



Article

Zearalenone Induces Oxidative Stress and Apoptosis in the Jejunum of Weaned Piglets via the p53/Nrf2 Signaling Pathway

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Abstract

This study investigated the mechanisms by which ZEA induces oxidative stress and apoptosis in the jejunum of piglets and explored the roles of the tumor suppressor gene p53 and nuclear factor E2-related factor 2 (Nrf2) signaling pathways. Twelve weaned piglets were randomized into Control (basal diet) and ZEA groups (basal diet + 1.0 mg/kg ZEA; 6 piglets/group). No differences were observed between the control and ZEA groups for all production performance indicators. Compared with the jejunum of the control group, the ZEA group exhibited reduced levels of total superoxide dismutase, glutathione peroxidase activity, and total antioxidant capacity, along with elevated malondialdehyde content. Morphological examination revealed increased crypt depth and decreased villus height and villus-to-crypt ratio, as well as swollen, vacuolated spherical mitochondria with disrupted cristae. Immunohistochemistry showed enhanced p53 and Nrf2 immunoreactivity. The relative mRNA levels of Nrf2, Ho1, Gpx1, Cyt1, p53, Caspase1, and Bax increased. The Bax/Bcl-2 ratio increased, and Keap1 and Bcl-2 mRNA levels decreased. The relative protein levels of Nrf2, p53, Bax, Caspase1, and Gpx1 increased, whereas that of Bcl-2 decreased. All differences were significant at $p < 0.05$. Dietary supplementation with ZEA altered the morphological structure of intestinal tissues and mitochondria. By affecting the expression of genes related to the p53 and Nrf2 signaling pathways, it induces intestinal oxidative stress and apoptosis, thereby impairing intestinal health in weaned piglets.

Keywords: apoptosis; oxidative stress; weaned piglets; zearalenone; jejunum



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1. Introduction

In livestock production, the mycotoxin zearalenone (ZEA) frequently contaminates feed ingredients. The toxic effects primarily manifest as the disruption of cellular homeostasis, the induction of inflammatory responses, and the promotion of apoptosis, causing damage to the liver, kidneys, intestines, and reproductive systems of animals [1–3]. Piglets exhibit high sensitivity to ZEA, and the ingestion of contaminated feed can readily damage the intestinal mucosa, disrupt intestinal flora, and impair immune function, severely compromising piglet health and incurring substantial economic losses in the swine industry [4].

Upon ingestion, zearalenone (ZEA) is readily absorbed via the intestinal tract and triggers multiple toxic responses that severely compromise intestinal health and development. ZEA disrupts intestinal morphological integrity, inducing villus shortening, reduced

epithelial cell counts, and mucosal erosion [5]. It also suppresses intestinal antioxidant enzyme activities [6,7], thereby weakening endogenous antioxidant defense and promoting reactive oxygen species (ROS) overaccumulation and oxidative stress [8,9]. Sustained oxidative stress further activates caspase-dependent mitochondrial or endoplasmic reticulum stress-mediated apoptotic pathways, regulates Bcl-2-family and other apoptosis-related genes, and ultimately leads to cell apoptosis [10].

Nrf2 is a key transcription factor that maintains cellular redox homeostasis by regulating the expression of antioxidant response-related genes [11]. p53, known as the “guardian of the genome,” orchestrates apoptotic processes via transcriptional regulation of pro-apoptotic gene expression [12]. Accumulating evidence has demonstrated crosstalk between the Nrf2-mediated oxidative stress and p53-mediated apoptotic pathways [13]. Moreover, ZEA induces intestinal oxidative stress in weaned piglets [14]. However, the mechanisms through which ZEA triggers oxidative stress and apoptosis via the Nrf2 and p53 pathways in the jejunum of weaned piglets remain largely unknown. Accordingly, this study aimed to clarify these regulatory mechanisms, provide a theoretical basis for elucidating ZEA-induced jejunal toxicity, and promote feed safety for more efficient animal production.

2. Results

2.1. Growth Performance

The effects of ZEA on the growth performance of weaned piglets are presented in Table 1. Compared with the control group, no significant differences were observed in the ZEA group in terms of initial weight, final weight, average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) ($p > 0.05$).

Table 1. Effect of zearalenone on the growth performance of weaned piglets.

Item	Control ⁽¹⁾	ZEA ⁽¹⁾	<i>p</i> -Value ⁽²⁾
Initial weight (Kg)	13.61 ± 0.56	14.00 ± 0.32	0.16
Final weight (Kg)	29.53 ± 0.66	30.30 ± 0.58	0.06
Average daily gain (Kg)	0.53 ± 0.01	0.55 ± 0.02	0.26
Average daily feed intake (Kg)	0.95 ± 0.02	0.94 ± 0.02	0.54
Feed conversion Ratio	1.79 ± 0.04	1.73 ± 0.07	0.06

⁽¹⁾ Control: fed the basal diet; ZEA1.0: fed the experimental diet supplemented with 1.0 mg/kg ZEA in the basal diet. ⁽²⁾ The difference was significant ($p < 0.05$), or the difference was not significant ($p > 0.05$).

2.2. Antioxidant Capacity

The effects of ZEA on the antioxidant capacity of the jejunum are shown in Table 2. Compared with the control group, the ZEA group exhibited reduced total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), and glutathione peroxidase (GSH-Px) activities ($p < 0.05$), and increased malondialdehyde (MDA) levels ($p < 0.05$).

Table 2. Effect of zearalenone on the antioxidant capacity of the jejunum of weaned piglets.

Item	Control ⁽¹⁾	ZEA1.0 ⁽¹⁾	<i>p</i> -Value ⁽²⁾
T-AOC, mmol/g	0.80 ± 0.02	0.56 ± 0.02	<0.01
T-SOD, U/mg prot	104.62 ± 4.08	82.12 ± 0.85	<0.01
GSH-Px, U/mg prot	250.13 ± 7.40	201.72 ± 7.01	<0.01
MDA, nmol/mg prot	1.76 ± 0.03	2.55 ± 0.03	<0.01

⁽¹⁾ Control: fed the basal diet; ZEA1.0: fed the experimental diet supplemented with 1.0 mg/kg ZEA in the basal diet. ⁽²⁾ The difference was significant ($p < 0.05$), or the difference was not significant ($p > 0.05$).

2.3. Mitochondrial Morphology

The effects of ZEA on the jejunal mitochondria of weaned piglets are shown in Figure 1. In the control group, the mitochondria exhibited regular morphology, appearing as short rod-shaped or tubular structures with intact and orderly arranged cristae (blue circles). In the ZEA group, mitochondrial morphology was significantly altered. Short rod-shaped mitochondria shrank into spherical forms, exhibiting marked swelling and vacuolation. Mitochondrial cristae structures were damaged, showing distinct discontinuities and disappearance (red circles).

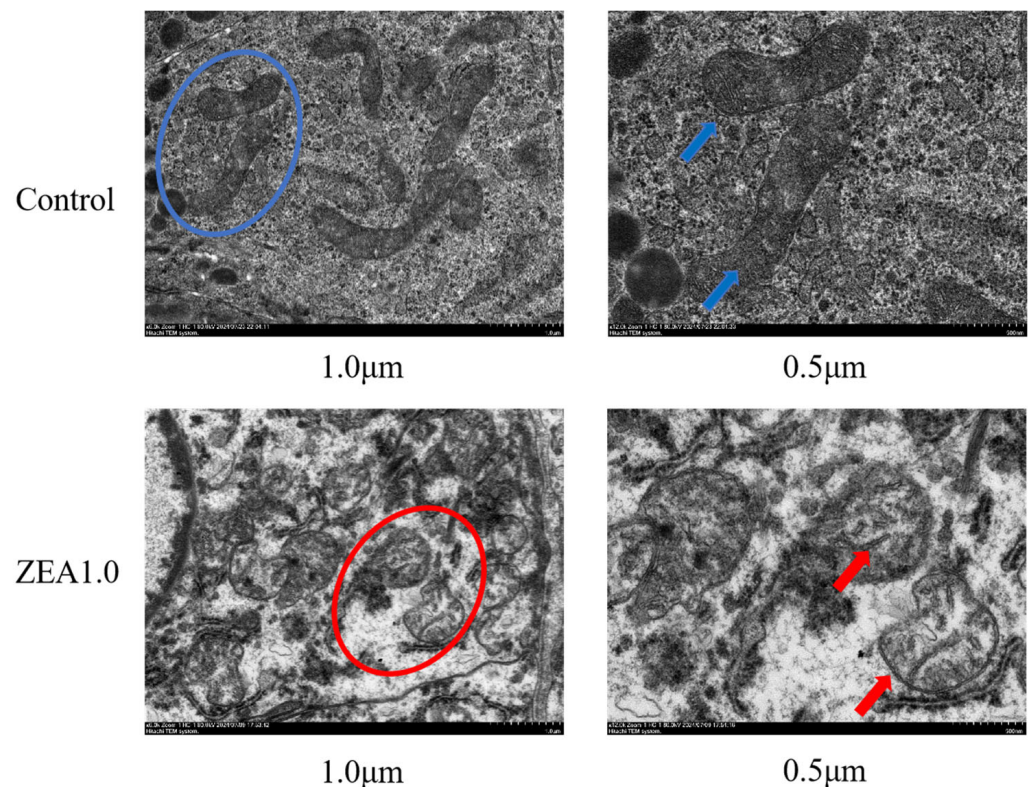


Figure 1. Effect of zearalenone on mitochondrial morphology and the structure of jejunum in weaned piglets. Control: fed the basal diet (blue circles and arrows); ZEA1.0: fed the experimental diet supplemented with 1.0 mg/kg ZEA in the basal diet (red circles and arrows).

2.4. Effects of Zearalenone on Jejunal Villus Height, Crypt Depth, and Villus-to-Crypt Ratio

The effects of ZEA on villus height, crypt depth, and villus-to-crypt ratio in weaned piglets are shown in Table 3. Compared with the control group, the ZEA group exhibited increased crypt depth ($p < 0.05$) and decreased villus height and villus-to-crypt ratio ($p < 0.05$).

Table 3. Effect of zearalenone on the jejunum of weaned piglets.

Item	Control ⁽¹⁾	ZEA1.0 ⁽²⁾	<i>p</i> -Value
Villus height (µm)	396.67 ± 22.97	376.05 ± 17.15	0.004
Crypt depth (µm)	281.13 ± 10.66	290.69 ± 9.87	0.009
Villus-to-crypt ratio	1.41 ± 0.11	1.29 ± 0.07	<0.01

⁽¹⁾ Control: fed the basal diet; ZEA1.0: fed the experimental diet supplemented with 1.0 mg/kg ZEA in the basal diet. ⁽²⁾ The difference was significant ($p < 0.05$), or the difference was not significant ($p > 0.05$).

2.5. Morphology and Structure of Jejunum

The effects of ZEA on the intestinal morphology of weaned piglets are shown in Figure 2. In the control group, the jejunal villi exhibited regular morphology with an orderly arrangement and clear boundaries between the intestinal epithelial cells. The ZEA group exhibited significant alterations in the villus morphology and crypt structure. Compared with the control group (blue circles), the ZEA group (red circles) showed blurred epithelial cell boundaries, shedding of the villus epithelium, and exposure of the lamina propria; and villus fragmentation, sparse arrangement, and increased length; along with deepened crypts.

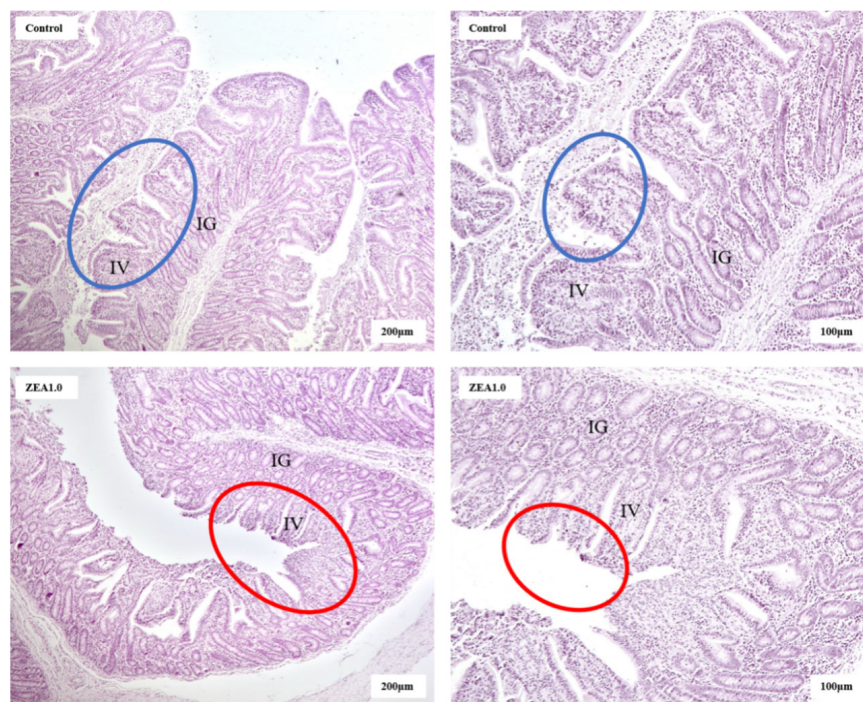


Figure 2. Effect of zearalenone on the jejunal morphology and structure of weaned piglets. IG: gland; IV: small intestinal villi; Control: fed the basal diet (blue circles); and ZEA1.0: fed the experimental diet supplemented with 1.0 mg/kg ZEA in the basal diet (red circles). 200 µm indicates photos taken at 10 × 4 magnification; 100 µm indicates photos taken at 10 × 10 magnification.

2.6. Jejunal Expression of p53 and Nrf2

The immunohistochemical effects of ZEA on p53 and Nrf2 expression in the jejunum of weaned piglets are presented in Figures 3 and 4. p53 immunopositive material was primarily distributed around the jejunal villus epithelium (LE), with faint immunopositivity observed in the intestinal glands (G) and lamina propria (S). Nrf2 immunopositive material was mainly distributed in the lamina propria (S), with faint immunopositivity observed in the intestinal glands (G) and jejunal villus epithelium (LE). In the control group, a faint brown p53 immunopositive stain was visible in the jejunal villus epithelium (LE) (Figure 3(A2,A3)). The ZEA group exhibited abundant p53 immunopositive material in the jejunal villus epithelium (LE) with intensified staining (Figure 3(B2,B3)), appearing dark brown. In the lamina propria (S) of the control group, a small amount of light-brown Nrf2 immunopositive material was visible (Figure 4(A2,A3)). Nrf2 immunopositive levels were enhanced in the lamina propria (S) of the ZEA group, with deeper staining (Figure 4(B2,B3)). Compared with the control group, the immunopositive reactions for p53 and Nrf2 were enhanced in the ZEA-treated group ($p < 0.05$; Table 4).

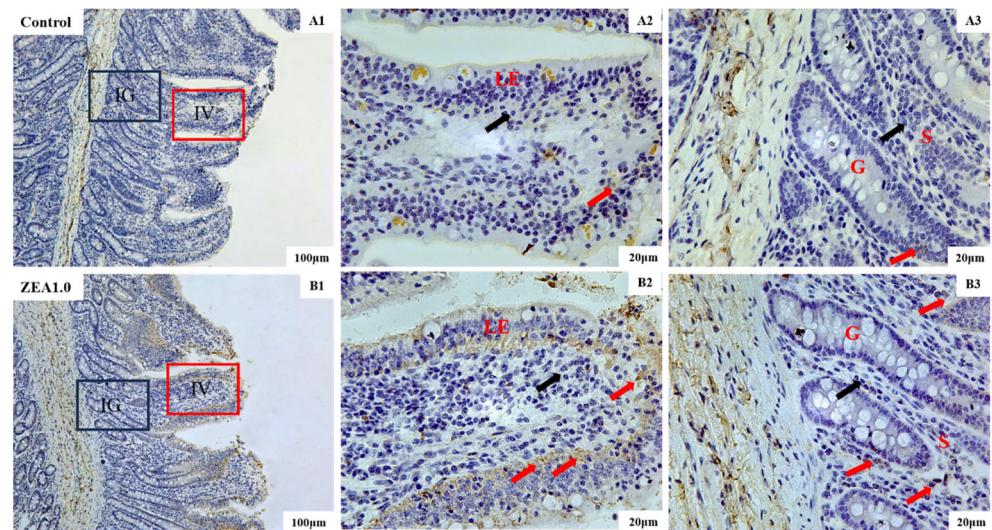


Figure 3. Effect of zearalenone on p53 in the jejunum of weaned piglets. Control group: Control, (A1–A3); Zearalenone 1.0 mg/kg: ZEA1.0, (B1–B3); G is the intestinal gland, S is the lamina propria, and LE is the small intestinal epithelial villi; the red and blue boxes indicate the regions magnified under 10×40 magnification. Red arrows are immunopositive cells, black arrows are negative. 100 μ m indicates photos taken at 10×10 magnification; 20 μ m indicates photos taken at 10×40 magnification.

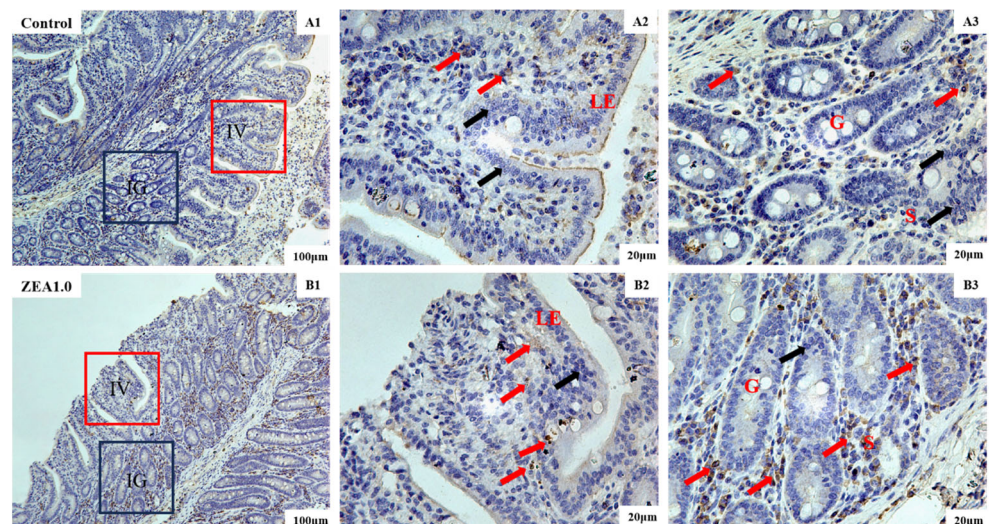


Figure 4. Effect of zearalenone on Nrf2 in the jejunum of weaned piglets. Control group: Control, (A1–A3); Zearalenone 1.0 mg/kg: ZEA1.0, (B1–B3); G is the intestinal gland, S is the lamina propria, and LE is the small intestinal epithelial villi; the red and blue boxes indicate the regions magnified under 10×40 magnification. Red arrows are immunopositive cells, black arrows are negative. 100 μ m indicates photos taken at 10×10 magnification; 20 μ m indicates photos taken at 10×40 magnification.

Table 4. Effect of zearalenone on the immunoreactive integrated optic density of p53 and Nrf2 in the small intestine of weaned piglets.

Item	Control ⁽¹⁾	ZEA1.0 ⁽¹⁾	<i>p</i> -Value ⁽²⁾
p53	115.10 $\pm$ 2.6	137.84 $\pm$ 1.63	<0.01
Nrf2	132.82 $\pm$ 2.8	151.51 $\pm$ 5.15	0.005

⁽¹⁾ Control: fed the basal diet; ZEA1.0: fed the experimental diet supplemented with 1.0 mg/kg ZEA in the basal diet. ⁽²⁾ The difference was significant ($p < 0.05$), or the difference was not significant ($p > 0.05$).

2.7. Relative Jejunal mRNA Levels of Keap1, Nrf2, Ho1, Gpx1 Bax, p53, Bcl-2, Cytc1, and Caspase1

Compared with the control group, the ZEA group exhibited increased relative mRNA levels of Nrf2, Ho1, Gpx1, Cytc1, p53, Caspase1, and Bax ($p < 0.05$), increased Bax/Bcl-2 ratio ($p < 0.05$), and decreased Keap1 and Bcl-2 mRNA levels ($p < 0.05$; Table 5).

Table 5. Relative expression of zearalenone mRNA in the jejunum of weaned piglets.

Item	Control ⁽¹⁾	ZEA1.0 ⁽¹⁾	<i>p</i> -Value ⁽²⁾
<i>Nrf2</i>	1.00 ± 0.05	1.98 ± 0.05	<0.01
<i>Keap1</i>	1.00 ± 0.05	0.68 ± 0.03	<0.01
<i>Ho1</i>	1.00 ± 0.05	1.31 ± 0.05	<0.01
<i>Gpx1</i>	1.00 ± 0.04	1.36 ± 0.05	<0.01
<i>Cytc1</i>	1.00 ± 0.07	1.40 ± 0.05	<0.01
<i>Caspase1</i>	1.00 ± 0.07	2.31 ± 0.13	<0.01
<i>p53</i>	1.00 ± 0.05	1.29 ± 0.05	<0.01
<i>Bcl2</i>	1.00 ± 0.05	0.56 ± 0.03	<0.01
<i>Bax</i>	1.00 ± 0.03	1.37 ± 0.05	<0.01
<i>Bax/Bcl2</i>	1.00 ± 0.07	2.46 ± 0.19	<0.01

⁽¹⁾ Control: fed the basal diet; ZEA1.0: fed the experimental diet supplemented with 1.0 mg/kg ZEA in the basal diet. ⁽²⁾ The difference was significant ($p < 0.05$), or the difference was not significant ($p > 0.05$).

2.8. Relative Expression of Intestinal Protein in Weaned Piglets

Compared with the control group, the ZEA-treated group exhibited increased protein levels of p53, Nrf2, Bax, Caspase1, and Gpx1 ($p < 0.05$), and decreased levels of Bcl-2 protein ($p < 0.05$; Figure 5).

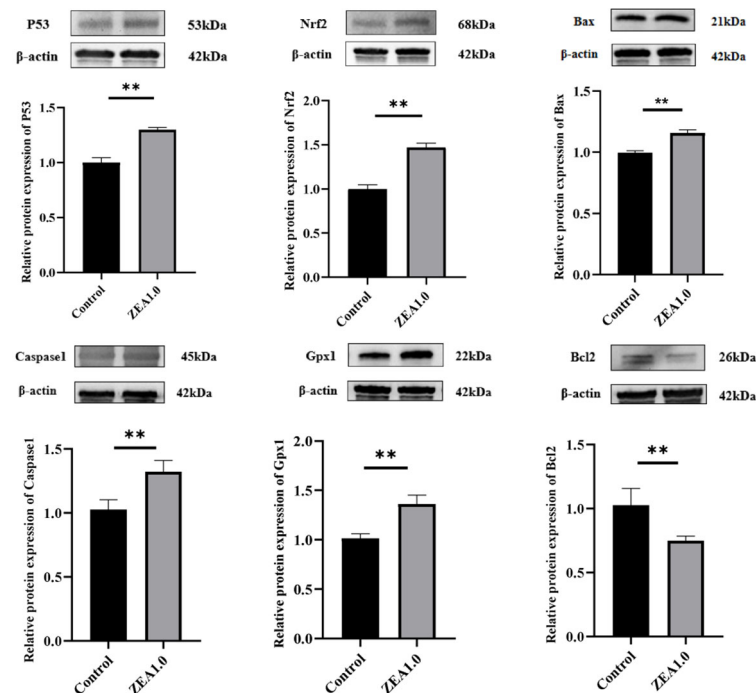


Figure 5. Effect of zearalenone on the relative expression levels of proteins in the jejunum of weaned piglets. Control: fed the basal diet; ZEA1.0: fed the experimental diet supplemented with 1.0 mg/kg ZEA in the basal diet. The difference was significant ($p < 0.05$), or the difference was not significant ($p > 0.05$). ** $p < 0.01$.

3. Discussion

Numerous studies have examined the effects of ZEA on swine production. However, the outcomes vary owing to various factors such as breed, ZEA dosage, and feeding period. Adding ZEA to diets does not significantly affect ADFI, ADG, or FCR in weaned piglets [15]. In fact, ZEA supplementation can increase body weight [16]. As the ZEA concentration in the diet increases (3.0–9.0 mg/kg), the average daily feed intake and weight gain of sows has been shown to decrease significantly [17]. This may be because of ZEA accumulation to toxic levels [18]. In the present study, 1 mg/kg dietary ZEA did not significantly affect the average daily feed intake, average daily weight gain, or feed-to-gain ratio of weaned piglets. This indicated that low concentrations of ZEA had no apparent impact on growth performance. Similarly, other studies have shown that 1 mg/kg ZEA had no significant effect on feed intake in pigs [7,19].

Oxidative stress arises from an imbalance between free radical and antioxidant production. Free radical accumulation above the capacity of the antioxidant system induces inflammatory responses and inhibits the activity of antioxidant enzymes, thereby causing oxidative stress that damages cells and tissues [20]. Oxidative stress alters the expression and activity of antioxidant enzymes, thereby weakening systemic antioxidant defense capabilities. Consequently, ROS accumulation disrupts cellular homeostasis and exacerbates oxidative damage [21,22]. Consistent with previous reports in IPEC-J2 cells and piglet models [14,23–25], our findings underscore that ZEA compromises the jejunal antioxidant barrier through the synchronized suppression of enzymatic defenses and the elevation of lipid peroxidation. This indicated that ZEA reduced the antioxidant capacity of the jejunum by affecting antioxidant enzyme activity, thereby inducing oxidative stress.

The gut—the largest interface between animal host and environment—is highly susceptible to disruption by ZEA [26]. Gut morphology is a key indicator of intestinal health [27], with villus shortening and crypt deepening ultimately reducing nutrient absorption and disease resistance [28]. ZEA disrupts the villus structure of the rat jejunum in a dose-dependent manner. Low concentrations of ZEA have no significant effect on rat intestinal morphology; however, high concentrations of ZEA damage mouse intestinal villi and crypt, reducing villus height and villus-to-crypt ratio, thereby impairing the intestinal barrier and decreasing the surface area available for nutrient absorption [29,30]. In this study, ZEA treatment significantly decreased villus height and the villus–crypt ratio, while increasing crypt depth in the jejunum. Histological observations further revealed ZEA-induced disruption of the villus structure and intestinal epithelial integrity. These morphological changes are consistent with previous studies showing that ZEA impairs jejunal mucosal architecture in weaned piglets [31,32], providing direct morphological evidence of jejunal damage caused by ZEA.

The mitochondrial electron transport chain is the primary source of intracellular ROS. Excessive ROS production triggers oxidative stress and thereby promotes mitochondrial dysfunction [33]. ZEA can reduce the mitochondrial membrane potential, thereby altering the permeability of the membrane and affecting ionic gradients and energy metabolism within the mitochondria. ZEA induces oxidative imbalance in IPEC-J2 cells with insufficient antioxidant defense, thereby impairing mitochondrial function [34]. Following ZEA treatment, chicken embryonic fibroblasts exhibit a loss of mitochondrial membrane potential, affecting the functional structure of mitochondria and disrupting normal cellular physiological functions [35]. In the present study, ZEA exposure induced obvious mitochondrial morphological damage in the jejunum. Mitochondria changed from short rod-shaped to spherical structures, accompanied by severe swelling, vacuolation, and disrupted or disappeared cristae. These alterations indicate that ZEA-induced oxidative stress impairs mitochondrial structural integrity and ultimately leads to mitochondrial dysfunction.

The regulation of cellular antioxidant stress defense systems is crucial for maintaining homeostasis. Nrf2 and p53, the two core transcription factors, exert both independent functions and complex interactions in response to oxidative stress. In particular, p53 regulates Nrf2 via indirect mechanisms: The p53 target gene p21 competes with Keap1 for Nrf2 binding, thereby blocking Keap1-mediated Nrf2 ubiquitination and degradation [36]. This demonstrates that both factors play synergistic roles in maintaining intracellular redox balance, jointly protecting cells from oxidative damage.

Nrf2, the core transcription factor of the cellular antioxidant defense system, plays a crucial role in maintaining cellular redox balance by regulating the expression of antioxidant genes [37]. Under normal conditions, Nrf2 is bound and degraded by Keap1; during oxidative stress, it dissociates from Keap1 and enters the nucleus to activate antioxidant gene expression, scavenge ROS, and protect cells from oxidative damage [38]. With increasing ZEA concentrations, Nrf2 levels increase, while Keap1 expression decreases in the jejunal tissues of weaned piglets and IPEC-J2 cells [9,14,23], activating the Nrf2-Keap1 pathway. In this study, ZEA treatment elevated Nrf2 immunoreactivity in the jejunal epithelium, lamina propria, and intestinal glands of weaned piglets, accompanied by upregulated mRNA and protein expression of Nrf2. The transcript levels of its downstream antioxidant genes Ho1 and Gpx1 were also significantly increased. These results demonstrate that ZEA-induced oxidative stress in the jejunum activates the Nrf2 signaling pathway, which may enhance antioxidant defense and alleviate ZEA-triggered oxidative damage.

Upon activation by oxidative stress and other signaling stimuli, p53 initiates apoptosis through a dual mechanism: it regulates pro- and anti-apoptotic genes via transcription-dependent pathways and directly binds to mitochondrial Bcl-2-family proteins through transcription-independent mechanisms [39]. ZEA induces apoptosis by elevating the relative intracellular expression of p53 [40,41]. ZEA increases p53 mRNA and protein levels in porcine endometrial epithelial and stromal cells [42,43]. In this study, ZEA-treated weaned piglets exhibited significantly increased p53 immunoreactive staining in the parietal epithelium, intestinal glands, and lamina propria of the jejunum. This finding aligns with the p53 mRNA and relative protein levels in the intestine, indicating that p53 was activated upon ZEA exposure. Notably, following the initiation of apoptotic signals by the dual mechanism, the downstream effector molecule of p53—apoptosis-related protein (ARTS) associated with the transforming growth factor- β (TGF- β) signaling pathway—synergistically amplifies apoptotic signals. This leads to mitochondrial outer membrane permeabilization, cytochrome c release, caspase cascade activation, and ultimately cell apoptosis [44]. Therefore, to investigate the mechanism by which ZEA induces apoptosis, we examined the expression of key genes involved in the apoptosis pathway. ZEA increased the levels of p53, Bax, and Caspase1 mRNA and proteins; increased Cyt c1 mRNA expression; and suppressed Bcl-2 mRNA and protein expressions. This indicates that ZEA activates the apoptotic signaling pathway in the jejunum by upregulating the expression of genes related to the p53 signaling pathway, thereby promoting apoptosis. Similarly, another study demonstrated that ZEA enhanced Bax mRNA expression and reduced Bcl-2 mRNA expression in the jejunum of weaned piglets [45]. Thus, ZEA induces the expression of apoptosis-related genes, causing damage to the intestines and mitochondria of weaned piglets and inducing intestinal cell apoptosis.

ZEA activated both the Nrf2 and p53 signaling pathways. Despite the activation of the Nrf2 pathway, intestinal and mitochondrial damage persisted. This may be because excessive doses or prolonged exposure generates ROS levels exceeding the Nrf2 pathway scavenging capacity, resulting in severe oxidative damage following the failure of this enhanced antioxidant protective mechanism. Simultaneously, the p53 signaling pathway was activated, thereby upregulating apoptosis-related gene expression to promote cell

death and eliminate severely damaged cells. Thus, in ZEA-induced oxidative stress in the jejunum, the Nrf2 and p53 pathways do not operate independently. However, whether they jointly determine jejunal cell survival or apoptosis through bidirectional regulation and mutual counterbalancing requires further investigation.

This study elucidated the roles of the p53 and Nrf2 pathways in ZEA-induced jejunal oxidative stress and apoptosis in weaned piglets, with several limitations remaining. Future work will focus on dose–response relationships, *in vivo*/*in vitro* validation, and crosstalk mechanisms between the two pathways. These findings provide theoretical and practical references for ZEA toxicity control, intestinal health improvement, and feed safety standard formulation in the swine industry.

4. Materials and Methods

4.1. Experimental Materials

ZEA (Z2125, purity $\geq 99.0\%$) was purchased from Sigma. The Total Antioxidant Capacity (T-AOC) Kit (A015-2), Glutathione Peroxidase (GSH-Px) Kit (A005), Total Superoxide Dismutase (T-SOD) Kit (A001), Malondialdehyde (MDA) Kit (A003), and Total Protein Quantification (TP) Kit (A045-4) were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. Rabbit anti-p53 polyclonal antibody (10442-1-AP), rabbit anti-Nrf2 recombinant antibody (80593-1-RR), and mouse anti-Bax monoclonal antibody (60267-1-Ig) were purchased from Wuhan Sanying Biotechnology Co., Ltd., Wuhan, China. Rabbit anti-Bcl2 monoclonal antibody (ET1603-11), mouse anti-Gpx1 monoclonal antibody (EM1701-96), and rabbit anti-Caspase1 monoclonal antibody (ET1608-69) were purchased from Hua'an Biotechnology Co., Ltd., Zhejiang, China. Goat anti-mouse IgG (LF101) and goat anti-rabbit IgG (LF102) were purchased from Shanghai Yamei Biotechnology Co., Ltd., Shanghai, China. The rabbit two-step detection kit (PV-9001) and DAB color development kit (ZLI-9018) were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China. AG RNAex Pro RNA extraction reagent (AG21102-C), Evo M-ML V reverse transcription premix kit (AG11728), and SYBR Green Pro Taq HS premix qPCR kit (AG11701) were purchased from Accurate Biotechnology (Hunan) Co., Ltd., Changsha, China.

4.2. Experimental Design

Twelve healthy 28-day-old three-way crossbred (Duroc $\times$ Landrace $\times$ Yorkshire) weaned piglets with a similar body weight were randomly allotted to two treatment groups, with six replicates per group and one piglet per replicate. The initial body weight exhibited no significant difference between the two groups ($p > 0.05$). The control group was fed a basal diet, whereas the ZEA group was offered the basal diet supplemented with 1.0 mg/kg ZEA. The trial included a 7-day adaptation period followed by a 28-day formal trial period. All experimental diets were prepared in a single batch at the initiation of the trial and stored in a dry, cool environment. The nutritional levels of the basal diet were formulated to meet the nutrient requirements of pigs as recommended by NRC (2012). The detailed composition and nutrient levels of the experimental diets are presented in Table 6.

Table 6. Ingredients and nutrient contents of the basal diet (% air-dry basis).

Ingredients	Content	Nutrients	Analyzed Values
Expanded corn	64.43	Metabolizable energy, MJ/kg	13.86
Whey powder, CP 3%	5.00	Crude protein	18.48
Fermented soybean meal	14.00	Calcium	0.74
Expanded soybean	8.50	Total phosphorus	0.62
Fish meal, CP 63.28%	4.00	STTD phosphorus	0.41

Table 6. *Cont.*

Ingredients	Content	Nutrients	Analyzed Values
CaHPO ₄	1.15	ATTD phosphorus	0.38
Pulverized limestone	0.70	Lysine	1.38
NaCl	0.20	Methionine	0.40
L-Lysine HCl	0.76	Sulfur amino acid	0.66
DL-Methionine	0.08	Threonine	0.85
L-Threonine	0.16	Tryptophan	0.23
L-Tryptophan	0.02		
Premix ⁽¹⁾	1.00		
Total	100.00		

⁽¹⁾ Supplied per kilogram of diet: Vitamin A, 3300 IU; vitamin D₃, 330 IU; vitamin E, 24 IU; vitamin K₃, 0.75 mg; vitamin B₁, 1.50 mg; vitamin B₂, 5.25 mg; vitamin B₆, 2.25 mg; vitamin B₁₂, 0.026 mg; pantothenic acid, 15.00 mg; niacin, 22.50 mg; biotin, 0.075 mg; folic acid, 0.45 mg; Mn (MnSO₄·H₂O), 4.00 mg; Fe (FeSO₄·H₂O), 90 mg; Zn (ZnSO₄·H₂O), 90 mg; Cu (CuSO₄·5H₂O), 6.00 mg; I (KIO₃), 0.14 mg; and Se (Na₂SeO₃), 0.30 mg.

4.3. Sample Collection

On day 35 of the experiment, all piglets were slaughtered. The abdominal cavity was opened, and mid-segment small intestinal samples were excised and divided equally into four portions. After rinsing the samples with physiological saline to remove blood and chyme, one portion was fixed in Bouin's solution for hematoxylin-eosin (HE) staining, intestinal morphology assessment, and immunohistochemical analysis. One portion was first processed with a mitochondrial extraction kit to isolate small intestinal epithelial cell mitochondria, then fixed in 1.5–2.5% glutaraldehyde solution for observation of mitochondrial structural changes. One sample was placed in a 2 mL sterile cryovial, rapidly frozen in liquid nitrogen, and stored at −80 °C for subsequent tissue antioxidant capacity assays and mRNA and protein relative expression measurements.

4.4. Determination of Antioxidant Capacity in Jejunum Tissue

The antioxidant capacity-related indices of jejunal tissue, including total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px) and total superoxide dismutase (T-SOD) activities, and malondialdehyde (MDA) content, were determined by commercial assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All experimental procedures were performed in strict accordance with the manufacturer's standard protocols.

4.5. Mitochondrial Morphology in Jejunum Tissue

After tissue sampling, specimens were fixed in 2.5% glutaraldehyde solution and stored at 4 °C. Glutaraldehyde-fixed tissues underwent acid fixation followed by PBS rinsing, then sequential alcohol dehydration. Subsequent steps included osmotization, embedding, sectioning, and staining, with final observation under a transmission electron microscope (JEM-1400 Plus, JEOL, Tokyo, Japan).

4.6. Hematoxylin-Eosin (HE) Staining and Intestinal Morphology Assessment

Tissues fixed in Bouin's solution were thoroughly rinsed under running tap water, and then processed for graded ethanol dehydration, xylene clearing and paraffin embedding successively to prepare paraffin-embedded tissue blocks. The blocks were sectioned into 5 µm-thick slices using a rotary microtome. Paraffin sections were dewaxed in xylene, dehydrated through a graded ethanol series and rehydrated with distilled water prior to staining. The rehydrated sections were stained with hematoxylin for 2 min, followed by a 2 min tap-water rinse. Subsequently, the sections were differentiated in 1% acid alcohol for 3 s, rinsed under running tap water for 5 min, and immersed in 70% and 80% ethanol

for 5 min each, respectively. The sections were then stained with 1% alcoholic eosin for 2 min. After staining, dehydration was performed in 90%, 95% and anhydrous ethanol for 10 min per gradient, followed by xylene clearing for 20 min, neutral balsam mounting and air drying. The stained sections were finally observed and photographed under a light microscope (Axio Scope.A1, Carl Zeiss, Oberkochen, Germany) for morphological analysis of jejunal tissue.

4.7. Immunohistochemical Analysis of p53 and Nrf2 Distribution in Jejunum Tissue

Paraffin sections were subjected to conventional xylene dewaxing followed by graded hydration. Heat-induced antigen retrieval was performed in citrate buffer (pH 6.0), and the sections were then washed three times with phosphate-buffered saline (PBS) for 3 min each (the same washing procedure was applied for all subsequent PBS rinses). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution at room temperature for 10 min, followed by three PBS washes. The sections were separately incubated with rabbit anti-p53 polyclonal antibody (1:300) and rabbit anti-Nrf2 recombinant antibody (1:100) at 37 °C for 30 min, and then rinsed three times with PBS. Subsequently, the enhancement solution was added to the sections, followed by three PBS washes. The enzyme-labeled goat anti-rabbit IgG polymer secondary antibody was then applied, and the sections were incubated at 37 °C for 20 min with three subsequent PBS washes. The sections were stained with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin, followed by graded ethanol dehydration, xylene clearing and neutral balsam mounting. The stained sections were finally observed under a light microscope. ImageJ 1.54g software was used to quantify the mean integrated optical density (IOD) of the jejunal cross-sections for statistical analysis. The endogenous peroxidase blocker, enhancement solution and enhanced enzyme-labeled goat anti-rabbit IgG polymer used in this experiment were all provided in the Rabbit Two-Step Immunohistochemistry Detection Kit (PV-9001, Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China).

4.8. Determination of Relative Gene Expression Levels in Jejunum Tissue

Jejunal tissue samples were retrieved from the −80 °C freezer, and 100 mg of each sample was weighed and transferred into a 2 mL RNase-free centrifuge tube containing 1 mL of RNA extraction reagent (RNAex). The samples were homogenized thoroughly using a tissue homogenizer on ice, vortexed vigorously for mixing, and then incubated at room temperature for 5 min. The homogenates were centrifuged at 12,000 × g and 4 °C for 15 min, and 200 µL of RNA extraction aid reagent was subsequently added to the tubes, followed by vortex mixing and a 5 min incubation at room temperature. The mixture was centrifuged again under the same conditions (12,000 × g, 4 °C) for 15 min. The resulting supernatant was carefully transferred into a new 1.5 mL RNase-free centrifuge tube, and 500 µL of isopropanol was added, vortexed, and incubated for 10 min at room temperature. After centrifugation at 12,000 × g and 4 °C for 10 min, the supernatant was discarded, and the RNA pellet was retained. The pellet was washed twice with 1 mL of pre-chilled 80% ethanol by centrifugation at 7500 × g and 4 °C for 5 min per wash, with the supernatant discarded after each centrifugation. The RNA pellet was air-dried and then gently resuspended in an appropriate volume of RNase-free DEPC-treated water. The concentration and purity of the extracted RNA were determined, and the qualified RNA samples were stored at −80 °C for subsequent experiments. Reverse transcription was performed using the Evo M-MLV Reverse Transcription Pre-mix Kit, and quantitative real-time PCR (qPCR) was conducted with the SYBR Green Pro Taq HS Pre-mix qPCR Kit, following the manufacturers' standard protocols strictly. All gene-specific primers were synthesized by Accurate Biotechnology (Hunan) Co., Ltd., Changsha, China. The relative

mRNA expression levels of target genes were calculated using the $2^{-\Delta\Delta C_t}$ method. The sequence of primers and amplicons lengths are presented in Table 7.

Table 7. Primer sequences for PCR reactions.

Target Gene	Primer Sequences (5' to 3')	Accession No.
<i>Keap1</i>	F:TAGGTGGCTCCCATGGCTGTA R:AACTGCGTAGAGCAGACGGTT	NM001114671.1
<i>Nrf2</i>	F:CACATCCCGTCAGAAACCAGT R:GTAGCCGAAGAAACCTCATTGTC	XM_021075131.1
<i>Ho1</i>	F:ACCGCTCCCGAATGAACACT R:CCTGCACCTCCTCAAACAGC	NM001004027.1
<i>p53</i>	F:TGGCAGCACTAAGCGAGCAC R:CACGGATCTGGAGGGTGAAATA	NM213824.3
<i>Cyt1</i>	F:CTTGGAGTTTGACGATGGCA R:GATGGTCGTGTTTCGGGTTC	XM003125443.6
<i>Bcl2</i>	F:TTTGCCGAGATGTCCAGCCA R:ACATGACCCCAACGAACTCAA	XM021099593.1
<i>Bax</i>	F:GCTGACGGCAACTTCAACTGG R:CCAGCCCATGATGGTCCTGAT	XM013998624.2
<i>Caspase1</i>	F:ACTCCGAAGAAGTCCCAGATGT R:CTTGCTTCTCACCACGGCAAG	NM214162.1
<i>Gpx1</i>	F:TGCAACCAGTTTGGACATCAGG R:GGGTGAGCATTTGCGCCATTC	NM214201.1
<i>GAPDH</i>	F:CGCCTGGAGAAACCTGCAAAA R:ACACAACCTGGTCCTCAGTGT	NM001206395.1

4.9. Determination of Relative Expression Levels of Jejunum Tissue-Associated Protein

Retrieve jejunal tissue from the -80°C freezer. Weigh the jejunal tissue and transfer it to a 2 mL enzyme-free centrifuge tube containing 1 mL Western and IP cell lysis buffer. Grind the tissue using a tissue homogenizer (Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China), then centrifuge at 4°C and $12,000 \times g$ for 15 min. Determine protein concentration using the TP kit and normalize protein concentrations. Add $5 \times$ SDS loading buffer to a final concentration of $1 \times$, then denature proteins by heating at 100°C for 10 min. Separate samples by electrophoresis and transfer to a membrane. After transfer, wash with $1 \times$ TBST (shaking) for 5 min, 3 times. Block with 5% nonfat dry milk for 2.5 h (shaking). Incubate with primary antibody overnight at 4°C . After primary antibody incubation, wash the PVDF membrane with $1 \times$ TBST (shaking) for 5 min, 3 times. Incubate with secondary antibody at 37°C for 1 h. After incubation, wash the PVDF membrane with $1 \times$ TBST (shaking) for 5 min, 3 times, then develop the membrane.

4.10. Statistics and Analysis

Data were analyzed using SPSS 26.0 software. The Shapiro–Wilk test and Levene’s test were applied to verify normality and homogeneity of variance, respectively. The independent-sample *t*-test was used for comparisons between the two groups. Results are presented as “mean $\pm$ standard deviation,” with $p < 0.05$ indicating statistically significant differences.

5. Conclusions

In piglets, adding 1.0 mg/kg ZEA to feed caused damage to jejunal tissue and mitochondria, reduced antioxidant enzyme activity, and induced oxidative stress in the jejunum. ZEA promoted apoptosis by upregulating the expression of the apoptosis-related genes p53, Bax, Cyt1, and Caspase1, suppressing Bcl-2 expression, and activating the p53 apoptosis

signaling pathway in the jejunum. Concurrently, ZEA activated the Nrf2 signaling pathway by upregulating the expression of the key genes Nrf2 and Gpx1 to counteract ZEA-induced oxidative damage. This study provides a theoretical basis and scientific reference for elucidating the key regulatory mechanisms underlying ZEA-induced oxidative stress and apoptosis in the jejunum of weaned piglets, and contributes to a better understanding of the molecular mechanisms of ZEA-induced intestinal toxicity.

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Institutional Review Board Statement: The experimental protocol complies with ethical standards and has been approved by the Animal Ethics Committee of the College of Animal Science and Technology, Qingdao Agricultural University (Approval No.: DKY20240412, Approval Date: 2024.4.12).

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Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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