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Gly-Pro-Ala peptide and FGSHF3 exert protective effects in DON-induced toxicity and intestinal damage via decreasing oxidative stress

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Graphical abstract

FGSHF3 and GPA peptide activate Nrf2 to up-regulate the expression of antioxidant enzymes, resulting in suppressing oxidative stress in intestinal. Ultimately, FGSHF3 and GPA maintain the intestinal barrier and inhibit DON-induced toxicity

Highlight

1. FGSHF3 and GPA treatment significantly attenuates DON-induced toxicity in mice.
2. FGSHF3 and GPA treatment significantly alleviates intestinal injury and maintains tight junction in mice intestinal cells and IPEC-J2 cells
3. FGSHF3 and GPA treatment significantly inhibits ROS and MDA production, and enhances antioxidant enzyme activity
4. FGSHF3 and GPA treatment promotes Nrf2 migration from the cytoplasm to the nucleus, resulting in activating the Nrf2 pathway to exert antioxidant

effects.

Abstract

Deoxynivalenol (DON), a common mycotoxin, usually induces oxidative stress and intestinal injury of humans and animals. This study aims to investigate the protective effect of Gly-Pro-Ala (GPA) peptide, isolated from fish skin gelatin hydrolysate fraction 3 (FGSHF3), on alleviating the toxicity and oxidative stress induced by DON in the mice and IPEC-J2 cells. DON treatment decreases average daily gain and feeds intake, which causes enlargement of the liver and spleen. FGSHF3 (200mg/kg) and GPA (200mg/kg) treatment significantly increase average daily gain and inhibits enlargement of the liver and spleen. Furthermore, FGSHF3 and GPA treatment significantly alleviates intestinal injury and maintains tight junction in mice and IPEC-J2 cells. FGSHF3 and GPA treatment significantly inhibits ROS and MDA production and enhances antioxidant enzyme activity, such as CAT, SOD-1, GCLM, GCLC, and GSH-PX. Furthermore, FGSHF3 and GPA treatment promote Nrf2 migration from the cytoplasm to the nucleus, resulting in exerting antioxidant effects. And its effects are abolished after Nrf2 is knockdown by siRNA. Overall, our results suggest GPA peptide may be a promising candidate for the alleviation of DON-induced toxicity in humans and animals.

Key words：GPA; DON; oxidative stress; toxicity; Nrf2

1. Introduction

Deoxynivalenol (DON), a type B trichothecene, is one of the most common mycotoxins in grains, mainly produced by *Fusarium graminearum and Fusarium culmorum* (Pestka and Smolinski, 2005). DON causes a broad range of toxic effects on human and animals, i.e. growth suppression, loss of appetite, weight loss, intestinal hemorrhage, and dysregulation of the immune system, and large doses of DON would cause animals to vomit (Hussein and Brasel, 2001; Pestka, 2007).

DON causes serious damage to cells with high cytotoxicity (Rotter et al., 1993). Besides, DON disrupts the synthesis of DNA, RNA, and proteins, and influences the distribution of cell cycle, inhibits mitochondrial function, and induce apoptosis (Zhou et al., 1999). DON stability and difficulty to degrade and its ability to reach the digestive tract through food, makes the intestine the primary organ damaged (Bhattacharyya et al., 2014). Meanwhile, in the gut, DON induces large amounts of reactive oxygen species (ROS), leading to severe oxidative stress in cells, which further induces protein, lipid, and DNA damage (Wang et al., 2016). Oxidative stress could be a direct contributor to cellular damage, including cell death and membrane injury (Babu et al., 2015). For instance, DON dramatically increases the production of malondialdehyde (MDA), a lipid peroxidation product, in Caco-2 cells (Kouadio et al., 2005). DON induces oxidative stress and oxidative damage in IEC-6, which is caused by nicotinamide adenine dinucleotide phosphate (NADPH) (Del Regno et al., 2015). Therefore, alleviating oxidative stress would be an effective way to attenuate the toxic effects of the DON, thereby reducing its damage to humans and animals.

Notably, cells have developed antioxidant defense systems to protect against oxidative stress (Kensler et al., 2007). Antioxidant defense system mainly consists of endogenous antioxidant enzymes, endogenous non-enzymatic antioxidants, and exogenous antioxidants (Bhattacharyya et al., 2014). Endogenous antioxidant enzymes are mainly composed of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), glutamate-cysteine ligase modifier (GCLM), glutamate-cysteine ligase catalyzes (GCLC), endogenous antioxidant enzymes are reported to be regulated by Keap1-Nrf2 (Mathers et al., 2004; Tkachev et al., 2011). Once stimulated, Nrf2

dissociates from Keap1, and then translocate into the nucleus to bind to antioxidant response element (ARE), a cis-acting DNA regulatory element that activates the promoter regions of many genes encoding antioxidants, is key to the activation of multiple antioxidant enzymes (Mitsuishi et al., 2012). Therefore, we assume that enhancing antioxidant enzyme activity through activating Nrf2 may be an effective way to inhibit DON-induced oxidative stress.

In recent years, numerous studies have indicated that some nutrients, food components, and bioactive peptides exert beneficial antioxidant properties *in vivo* and *in vitro* (Chalamaiah et al., 2018; Martinez-Alvarez et al., 2015). And soy hydrolysate was reported to alleviate DON-induced damage of the epithelial barrier in vitro (Kiewiet et al., 2018). However, the effects of peptides on DON in vivo are unclear. We previously found that fish skin gelatin hydrolysate fraction 3 (FGSHF3), isolated from fish skin gelatin hydrolysate, contained several peptides, i.e. Gly-Pro-Ala, Gly-Pro-Arg, Gly-Pro-Hyp, Leu-Hyp-Gly, and Hyp-Pro, these peptides could activate Nrf2/ARE transcriptional activity to exert antioxidant effects (Zheng et al., 2018a; Zheng et al., 2018c). Among them, peptide Gly-Pro-Ala (GPA) results in the highest ARE-luciferase activity in IPEC-J2 cells (Zheng et al., 2018c). However, the antioxidant effects of FGSHF3 and GPA peptide in DON-induced oxidative stress and toxicity remain unknown. The objectives of this study are to investigate the effect of FGSHF3 and GPA in DON induced oxidative stress and toxicity. In the current study, we found that, through activating Nrf2, GPA up-regulates the expression of antioxidant enzymes and suppresses oxidative stress, thereby maintaining the tight junction, resulting in alleviating DON-induced toxicity. Taken together, these findings may provide an effective way to alleviate the harm caused by DON in humans and animals.

2. Materials and methods

2.1 Chemicals and reagents

Synthetic GPA peptides were purchased from Top-peptide Biotechnology Co., Ltd (Shanghai, China). FSGHF3 and GPA were prepared according to the previous method (Deng et al., 2020b; Deng et al., 2020c; Deng et al., 2020d; Zheng et al., 2018a; Zheng

et al., 2018c). Protease Inhibitor Cocktail (C0001) were purchased from Target Mol (Topscience, Shanghai, China). Cell lysis buffer for Western analysis (P0013), and phenylmethanesulfonyl fluoride (PMSF) (ST505) were purchased from Beyotime (Shanghai, China). Deoxynivalenol (D0156) was purchased from Sigma-Aldrich. The antibodies against PCNA (A13336) and β-actin (AC026) were purchased from Abclonal (Wuhan, China). The antibodies against Nrf2(12721), Keap1 (8047) and SOD1 (4266) were purchased from Cell Signaling Technology (Danvers, USA). The antibodies against GCLM (DF7268), GCLC (DF8550), ZO-1 (AF5145) and occludin (DF7504) were purchased from Affinity (Cincinnati, USA). MultiF Seamless Assembly Mix was purchased from Abclonal (Wuhan, China).

2.2 Cell culture

IPEC-J2 cells were obtained from BeNa Culture Collection (Beijing, China), which were cultured in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin under a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

DON was dissolved in ethanol for 10 mg/ml, then was diluted by PBS, IPEC-J2 cells were treated with DON (1μg/ml) to induced intestinal injury and oxidative stress.

2.3 Animals and treatments

5 weeks old male C57BL/6 mice were purchased from Hubei Provincial Center for Disease Control and Prevention. The mice were housed under specific pathogen-free conditions in an airconditioned room at 23±2°C. Food and water were supplied ad libitum. Animal welfare and experimental procedures were carried out in accordance with the criteria outlined in the Guide FOR THE CARE AND USE OF LABORATORY ANIMALS (Eight Edition) and the related ethical regulations of Huazhong Agricultural University.

Mice intestinal injury model was induced by DON (2mg/kg), which is continuously gavaged for 6 days. The experiment was randomly divided into 6 groups (n=12/group): control group, DON (2mg/kg) group, FGSHF3 (100 mg/kg) + DON (2mg/kg) group, FGSHF3 (200 mg/kg) + DON (2mg/kg) group, GPA (100 mg/kg) + DON (2mg/kg)

group, and GPA (200 mg/kg) + DON (2mg/kg) group. FGSHF3 or GPA was administered for 8 days before and during DON treatment via oral gavage once per day. After 14 days, the mice were humanely euthanized, and the colons were excised, measured, and sectioned for further analysis. The dose of GPA and FGSHF3 was based on previous studies (Deng et al., 2020a; Zhang et al., 2015; Zhang et al., 2018).

2.4 Cell Viability and membrane integrity assay

Cell viability and membrane integrity were analyzed by an MTT colorimetric assay and lactate dehydrogenase (LDH) release assay, respectively. Briefly, IPEC-J2 cells (2×10^4 cells/well in a 96-well plate) were incubated with 1 μg/ml of DON for 24 h either after or without pretreatment with FGSHF3 and GPA (1-5 mg/ml, for 24 h). After incubation, cells were washed with PBS and then incubated with MTT solution (0.5 mg/mL) for 4 h. The supernatant was then removed, and dimethyl sulfoxide was added to dissolve the formazan. The absorbance at 490 nm was detected by a Thermo BioMate 5 spectrophotometer, results were expressed as a percent of control values, MTT (M-KMLJM220123m) was purchased from Nanjing Camilo biological engineering co.LTD (Nanjing, China). The LDH release was determined using a cytotoxicity detection kit (P-KMLJ942260p, Nanjing Camilo biological engineering co.LTD, Nanjing, China). Three independent experiments (n=8/group).

2.5 Measurement of ROS production

The fluorescent probe $DCFH_2$-DA was used to detect the formation of intracellular ROS (P-KMLJ942013p, Nanjing Camilo biological engineering co.LTD, Nanjing, China). Briefly, IPEC-J2 cells (1×10^5 cells/well) were seeded in a 12-well plate to reach 80% confluence. After FGSHF3 and GPA pretreatment for 24 h followed by DON incubation for 24 h, the cells were incubated with 10 μM $DCFH_2$-DA at 37°C for 30 min. Finally, cells were washed with PBS for three times, and the fluorescence was quantified on a FACS Calibur cytometry system (BD Biosciences) with excitation at 488 nm and emission at 530 nm. Three independent experiments (n=6/group).

2.6 Determination of transepithelial electrical resistance

IPEC-J2 cells (2×10^5 cells/well) were seeded in transwell inserts (membrane area 0.33 cm^2, pore size 0.4 μm) placed in 24-well plates. Transepithelial electrical resistance (TEER) was monitored daily using an EVOM volt ohmmeter with STX2 electrodes (World Precision Instruments). When the resistance approached 500 Ω cm^2, cells were pretreated with or without FGSHF3 and GPA for 24 h followed by treatment with DON. TEER was measured every three hours during 12 h DON treatment. Three independent experiments (n=6/group).

2.7 RNA isolation and quantitative real-time PCR

IPEC-J2 cells (1 × 105 cells/well) were seeded in a 12-well plate and incubated with or without FGSHF3 or GPA for 12 h. Total RNA was extracted using Trizol reagent (Takara), according to the manufacturer's instructions, and quantified by a NanoDrop ND-1000 spectrophotometer. A total of 1 μg of RNA was then reverse-transcribed into cDNA using a Prime Script RT reagent kit (Bimake, Shanghai, China). And the primers are given in **Table 1**. The relative gene expression was adjusted with β-actin using the $2^{-\Delta\Delta CT}$ method and normalized to that of the control group. Three independent experiments (n=6/group).

2.8 Western blotting

After treatment, IPEC-2 cells were collected and whole-cell lysates were prepared using RIPA buffer containing a protease inhibitor cocktail. Nuclear and cytosol fractions were obtained using a nuclear/cytosol fractionation kit (BestBio, Shanghai, China). Western blotting was analyzed by ImageJ software. Three independent experiments (n=3/group).

2.9 Determination of antioxidant enzymes activity

After cell treatment, the medium was discarded, the cells were collected and dissolved in PBS, and the cells were broken by ultrasonic crushing apparatus. The jejunal tissues were homogenate and dissolved in PBS. According to the use method of

BCA kit (R-KMLJr31335, Nanjing Camilo biological engineering co.LTD), the protein concentration was determined. The activity of mouse-MDA (2M-KMLJM219464m), mouse-SOD1 (M-KMLJM220573m), mouse-GSH-Px (M-KMLJM220779m),pig-MDA (P-KMLJ941963p), pig-SOD1 (P-KMLJ942723p), pig-GSH-Px (P-KMLJ9427321p) in intracellular, plasma, and jejunal tissue were determined by the commercially available kit (Nanjing Camilo biological engineering co.LTD, Nanjing, China).

2.10 Morphological analysis of jejunum

The jejunal transected sample is put into the pre-formed 4% formalin fixative to denature and solidify the protein of the tissue. Sections of 5-mm thickness were deparaffinized in xylene and stained with H&E. Images were obtained using a DM3000 microscope (Leica Microsystems, Wetzlar, Germany). The villous height and crypt depth of the jejunum were measured by Image-Pro Plus 6.0 software.

2.11 Determination of Scavenging Activities for DPPH and Hydroxyl Radicals.

The DPPH radical scavenging activity was determined as follows, briefly, 0.5 mL sample was added to 2.0 mL of DPPH (0.1mM) in methanol. The mixture was left in dark for 30min at room temperature. The absorbance of the resulting solution was measured at 517 nm. The scavenging effect for DPPH radical was expressed as follows: scavenging activity (%) = [(control + blank − sample)/A control] ×100%.

Hydroxyl radical scavenging activity was determined as described as follows: scavenging activity (%) = [(sample − blank)/(control − blank)]×100%.

The IC_{50} value was defined as the concentration of sample required to scavenge 50% of the radical activity

2.12 RNA interference and transfection

Synthetic siRNA oligonucleotides specific for regions in the pig Nrf2 were designed and synthesized by GenePharma (Shanghai, China). The siRNA primers used are pig-Nrf2 siRNA, sense 5′-GCCCAUUGAUCUCUCUGAUTT-3′, and antisense 5′

-AUCAGAGAGAUCAAUGGGCTT-3 ′. Cells were transfected at 70%–80% confluence with siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions.

2.13 Statistical analyses

Values are expressed as means ± standard error of measurement (SEM) in figures, values are expressed as means ± standard deviation (SD) in tables. Before data analysis, the normality and variance homogeneity of results were checked. Differences between group means were determined by one-way ANOVA using SAS 8.0 software. The Tukey post hoc multiple comparison test was performed to compare significant variations. Differences were considered significant at $P < 0.05$.

3. Results

3.1 Protective effects of FGSHF3 and GPA on DON-induced toxicity and intestinal injury

We previously found that FGSHF3, isolated from fish skin gelatin hydrolysate, contained several peptides (Zheng et al., 2018c). Then we evaluated its antioxidant activities by DPPH and hydroxyl radical scavenging, the results showed FGSHF3 and peptides had antioxidant properties (Fig. S1AB). Subsequently, we evaluated antioxidant activities of FGSHF3 and peptides in IPEC-J2 cells, we found that FGSHF3 and peptides significantly increased ARE-Luc activity and inhibited H_2O_2-induced ROS (Fig. S1CD). Among peptides, peptide GPA results in the highest antioxidant activities activity in IPEC-J2 cells (Fig. S1CD), and GPA peptide was chosen in subsequent experiments. These results indicated FGSHF3 and peptides could exert protective effects on oxidative stress in vitro.

To evaluate the protective effects of FGSHF3 and GPA on DON-induced toxicity in vivo, we established a model of DON-induced toxicity in mice. Mice were treated with DON (2mg/kg) via oral gavage for 6 days to induce acute toxicity, and FGSHF3 (100, 200mg/kg) or GPA (100, 200mg/kg) were administered for 14 days before and during DON treatment via oral gavage once per day, mice were sacrificed at day 15

after toxicity induction.

As shown in Fig. 2, compared to the control group, average daily gain, and average daily feed intake were significantly decreased after DON administration for 6 consecutive days. FGSHF3 (200 mg/kg) or GPA (200 mg/kg) treatment for 14 days significantly increased average daily gain, compared to the DON group (Fig. 1A), while it did not affect average daily feed intake (Fig. 1B). It indicated that FGSHF3 and GPA inhibit the decrease of average daily weight gain without affecting feed intake.

For the visceral organ index, DON exerted toxicity in liver and kidney, compared to the control group, DON treatment significantly increased the weight of liver and kidney, without affected spleen weight. Meanwhile, FGSHF3 (200 mg/kg) or GPA (100, 200 mg/kg) treatment significantly alleviated DON-induced hepatomegaly, and FGSHF3 (200 mg/kg) or GPA (200 mg/kg) treatment significantly alleviated DON-induced renal enlargement (Table. 2). These results indicated that FGSHF3 and GPA exert a protective effect in DON-induced toxicity in vivo.

Mice treated with DON caused jejunum injuries, included in the length of villi in the proximal small intestine of mice was significantly reduced (P et al., 2015). The depth of the intestinal crypt represents the cell generation rate, the shallower the crypt, the better the cell maturity and the better the secretion function; and villi of the small intestine could increase the absorption area of the small intestine, which absorbs nutrients and as a filter (Tan and Barker, 2014). In the current study, DON significantly caused structural damage to the jejunum, and FGSHF3 or GPA treatment significantly alleviated DON-induced jejunum injuries (Fig. 1C). Furthermore, we detected villi length and crypt depth of jejunum, and the ratio of villi length to crypt depth. The results showed that DON significantly reduced villi length and increased crypt depth, compared to the control group. And compared to the DON group, GPA (200 mg/kg) treatment significantly increased villi length, and reduced crypt depth (Table. 3). At the same time, DON significantly reduced the ratio of villi length to crypt depth, and FGSHF3 (200 mg/kg) or GPA (200 mg/kg) treatment significantly alleviated DON-induced the decrease of the ratio of villi length to crypt depth (Table. 3). Overall, the results indicated that FGSHF3 and GPA maintain the intestinal structure and alleviate

jejunum injury.

3.2 FGSHF3 and GPA inhibited DON-induced cytotoxicity and maintain barrier function

Next, to confirm the antioxidant effects of FGSHF3 and GPA, we used DON to induce the oxidative stress model in IPEC-J2 cells. DON (1μg/ml) significantly reduced cell viability and increased cytotoxicity in IPEC-J2 cells, fortunately, pretreated with FGSHF3 (1-5mg/ml) or GPA (1-5mg/ml) significantly alleviated the decrease of cell viability, and inhibited cytotoxicity (Fig. 2AB).

Epithelial tight junction (TJ) proteins are an especially important aspect of the mechanical barrier, preventing harmful substances from breaching the mucosa, maintaining cellular integrity and permeability, and ensuring a relatively stable internal environment (Park et al., 2010). As shown in the result, TEER exhibited a marked reduction in a time-dependent manner after DON treatment, while it was alleviated by pretreatment with 2.5 mg/mL of FGSHF3 and GPA (Fig. 2C). And the tight junction proteins of Occludin and ZO-1 play an important role in maintaining barrier function in intestinal epithelial cells (Bashashati et al., 2015; McGuckin et al., 2009). Then we detected Occludin and ZO-1 expression by western blot, the results showed DON treatment significantly reduced the level of Occludin and ZO-1, while compared with DON group, 2.5 mg/mL of FGSHF3 and GPA pretreatment significantly increased the protein expression of Occludin and ZO-1 in IPEC-J2 cells (Fig. 2D). And in vivo, FGSHF3 and GPA pretreatment significantly increased the protein expression of Occludin and ZO-1 in jejunum tissues (Fig. 3EF). The results indicated that FGSHF3 and GPA maintain the intestinal barrier to reduce DON-induced cytotoxicity.

3.3 FGSHF3 and GPA enhanced antioxidant enzyme activity to relieve oxidative stress

Malondialdehyde (MDA) is a product of lipid peroxidation, and it is an effective indicator of oxidative damage. Then we detected the level of MDA in serum and jejunal. The results showed DON treatment increased level of MDA, and FGSHF3 (200

mg/kg) or GPA (200 mg/kg) treatment significantly inhibited MDA in serum and jejunal (Fig. 3AD).

Excessive generation of intracellular ROS causes cellular damage and disrupts the intestinal barrier, it is vital to inhibit ROS overproduction. Then we detected whether FGSHF3 and GPA could block the production of ROS caused by DON. The results showed DON caused a significant increase in ROS production, which was attenuated by FGSHF3 and GPA pretreatment in IPEC-J2 cells (Fig. S2A). The same with ROS, FGSHF3 and GPA pretreatment inhibited the level of MDA, caused by DON, in IPEC-J2 cells (Fig.S2B). It indicated that FGSHF3 and GPA alleviate DON-induced oxidative stress in vitro.

Antioxidant enzymes are a key driver of eliminating oxidative stress, then we detected antioxidant enzymes in vivo and in vitro. The results showed that DON inhibited antioxidant enzyme activity, such as T-SOD and GSH-PX, and FGSHF3 or GPA treatment significantly enhanced the activity of T-SOD and GSH-PX in serum and jejunal (Fig. 3BCEF). The results demonstrated that FGSHF3 or GPA may inhibit oxidative stress by enhancing antioxidant enzyme activity in vivo.

Next, we detected whether FGSHF3 and GPA could enhance antioxidant enzyme activity in IPEC-J2 cells. Then we detected the level of the antioxidant enzymes, such as CAT, SOD-1, GCLM, GCLC, by qPCR and western blot. As expected, treatment with FGSHF3 and GPA increased the expression of levels of CAT, SOD-1, GCLM, GCLC in IPEC-J2 cells (Fig. 5C-I). The results demonstrated that FGSHF3 or GPA may enhance antioxidant enzyme activity to suppress oxidative stress in vitro.

3.4 FGSHF3 and GPA enhanced the level of Nrf2 to enhance antioxidant enzyme activity.

Nuclear factor E2-related factor-2 (Nrf2) plays a critical role in protecting various tissues against oxidative stress through enhancing antioxidant enzyme activity (Kim et al., 2010). Then we detected the level of Nrf2 by qPCR and western blot. The results showed that FGSHF3 and GPA markedly increased the expression of Nrf2 in IPEC-J2 cells (Fig. 4AB). Besides, FGSHF3 and GPA markedly increased the ratio of Nrf2 to

Keap1 (Fig. 4B). And FGSHF3 and GPA also increased the expression of Nrf2 in jejunum tissues (Fig. 4C). Subsequently, we detected whether FGSHF3 and GPA could stimulate the translocation of Nrf2 from the cytosol into the nucleus. The results showed that the protein level of Nrf2 in the nuclear fractions was markedly elevated by FGSHF3 and GPA treatment (Fig. 4D), while there was no improvement in the cytosolic fractions (Fig. 4E). Based on the protein expression of Nrf2 in the total protein of IPEC-J2 cells, FGSHF3 and GPA effectively promoted the translocation of Nrf2 from the cytosol into the nucleus. Then we knockdown Nrf2 by siRNA in IPEC-J2 cells (Fig. 4E), we found that the effects on ROS and antioxidant enzyme activity of FGSHF3 and GPA were blocked after Nrf2 was knockdown (Fig. 4G-I). It indicated that FGSHF3 and GPA could alleviate oxidative stress by Nrf2 activation.

Discussion

DON has an extensive range of toxic effects on humans and animals. DON is known to be a deleterious agent, and cytotoxicity associated with DON is thought to occur mainly through changes in the expression of apoptosis-associated genes and proteins (Hussein and Brasel, 2001; Pestka, 2007). Present methods cannot completely remove DON from feed and grain (Bocai et al., 2010). Then it is vital to find an effective way to cope with it.

There have been increasing applications of biologically active peptides as a potential source of functional food ingredients for health promotion in recent years; they may be involved in the modulation of the immune response and suggested to be an attractive alternative to therapy against oxidative stress (Chalamaiah et al., 2018; Torres and Rios, 2008). However, the effects of peptides on DON-induced toxicity are unclear. In the present study, we found that, through activating Nrf2, FGSHF3 and GPA up-regulate the expression of antioxidant enzymes and suppress oxidative stress, thereby maintaining tight junction, inhibiting DON-induced intestinal injury and toxicity.

DON administration significantly decreases average daily gain and average daily feed intake, and FGSHF3 or GPA treatment significantly increase average daily gain,

while it did not affect average daily feed intake. It indicates that FGSHF3 and GPA inhibit the decrease of average daily weight gain without affecting feed intake. This may be because peptides alleviate oxidative stress and protect the intestinal structure, enabling mice to absorb nutrients and promote growth. Furthermore, FGSHF3 and GPA exert a protective effect in DON-induced jejunum injury and enlargement of the liver and spleen. Meanwhile, FGSHF3 is composed of peptide or amino acids, which are common nutrients without cytotoxic effect in vitro. Therefore, it supports that FGSHF3 and GPA may be an optional agent or at least a nutritional supplement to support the prevention of DON-induced intestinal injury and toxicity.

Numerous studies have reported that the intestinal barrier plays a key role in maintaining intestinal health (Bashashati et al., 2015; McGuckin et al., 2009). The structural abnormalities in tight junction proteins are the major cause of altered the intestinal barrier in oxidative stress (Kucharzik et al., 2001). Tight junction proteins are capable of decreasing the permeability of intestinal mucosa and restraining foreign substances from crossing the intestinal mucosa, which results in suppression of ROS overproduction (Jeong et al., 2015; Suzuki, 2013). In the current study, we found that FGSHF3 and GPA exert a protective effect on barrier integrity by maintaining the expression of ZO-1 and Occludin in vivo and in vitro. It suggests an effective way, through maintaining tight junction, to alleviate DON-induced intestinal injury and toxicity.

Oxidative stress is usually induced by an imbalance of ROS, which leads to intestinal disorders via damage to the intestinal barrier (Vergauwen et al., 2015). Overproduction of ROS or lack of antioxidants usually results in oxidative stress and even cell death (Nathan and Cunningham-Bussel, 2013). More important, ROS, as a signal molecule, activates several typical inflammatory signaling pathways, leading to an inflammatory response and further damage intestinal barrier (Ray et al., 2012; Zhang et al., 2017). Then it is effective to maintain the intestinal barriers by inhibiting oxidative stress. Notably, cells have developed antioxidant defense systems to guard against oxidative stress (Kensler et al., 2007). Therefore, increasing the expression of antioxidant enzymes could be considered as an effective target for preventing oxidative

stress. And Nrf2, a redox-sensitive master-regulated transcription factor, plays an important role in cellular defenses against oxidative through mediating the expression of various antioxidant enzymes (Kensler et al., 2007). Combined with previous studies, we suggest that FGSHF3 and GPA suppress oxidative stress by activating Nrf2 to up-regulate the expression of antioxidant enzymes.

In conclusion, FGSHF3 and GPA peptide activate Nrf2 to up-regulate the expression of antioxidant enzymes, resulting in suppressing oxidative stress in intestinal. Ultimately, FGSHF3 and GPA maintain the intestinal barrier and inhibit DON-induced toxicity. Therefore, our results suggest that GPA peptide could potentially be used for the prevention of DON-induced toxicity.

Conflict of interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Ethics statement

Our study was approved by the Ethics Committee of Huazhong Agricultural University, Wuhan City, Hubei Province, China.

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Figures and tables legends

Figure 1. Protective effects of FGSHF3 and GPA on DON-induced toxicity and jejunum injury in vivo

Mice intestinal injury model was induced by DON (2mg/kg), which is continuously gavaged for 6 days. FGSHF3 or GPA was administered for 8 days before and during DON treatment via oral gavage once per day. After 14 days, the mice were humanely euthanized. Average daily gain of C57BL/6 mice (A). Average daily feed intake of C57BL/6 mice (B). The jejunums from each experimental group were processed for histological evaluation (hematoxylin–eosin staining, 100×, scale bar, 100μm) (C). Data are expressed as means ± SEM; n = 12/group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Figure 2. FGSHF3 and GPA inhibit DON-induced cytotoxicity and maintain barrier function in vitro and in vivo

Cells were preincubated with FGSHF3 and GPA for 24 h and then incubated with 1μg/ml DON for 24h. Cell viability and membrane integrity were measured by MTT assay and lactate dehydrogenase (LDH) release assay, respectively (A, B). Data are expressed as means ± SEM, three independent experiments (n=8/group).

Cells were preincubated with FGSHF3 and GPA for 24 h and then incubated with 3μg/ml DON for 3-12 h. Transepithelial electrical resistance (TEER) was determined every three hours (C). Data are expressed as means ± SEM three independent experiments (n=6/group).

Cells were preincubated with FGSHF3 and GPA for 24 h and then incubated with 1μg/ml DON for 6h. Protein expression of Occludin and ZO-1 were estimated by Western blot (D). Data are expressed as means ± SEM, three independent experiments (n=3/group).

mRNA levels Occludin and ZO-1 were estimated by qPCR in jejunum tissues (E, F). Data are expressed as means ± SEM (n=12/group). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Figure 3. FGSHF3 and GPA enhance antioxidant enzyme activity to relieve oxidative stress in vivo.

Content of MDA, T-SOD, and GSH-Px in mice serum were measured by kit (ABC), Data are expressed as means ± SEM (n=12/group).

Content of MDA, T-SOD, and GSH-Px in mice jejunal tissues were measured by kit (ABC), Data are expressed as means ± SEM (n=12/group). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Figure 4. FGSHF3 and GPA enhance the level of Nrf2 to enhance antioxidant enzymes activity.

Cells were preincubated with FGSHF3 and GPA for 24 h and then incubated with 1μg/ml DON for 6h. Relative mRNA expression of Nrf2 was quantified using qPCR (n=6/group) (A). Protein expression of total Nrf2 and Kelch-like ECH-associated protein 1 (Keap1) was estimated by Western blot (n=3/group) (B). mRNA levels Nrf2 was estimated by qPCR in mice jejunum tissues (n=12/group) (C). Nrf2 in the nuclear and cytosolic fractions was isolated, and protein levels were estimated by Western blot in IPEC-J2 cells (n=3/group) (DE).

Cells were transfected with control or siRNA-Nrf2, then preincubated with FGSHF3 and GPA for 24 h and then incubated with 1μg/ml DON for 6h. Relative mRNA expression of Nrf2, GCLM and GCLC were quantified using qPCR (FGH), the intracellular ROS levels were measured by DCF fluorescence microscopy (n=6/group) (I). Data are expressed as means ± SEM, three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Figure S1. Protective effects of FGSHF3 and peptide on oxidative stress in vitro

The effects of FGSHF3 and peptide on DPPH radical scavenging activity and hydroxyl radical scavenging activity (AB). IPEC-J2 cells were preincubated with FGSHF3 (2.5mg/ml) and peptide (2.5mg/ml) for 24 h, then ARE-luciferase activity was measured (C). IPEC-J2 cells were preincubated with FGSHF3 (2.5mg/ml) and peptide

(2.5mg/ml) for 24 h, followed by incubation with 0.5 mM H_2O_2 for 12h, then ROS was measured (D). Data are expressed as means ± SEM; three independent experiments (n=6/group). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Figure S2. FGSHF3 and GPA enhance antioxidant enzyme activity to inhibit ROS production in IPEC-J2 cells.

IPEC-J2 cells were preincubated with FGSHF3 and GPA for 24 h and then incubated with 1μg/ml DON for 24h. The intracellular ROS levels were measured by DCF fluorescence microscopy (A). And the content of MDA was determined (B). IPEC-J2 cells were preincubated with FGSHF3 and GPA for 24 h and then incubated with 1μg/ml DON for 6h. Relative mRNA expression of antioxidant enzymes was quantified using real time RT-PCR (C-F). Data are expressed as means ± SEM; three independent experiments (n=6/group).

Protein expression of antioxidant enzymes was estimated by Western blot, three independent experiments (n=3/group) (G). Intracellular glutathione (GSH) and SOD-1 enzymatic activity were determined (HI). Data are expressed as means ± SEM; three independent experiments (n=8/group). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Table 1 Primer sequences used for the real-time PCR analysis

Table 2. Effects of FGSHF3 and GPA on the relative weight of organs in C57BL/6 mice.

Data are expressed as means ± SD (n = 12/group). $^{a\text{-}b}$ Values within a row with different lowercase letters differ significantly in lactation time at $P < 0.05$.

Table 3. Effects of FGSHF3 and GPA on jejunal histomorphology in C57BL/6 mice.

Data are expressed as means ± SD (n = 12/group). $^{a\text{-}c}$ Values within a row with different lowercase letters differ significantly in lactation time at $P < 0.05$.