sun, L.H. Selenium Deficiency Aggravates Aflatoxin B$_1$-Induced Immunotoxicity in Chick Spleen by Regulating 6 Selenoprotein Genes and Redox/Inflammation/Apoptotic Signaling. *J. Nutr.* **2019**, *149*, 894–901. [CrossRef] [PubMed]

33. Kasmani, F.B.; Torshizi, M.A.K.; Allameh, A.; Shariatmadari, F. A novel aflatoxin-binding Bacillus probiotic: Performance, serum biochemistry, and immunological parameters in Japanese quail. *Poult. Sci.* **2012**, *91*, 1846–1853. [CrossRef]

34. Khaleghipour, B.; Khosravinia, H.; Toghiyani, M.; Azarfar, A. Effects of silymarin on productive performance, liver function and serum biochemical profile in broiler Japanese quail challenged with dietary aflatoxins. *Ital. J. Anim. Sci.* **2019**, *18*, 564–573. [CrossRef]

35. Chen, R.; He, J.; Tong, X.; Tang, L.; Liu, M. The Hedyotis diffusa Willd. (Rubiaceae): A Review on Phytochemistry, Pharmacology, Quality Control and Pharmacokinetics. *Molecules* **2016**, *21*, 710. [CrossRef] [PubMed]

36. Gao, X.; Li, C.; Tang, Y.L.; Zhang, H.; Chan, S.W. Effect of Hedyotis diffusa water extract on protecting human hepatocyte cells (LO$_2$) from H$_2$O$_2$-induced cytotoxicity. *Pharm. Biol.* **2016**, *54*, 1148–1155. [PubMed]

37. Dinkova-Kostova, A.T.; Talalay, P. NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. *Arch. Biochem. Biophys.* **2010**, *501*, 116–123. [CrossRef] [PubMed]

38. Loboda, A.; Damulewicz, M.; Pyza, E.; Jozkowicz, A.; Dulak, J. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: An evolutionarily conserved mechanism. *Cell. Mol. Life Sci.* **2016**, *73*, 3221–3247. [CrossRef] [PubMed]

39. Zhou, Y.; Jin, Y.; Yu, H.; Shan, A.; Shen, J.; Zhou, C.; Zhao, Y.; Fang, H.; Wang, X.; Wang, J.; et al. Resveratrol inhibits aflatoxin B1-induced oxidative stress and apoptosis in bovine mammary epithelial cells and is involved the Nrf2 signaling pathway. *Toxicon* **2019**, *164*, 10–15. [CrossRef]

40. Ji, Y.; Nyamagoud, S.B.; SreeHarsha, N.; Mishra, A.; Gubbiyappa, S.K.; Singh, Y. Sitagliptin protects liver against aflatoxin B1-induced hepatotoxicity through upregulating Nrf2/ARE/HO-1 pathway. *Biofactors* **2020**, *46*, 76–82. [CrossRef]

41. Lu, S.C. Glutathione synthesis. *Biochim. Biophys. Acta.* **2013**, *1830*, 3143–3153. [CrossRef]

42. Sriwattanapong, K.; Slocum, S.L.; Chawanthayatham, S.; Fedeles, B.I.; Egner, P.A.; Groopman, J.D.; Satayavivad, J.; Croy, R.G.; Essigmann, J.M. Editor's Highlight: Pregnancy Alters Aflatoxin B1 Metabolism and Increases DNA Damage in Mouse Liver. *Toxicol. Sci.* **2017**, *160*, 173–179. [CrossRef] [PubMed]

43. Guo, Y.; Balasubramanian, B.; Zhao, Z.H.; Liu, W.C. Marine algal polysaccharides alleviate aflatoxin B1-induced bursa of Fabricius injury by regulating redox and apoptotic signaling pathway in broilers. *Poult. Sci.* **2021**, *100*, 844–857. [CrossRef] [PubMed]

44. Zhang, N.Y.; Qi, M.; Gao, X.; Zhao, L.; Liu, J.; Gu, C.Q.; Song, W.J.; Krumm, C.S.; Sun, L.H.; Qi, D.S. Response of the hepatic transcriptome to aflatoxin B$_1$ in ducklings. *Toxicon* **2016**, *111*, 69–76. [CrossRef] [PubMed]

45. Zhang, N.Y.; Qi, M.; Zhao, L.; Zhu, M.K.; Guo, J.; Liu, J.; Gu, C.Q.; Rajput, S.A.; Krumm, C.S.; Qi, D.S.; et al. Curcumin Prevents Aflatoxin B$_1$ Hepatoxicity by Inhibition of Cytochrome P450 Isozymes in Chick Liver. *Toxins* **2016**, *8*, 327. [CrossRef] [PubMed]