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Original Research Article

# Phenethyl isothiocyanate as an anti-nutritional factor attenuates deoxynivalenol-induced IPEC-J2 cell injury through inhibiting ROS-mediated autophagy

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ABSTRACT

Deoxynivalenol (DON) is considered to be the most harmful mycotoxin that affects the intestinal health of animals and humans. Phenethyl isothiocyanate (PEITC) in feedstuff is an anti-nutritional factor and impairs nutrient digestion and absorption in the animal intestinal. In the current study, we aimed to explore the effects of PEITC on DON-induced apoptosis, intestinal tight junction disorder, and its potential molecular mechanism in the porcine jejunum epithelial cell line (IPEC-J2). Our results indicated that PEITC treatment markedly alleviated DON-induced cytotoxicity, decreasing the apoptotic cell percentage and pro-apoptotic mRNA/protein levels, and increasing zonula occludens-1 (ZO-1), occludin and claudin-1 mRNA/protein expression. Meanwhile, PEITC treatment ameliorated DON-induced an increase of the inducible nitric oxide synthase (*iNOS*) and cyclooxygenase 2 (*COX-2*) mRNA levels and intracellular reactive oxygen species (ROS) level, and a decrease of glutathione peroxidase 1 (*GPx1*), superoxide dismutase 2 (*SOD2*), catalase (*CAT*) and heme oxygenase 1 (*HO-1*) mRNA levels. Additionally, PEITC treatment significantly down-regulated autophagy-related protein 5 (ATG5), beclin-1 and microtubule-associated protein 1 light chain 3B (LC3-II) mRNA/protein levels, decreased the number of green fluorescent protein-microtubule-associated protein 1 light-chain 3 (GFP-LC3) puncta and phosphatidylinositol 3 kinase (PI3K) protein expression, and up-regulated phospho-protein kinase B (p-Akt) and phospho-mammalian target of rapamycin (p-mTOR) protein expression against DON. However, the activation of autophagy by rapamycin, an autophagy agonist, abolished the protective effects of PEITC against DON-induced cytotoxicity, apoptosis and intestinal tight junction disorder. Collectively, PEITC could confer protection against DON-induced porcine intestinal epithelial cell injury by suppressing ROS-mediated autophagy.



## 1. Introduction

Mycotoxins belong to secondary metabolites of fungi, which frequently contaminate feed, cereal crops and foods worldwide, and pose potential health hazards to animals and humans. Globally, approximately 25% of crops such as rice, cereals and nuts are contaminated with mycotoxins, which are considered as a major safety risk factor for animal and human health (Berthiller et al., 2013; Streit et al., 2013). Deoxynivalenol (DON), a mycotoxin mainly synthesized by *Fusarium* species in wheat, corn and barley,

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also known as vomiting toxin, has the highest occurrence and over-standard rate (Pestka, 2010; Wu et al., 2016). The toxicity caused by DON can be classified as acute or chronic. Acute exposure to DON can induce vomiting, diarrhea, circulatory shock, gastrointestinal bleeding and even death with extremely high exposure (Mishra et al., 2020; Pestka, 2010). At a chronic low dose, DON exposure over a long period time can cause growth retardation, immune system disorder and intestinal barrier disruption (Liao et al., 2018; Vignal et al., 2018; Wang et al., 2019). Therefore, the adverse effects of acute and chronic DON consumption on human and animal health have become a global concern for researchers.

The intestine is the first defensive barrier and the most exposed organ against the luminal content, which includes toxic substances. DON efficiently crosses the small intestinal epithelial barrier by passive diffusion, and the small intestinal cells display the most sensitivity to DON (Videmann et al., 2007). In vitro, intestinal porcine epithelial cell line (IPEC-J2 cell), separated from the jejunum of newborn piglets, is widely used to study the effects of toxins on intestinal function. Our previous studies have revealed that DON exposure reduced the cell viability of IPEC-J2 in a dose- and time-dependent manner (Ge et al., 2020; Ying et al., 2019). DON also impaired intestinal nutrition transport, affected the structure of the intestine, decreased the production of mucus, and impaired the tight junction proteins expression, leading to disruption of the intestinal barrier function (Li et al., 2018; Pasternak et al., 2018; Wang et al., 2020). Further, increasing evidence has indicated that oxidative stress plays a key role in DON-induced intestinal toxicity, apoptosis and inflammation in vivo and vitro (Broom, 2015; Kang et al., 2019; Osselaere et al., 2013). When the reactive oxygen species (ROS) attain a maximum resistance threshold, it will lead to organelle damage and eventually cell death.

Phenethyl isothiocyanate (PEITC), belonging to glucosinolates (Gls), is a secondary organosulfur plant metabolite that exists extensively in all Brassica-originated fodders. PEITC is produced by the breakdown of gastrointestinal microbiota, which belong to an anti-nutritional factor in feedstuff (Dinkova-Kostova and Kostov, 2012; Liu et al., 2017). It has been proven that a substantial amount of PEITC ingestion might be deleterious to animal health and production by reducing digestibility and nutrient utilization which might be associated with the drastic endocrine disturbance induced by PEITC, especially in pigs (Tripathi and Mishra, 2007). High PEITC intake reduced palatability (due to their bitter taste), stimulated the mucosa to produce gastroenteritis, inhibited the synthesis of iodine, caused liver, kidney and thyroid hypertrophy and increased mortality (Burel et al., 2000; Duchamp et al., 1996; Mawson et al., 1994; Tripathi and Mishra, 2007). It has previously been observed that a high dose of PEITC had a cytotoxic by inducing oxidative stress and apoptosis in vitro (Liu et al., 2019; Zhu et al., 2020). Further, several studies have demonstrated that PEITC could exert an anti-inflammatory and antioxidant capacity in some cancer cells by inhibiting NF-κB or activating the Nrf2 signaling pathway (Krajka-Kuźniak et al., 2020; Liu, 2017; Okubo et al., 2010).

DON-contaminated feedstuffs primarily affect the intestinal health of animals. And DON and PEITC can exist in feedstuffs simultaneously. But there is little study regarding the effect of PEITC on DON-mediated intestinal injury. Hence, the present study aimed to explore the protective effects of PEITC on oxidative stress, apoptosis and intestinal tight junction disorder induced by DON and its potential mechanism using the IPEC-J2 cell as a model, which will provide a theoretical basis for the prevention and control of DON in feedstuff.

## 2. Materials and methods

### 2.1. Chemical and antibodies

DON (purity >99%), PEITC (purity >99%), rapamycin (Rapa), dimethyl sulfoxide (DMSO, purity >96%), and rabbit polyclonal microtubule-associated protein 1 light chain 3B (LC3B) antibody were purchased from Sigma−Aldrich (St. Louis, USA). Rabbit autophagy-related protein 5 (ATG5) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Rabbit phosphoprotein kinase B (p-Akt), rabbit phospho-mammalian target of rapamycin (p-mTOR), rabbit phosphatidylinositol 3 kinase (PI3K), mouse β-actin and horseradish peroxidase (HRP)-conjugated goat anti-rabbit or -mouse secondary antibodies were purchased from Cell signaling Technology (Boston, USA). Rabbit zonula occludens-1 (ZO-1), rabbit occludin, rabbit Akt and rabbit mTOR antibodies were purchased from Abcam (Cambridge, UK). Rabbit caspase-3 and rabbit caspase-9 antibodies were purchased from Abmart Shanghai Co.,Ltd (Abmart, China).

### 2.2. Cell culture

The IPEC-J2 cell line was used between passages 10 to 30, and grown in DMEM/F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) (Gibco, USA) at 37 °C under a humidified atmosphere containing 5% $CO_2$ in a cell culture incubator (Thermo Fisher, USA).

### 2.3. MTT assay

Cell viability was detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyltetrazolium bromide (MTT, Sigma, USA) as previously described (Liu et al., 2021). Briefly, IPEC-J2 cells were cultured in 96-well plates at a concentration of $4 \times 10^3$ cells/well and exposed to DON or PEITC alone and in combination for 24 h. Then, 15 μL of MTT (5 mg/mL) was added into each well. After 4 h incubation, the supernatant was removed and added 150 μL DMSO to dissolve the precipitate. The absorbance at 490 nm was determined using a Microplate Reader (Bio-Rad, USA). All tests were performed 3 times.

### 2.4. Lactate dehydrogenase (LDH) activity assay

IPEC-J2 cells were seeded in 96-well plates at a density of $4 \times 10^3$ cells/well with corresponding treatments. Then the supernatant was collected and centrifuged at 12,000 × *g* for 10 min at 4 °C. LDH activity was measured by using LDH kits according to the manufacture's instructions (Jiancheng, China).

### 2.5. Analysis of apoptosis by Hoechst 33,258 staining

IPEC-J2 cells were cultured in the 20-mm diameter round glass coverslips (WHB, China), which were placed in a 12-well plate at a density of $1 \times 10^5$ cells/well. After treatment, cells were stained with Hoechst 33,258 (Beyotime, China) for 10 min. Finally, the slides were washed 3 times with phosphate buffer solution (PBS) and scanned with a fluorescence microscope (Zeiss, Germany).

### 2.6. Annexin V-FITC apoptosis detection

IPEC-J2 cells were cultured at a concentration of $2 \times 10^5$ cells/well in 6-well plates with the corresponding treatment. Then, cell apoptosis ratio was detected according to Annexin after the cells were digested with trypsin without ethylene diamine tetraacetic

acid (EDTA). Annexin V-FITC apoptosis detection kit (BD Pharmingen, USA) was used to measure the cell apoptosis. The obtained cells were washed twice with PBS and centrifuged for 5 min at 1,000 × *g*, then resuspended with 100 μL of binding buffer and stained with 5 μL of annexin V-FITC and 5 μL of PI in the dark at room temperature for 15 min. Finally, 400 μL binding buffer was added into each sample for flow cytometric analysis (FASC Calibur, USA).

### 2.7. Intracellular ROS assay

Intracellular ROS in IPEC-J2 cells were quantified by using an $H_2$DCF-DA probe (Beyotime, China). IPEC-J2 cells were plated in a 12-well plate at a density of $1 \times 10^5$ cells/well. After treatment, the cells were rinsed twice with PBS and incubated with $H_2$DCF-DA (10 μmol/L) at 37 °C for 30 min in the dark. The fluorescence signals were detected by fluorescence microscopy at 488 (excitation) and 581 nm (emission).

### 2.8. Quantitative real-time PCR (qRT-PCR) analysis

The RNA isolation and qRT-PCR were determined as our previous study using Trizol reagent (TaKaRa, China) (Liu et al., 2021). cDNA was synthesized using the Prime Script RT Master Mix Kit (Takara, China) according to the manufacturer's instructions. The relative mRNA levels of target genes were determined using the $2^{-\Delta\Delta Ct}$ method with β-actin serving as a reference gene. The primers used were synthesized by Sangon Biotech (Shanghai, China) and are shown in Table 1.

### 2.9. Western blotting analysis

After corresponding treatment, the cells were washed with cold PBS and lysed with cold RIPA buffer (Beyotime, China) with a protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Next, the total protein concentration was assessed by the BCA protein assay kit (Beyotime, China). Equal amounts of denatured protein were loaded on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, France). The membranes were incubated with primary antibodies overnight at 4 °C, followed by a 1 h incubation with respective secondary antibodies at room temperature. The protein bands were visualized by using Image Quant LAS 4000 (GE Healthcare Life Sciences, USA) and quantified by using Image Pro-Plus 6.0 software (Media Cybernetics. Sarasota, USA).

### 2.10. Confocal immunofluorescence

IPEC-J2 cells were grown on coverslips to approximately 60% to 70% confluence in a 12-well plate, and transfected with the GFP-LC3 plasmid using a X-tremeGENE HP DNA transfection reagent (Roche, USA) according to the manufacturer's protocols for 12 h. Then, the cells were fixed with 4% paraformaldehyde for 15 min and nuclei were stained with DAPI (Beyotime, China) for 5 min. The localization of LC3 was visualized using a Zeiss LSM710 confocal microscope (Zeiss, Germany).

### 2.11. Statistics analysis

All experiments have been performed a minimum of 3 times with similar results. All data were analyzed by one-way analysis of variance (ANOVA) using SPSS 20.0 for Windows, and the data were presented as the mean ± SEM of the indicated number of replicates.

## 3. Results

### 3.1. PEITC treatment attenuated DON-induced cytotoxicity in IPEC-J2 cells

First, the cytotoxicity effect of DON (0.5 to 16 μmol/L) and PEITC (0 to 40 μmol/L) on IPEC-J2 cell viability were measured by MTT and LDH release assay. As shown in Fig. 1A the cell viability of IPEC-J2 cells was decreased linearly with increasing concentration of DON. Furthermore, 2 to 16 μmol/L of DON treatment significantly increased linearly the LDH activity ($P < 0.01$, Fig. 1B). As shown in Fig, 1B and E, PEITC displayed no cytotoxicity to IPEC-J2 cells except at the concentrations beyond 5 μmol/L. Thus, 1 μmol/L of DON and 1.25, 2.5, 5 μmol/L of PEITC was chosen in the subsequent experiment. To assess the effects of PEITC on DON-induced cytotoxicity, we measured the viability and the LDH release of IPEC-J2 cells pretreated with PEITC in the presence of DON. As shown in Fig. 1C, F 2.5, 5 μmol/L PEITC significantly suppressed DON-induced decrease in cell viability and DON-induced increase in LDH activity ($P < 0.01$). These results suggested that PEITC could protect against DON-induced cytotoxicity in IPEC-J2 cells.

### 3.2. PEITC treatment alleviated DON-induced apoptosis in IPEC-J2 cells

To determine the effects of PEITC on DON-induced apoptosis, we measured the apoptosis-related mRNA/protein expression and the apoptotic cell percentage in IPEC-J2 cells. As shown Fig. 2A–F, DON exposure significantly enhanced the mRNA expression of *Bax* and *caspase-3*, up-regulated the protein expression ratios of cleaved caspase-3/pro caspase-3 and cleaved caspase-9/pro caspase-9, decreased the mRNA expression of *Bcl-2* ($P < 0.01$). But these changes were markedly reversed by 1.25, 2.5 and 5 μmol/L PEITC treatment in a dose-dependent manner. Meanwhile, DON alone induced nuclear condensation, and enhanced the number of apoptotic body and the total apoptosis ratio (Fig. 2G–I). But the increases were markedly reduced by PEITC treatment ($P < 0.01$). Thus, these results showed that PEITC protected against DON-induced apoptosis in IPEC-J2 cells.

### 3.3. PEITC treatment alleviated DON-induced tight junction disorder in IPEC-J2 cells

To evaluate the potential effects of PEITC on DON-induced intestinal tight junction dysfunction, we assessed the mRNA expression of *ZO-1*, *occludin* and *claudin*-1 by qRT-PCR, and the protein expression of ZO-1 and occludin by Western blotting. As presented in Fig. 3, DON exposure significantly decreased the ZO-1, occludin and claudin-1 mRNA and protein expressions compared with the control group ($P < 0.01$). However, PEITC treatment could reverse DON-induced decreases of above mRNA and protein levels. Thus, these results suggest that PEITC treatment had a beneficial effect on DON-induced tight junction of intestinal in IPEC-J2 cells.

### 3.4. PEITC treatment alleviated DON-induced oxidative stress in IPEC-J2 cells

To assess the effects of PEITC on DON-induced oxidative stress, we measured the mRNA levels of inducible nitric oxide synthase (*iNOS*), cyclooxygenase 2 (*COX-2*), glutathione peroxidase 1 (*GPx1*), superoxide dismutase 2 (*SOD2*), catalase (*CAT*) and heme oxygenase 1 (*HO-1*) by qRT-PCR, and intracellular ROS by using an $H_2$DCF-DA probe in IPEC-J2 cells. As shown in Fig. 4, DON-treated cells showed a significant increase in *iNOS* and *COX*-2 mRNA levels, and intracellular ROS level ($P < 0.01$). Meanwhile, DON exposure markedly

**Table 1**
Primers used for qRT-PCR.

| Gene | Accession number | Primer sequence (5′–3′) | Product, bp |
|---|---|---|---|
| *HO-1* | NM_001004027.1 | F: AGCTGTTTCTGAGCCTCCAA<br>R: CAAGACGGAAACACGAGACA | 130 |
| *iNOS* | NC_010454.4 | F: GGGTCAGAGCTACCATCCTC<br>R: CGTCCATGCAGAGAACCTTG | 141 |
| *COX-2* | NC_010451.4 | F: TGCGGGAACATAATAGAG<br>R: GTATCAGCCTGCTCGTCT | 90 |
| *GPx1* | NM_214201.1 | F: CCTAGCAGTGCCTAGAGTGC<br>R: CGCCCATCTCAGGGGATTTT | 143 |
| *SOD2* | NM_214127.2 | F: GGCCTACGTGAACAACCTGA<br>R: TGATTGATGTGGCCTCCACC | 126 |
| *CAT* | NM_214301.2 | F: CCTGCAACGTTCTGTAAGGC<br>R: GCTTCATCTGGTCACTGGCT | 72 |
| *Bax* | XM_003355975.1 | F: GCCCTTTTGCTTCAGGGGATG<br>R: GGCAAAGTAGAAAAGCGCGA | 135 |
| *Bcl-2* | XM_003122573.2 | F: AACTAGGGCTGGGCTCCTTTA<br>R: TCCTGCTCACTCTGCTCAAAC | 120 |
| *Caspase-3* | NM_214131.1 | F: TGGGATTGAGACGGACAGTG<br>R: CGCTGCACAAAGTGACTGGA | 157 |
| *ZO-1* | XM_021098848.1 | F: ATGAGCAGGTCCCGTCCCAAG<br>R: GGCGGAGGCAGCGGTTTG | 142 |
| *Occludin* | XM_005672522.3 | F: GACAGACTACACAACTGGCGG<br>R: TGTACTCCTGCAGGCCACTG | 134 |
| *Claudin-1* | NM_001244539.1 | F: CCATCGTCAGCACCGCACTG<br>R: CGACACGCAGGACATCCACAG | 107 |
| *LC3B* | NM_001170827.1 | F: TGTCAACATGAGCGAGTTGG<br>R: TCACCATGCTGTGCTGGTTC | 98 |
| *ATG5* | NM_001037152.2 | F: TTGCTCCTGAAGATGGGGAA<br>R: TATCCGGGTAGCTCAGATGT | 102 |
| *Beclin-1* | NM_001044530.1 | F: ACTTGTTCCCTATGGAAACCATTC<br>R: CTTTCTCCACATCCATCCTGTAAG | 215 |
| *β-actin* | XM_021086047.1 | F: CTGCGGCATCCACGAAACT<br>R: AGGGCCGTGATCTCCTTCTG | 147 |

*HO-1* = heme oxygenase 1; *iNOS* = inducible nitric oxide synthase; *COX-2* = cyclooxygenase 2; *GPx1* = glutathione peroxidase 1; *SOD2* = superoxide dismutase 2; *CAT* = catalase; *ZO-1* = zonula occludens-1; *LC3B* = microtubule-associated protein 1 light chain 3B; *ATG5* = autophagy-related protein 5.

decreased the mRNA levels of *GPx1*, *SOD2*, *CAT* and *HO-1*. However, PEITC treatment could suppress DON-induced increase in the oxidative stress-related mRNA levels. 1.25, 2.5 and 5 μmol/L PEITC showed the ability to reduce the production of intracellular ROS induced by DON, and increased the mRNA levels of antioxidant genes markedly. Thus, these results revealed that PEITC might alleviate DON-induced oxidative stress in IPEC-J2 cells.

### 3.5. PEITC treatment inhibited DON-induced autophagy in IPEC-J2 cells

Fig. 5 elaborated the effects of PEITC on cellular autophagy induced by DON. Treatment with 1 μmol/L DON had higher *ATG5*, *beclin-1* and *LC3B* mRNA expressions than that in the control group ($P < 0.01$, Fig. 5A–C). To further identity whether autophagy was triggered by DON treatment, green fluorescent protein-microtubule-associated protein 1 light-chain 3 (GFP-LC3), a specific marker of autophagic vesicles and autophagic activity was transfected to IPEC-J2 cells. As presented in Fig. 5G 1 μmol/L DON exposure had higher the number of GFP-LC3 puncta than the control cell ($P < 0.01$). The results from Fig. 5D–F revealed that DON exposure markedly increased the PI3K, ATG5 and LC3-II protein expression, and decreased the protein expression of p-Akt and p-mTOR. However, PEITC supplementation significantly reduced DON-induced increase in the mRNA levels of *ATG5*, *beclin-1* and *LC3B* in a dose-dependent manner. Meanwhile, 5 μmol/L PEITC treatment markedly suppressed the protein expression of PI3K, ATG5 and LC3-II and the number of GFP-LC3 puncta, and increased the protein expression of p-Akt and p-mTOR compared to the 1 μmol/L DON-treated group. Thus, these results suggested that activating PI3K/Akt/mTOR signaling pathway might be required for PEITC to alleviate the autophagy induced by DON.

### 3.6. Activation of autophagy by rapamycin decreased the protection provided by PEITC against DON-induced intestinal tight junction disorder in IPEC-J2 cells

To further explore whether autophagy played a crucial role in the effects of PEITC on the DON-induced porcine intestinal epithelial cell injury in vitro, IPEC-J2 cells were pre-treated with rapamycin (50 nmol/L) for 2 h before exposure to DON and PEITC combined. Rapamycin was an autophagy agonist and promoted cell autophagy by inhibiting mTOR pathway. As shown in Fig. 6A, E, rapamycin pretreatment significantly enhanced *ATG5*, *beclin-1* and *LC-3B* mRNA levels and the number of GFP-LC3 puncta compared with the control group ($P < 0.01$). Furthermore, rapamycin pretreatment significantly decreased the ratio of p-mTOR/mTOR, and increased ATG5 and LC-3 II protein expression (Fig. 6D, F). However, the effects of PEITC on the autophagy-related mRNA/protein levels were significantly exacerbated by rapamycin (Fig. 6A, D and F). Rapamycin reversed the effects of PEITC on the apoptosis-related mRNA/protein levels and intestinal tight junction mRNA/protein levels (Fig. 6B–D and G). Meanwhile, pretreatment with rapamycin showed a lowered cell viability of IPEC-J2 cells and higher LDH activity than the combination of DON and PEITC group ($P < 0.05$, Fig. 6H, I). These results suggested that PEITC protected against DON-induced intestinal tight junction disorder through inhibiting autophagy via activation of mTOR in IPEC-J2 cells.

## 4. Discussion

Deoxynivalenol (DON), as the mycotoxin with the highest detected ratio in feedstuff, seriously threatens animal and human health (Wu et al., 2016). Swine are the most susceptible animals to

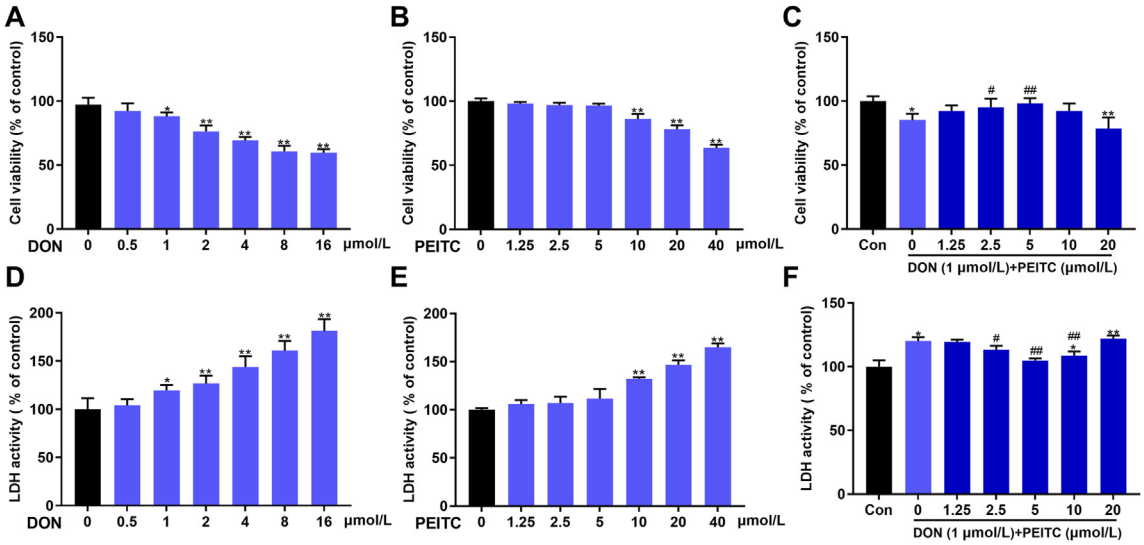


**Fig. 1. Effects of various concentrations of DON and PEITC alone or combination on IPEC-J2 cells.** The cell viability (A–C) and LDH activity (D–F) were assayed. Date are presented as mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the DON alone group. DON = deoxynivalenol; PEITC = phenethyl isothiocyanate.

DON exposure. Numerous studies have confirmed that DON could affect nutrient absorption, disrupt the intestinal barrier integrity, and induce the oxidative stress and inflammation response of pigs (Liao et al., 2020; Martinez et al., 2019; Pasternak et al., 2018). Regardless of several antioxidants being reported to alleviate DON-induced cytotoxicity, effective treatments for DON oxidative stress and intestinal tight junction require further investigation (Long et al., 2021).

Phenethyl isothiocyanate (PEITC) is a natural plant metabolite that widely existed in its glucosinolate precursor form in cruciferous vegetables. It was reported that PEITC could attenuate dextran sodium sulfate (DSS)-induced ulcerative colitis by modulation of nuclear factor kappa-B (NF-κB) signaling (Liu, 2017). Nevertheless, the protective effects of PEITC against DON-induced cell damage is not clear. Therefore, the present study used the IPEC-J2 cell line as a model to explore the potential protective effect and molecular mechanism of PEITC on alleviating cell damage, oxidative stress and intestinal tight junction disorder caused by DON. Some previous studies have demonstrated that DON exposure could affect cell proliferation, damage intestinal barrier function and apoptosis in IPEC-J2 cells (Ge et al., 2020; Kang et al., 2019; Liao et al., 2017). These results are consistent with our findings that DON exposure reduced the cell viability, and increased the LDH activity in IPEC-J2 cells in a dose-dependent manner. However, the adverse effects were markedly reversed by PEITC treatment. Overall, our results proved that PEITC has the ability to protect against DON-induced cytotoxicity in IPEC-J2 cells.

Oxidative stress is usually induced by an imbalance in the accumulation of ROS and the antioxidant capability of the cells (Birben et al., 2012). ROS are produced by normal cellular metabolism and environmental factors, which act as a double-edged sword. Low levels of ROS as a signaling messenger can promote proliferation, mitosis, innate immune responses and stress–responsive pathways (Chen et al., 2016; Sena and Chandel, 2012), whereas high levels of ROS may change DNA structure, affect modification of lipids and proteins, induce the production of proinflammatory cytokines and apoptosis which ultimately induces cell death (Gorrini et al., 2013; Wiseman and Halliwell, 1996). Meanwhile, oxidative stress results in intestinal tight junction disorder by disrupting the intestinal barrier, and inducing inflammation and apoptosis in the intestinal epithelium (Adesso et al., 2017; Vergauwen et al., 2015). According to a previous study, DON exposure enhanced the intracellular ROS levels, reduced CAT, SOD and GSH-Px activities, and induced apoptosis in IPEC-J2 cells (Kang et al., 2019). In this present study, we measured the activities of antioxidant enzymes by qRT-PCR, and detected the ROS levels by using an $H_2$DCF-DA probe. Our results showed that DON exposure induced the production of intracellular ROS, and oxidative stress-related genes, such as *GPx1*, *iNOS*, *SOD2*, *CAT*, *COX-2* and *HO-1*, also exhibited changes in RNA expression, indicating that strong oxidation takes place in the IPEC-J2 cell. Meanwhile, several studies have reported that oxidative stress generally brings about apoptosis in vivo or vitro (Kang et al., 2019; Vergauwen et al., 2015). Similarly, after DON treatment, the apoptosis-related mRNA/protein such as Bax, caspase-3, and caspase-9, and the apoptosis ratio were increased in our results. Besides, some reports have found that PEITC could induce antioxidant enzyme production and phase II detoxification by activating Nrf2 or MAPK signaling pathway in vitro and in vivo (Krajka-Kuźniak et al., 2020; Xu et al., 2006). While PEITC treatment suppressed ROS generation, *GPx1*, *SOD2*, *CAT* and *HO*-1 mRNA levels were significantly increased, which indicates that PEITC may reduce intracellular ROS levels in DON-exposed IPEC-J2 cells via enhancing the production of glutathione and catalase. In the current study, PEITC treatment could inhibit apoptosis via decreasing pro-apoptotic mRNA/protein levels (Bax, caspase-3 and caspase-9) and increasing *Bcl*-2 mRNA levels in IPEC-J2 cells. Hence, these results demonstrated that PEITC protects IPEC-J2 cells from DON exposure contributing to activating antioxidant adaptive response.

Intestinal epithelium maintains an effective barrier separating the internal lumen of the host, capable of protecting the intestinal internal environment from gut microbiota and exogenous toxins, while simultaneously allowing the selective intake of nutrients and fluids. If the integrity of the intestinal tract is broken, it will lead to many serious intestinal inflammatory disorders (Holmberg et al., 2018). The expression and distribution of tight junction proteins play a crucial role in intestinal barrier function. Some studies have demonstrated that oxidative stress could lead to intestinal barrier injury (Vergauwen et al., 2015). Therefore, we hypothesized that PEITC may alleviate intestinal dysfunction by inhibiting the production of intracellular ROS. In the current study, we have indicated

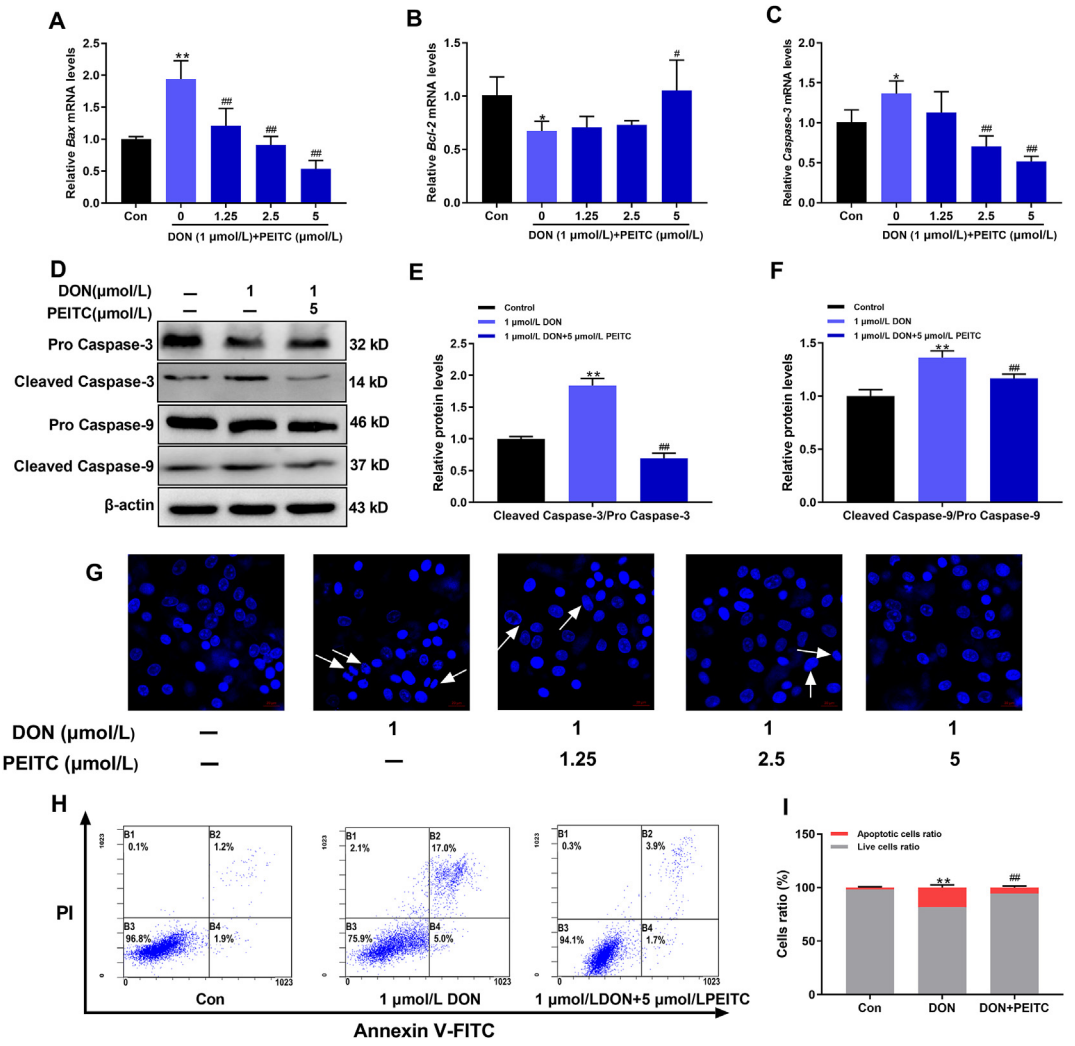


**Fig. 2. Effect of PEITC on DON-induced apoptosis in IPEC-J2 cells.** (A–C) The mRNA levels of *Bax*, *Bcl-2* and *caspase-3* were detected by qRT-PCR. (D–F) Apoptosis-related proteins (pro-caspase-3/-9 and cleaved caspase-3/-9) expression was analyzed by Western blotting. β-actin was used as the loading control. (G) Hoechst 33,258 staining (Scale bar: 10 μm) was used to detect apoptosis, white arrows denote condensed and broken nucleus. (H–I) Annexin V-binding was assayed using flow cytometry. Date are presented as mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the DON alone group.

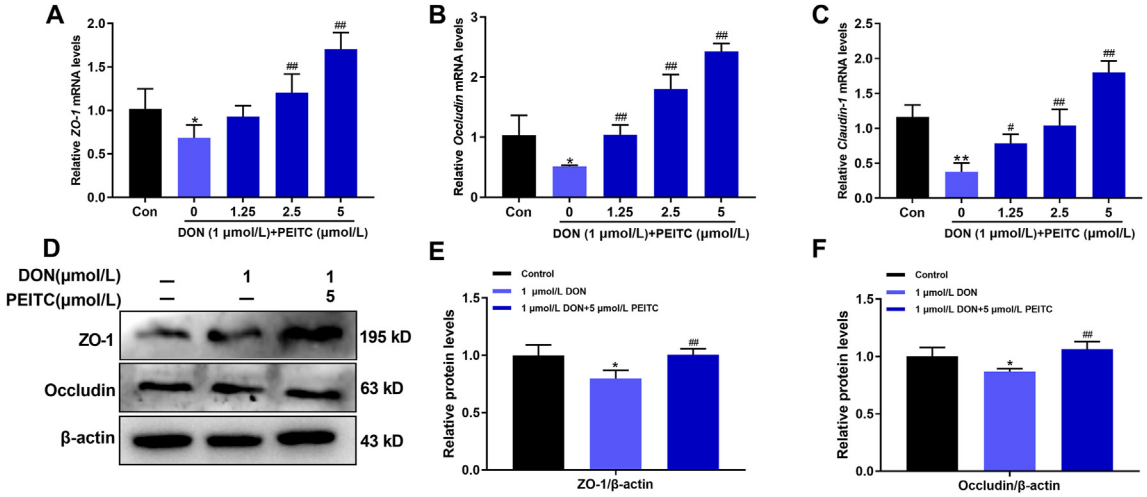


**Fig. 3. Effect of PEITC on DON-induced tight junction disorder in IPEC-J2 cells.** (A–C) The mRNA levels of *ZO-1*, *occludin* and *claudin-1* were measured by qRT-PCR. (D–F) Tight junction proteins (ZO-1 and occludin) expression were analyzed by Western blot. β-actin was used as the loading control. Date are presented as mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the DON alone group. *ZO-1* = zonula occludens-1.

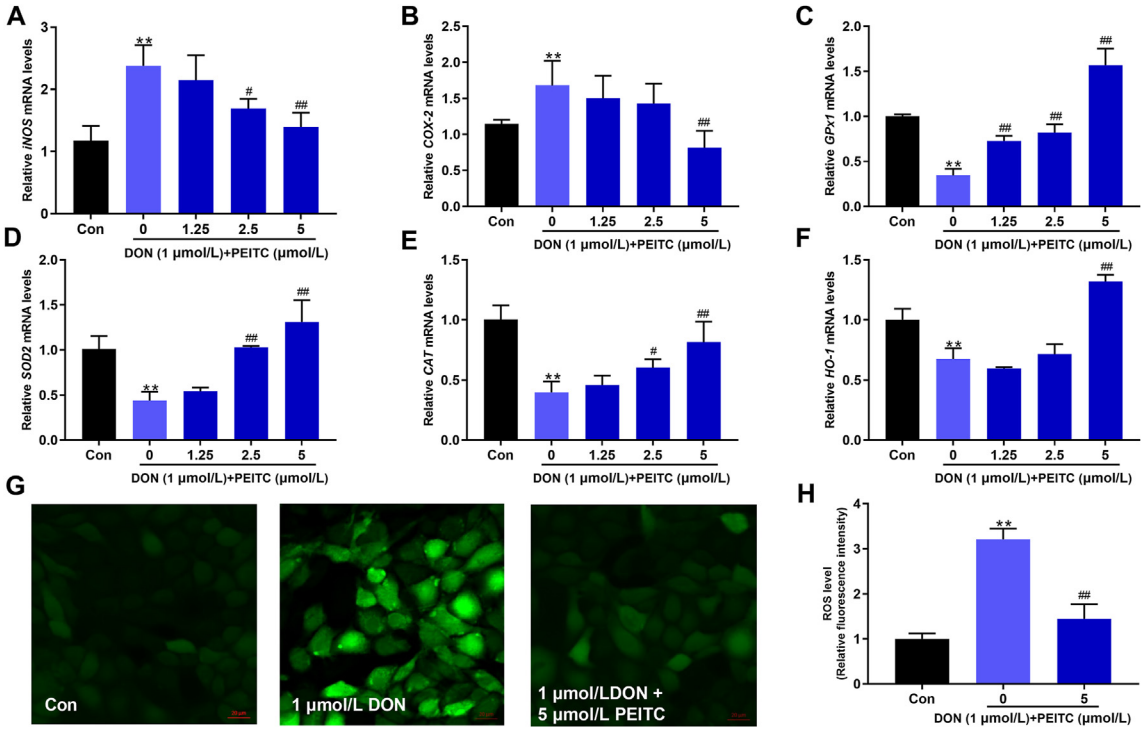


**Fig. 4. Effect of PEITC on DON-induced oxidative stress in IPEC-J2 cells.** (A–F) The mRNA levels of *iNOS*, *COX-2*, *GPx1*, *SOD2*, *CAT* and *HO-1* were measured by qRT-PCR. (G–H) The ROS levels were detected by using $H_2$DCF-DA probe (Scale bar: 50 μm), and the average fluorescence intensity was analyzed by Image-Pro Plus software. Date are presented as mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the DON alone group. *iNOS* = inducible nitric oxide synthase; *COX-2* = cyclooxygenase 2; *GPx1* = glutathione peroxidase 1; *SOD2* = superoxide dismutase 2; *CAT* = catalase; *HO-1* = heme oxygenase 1.

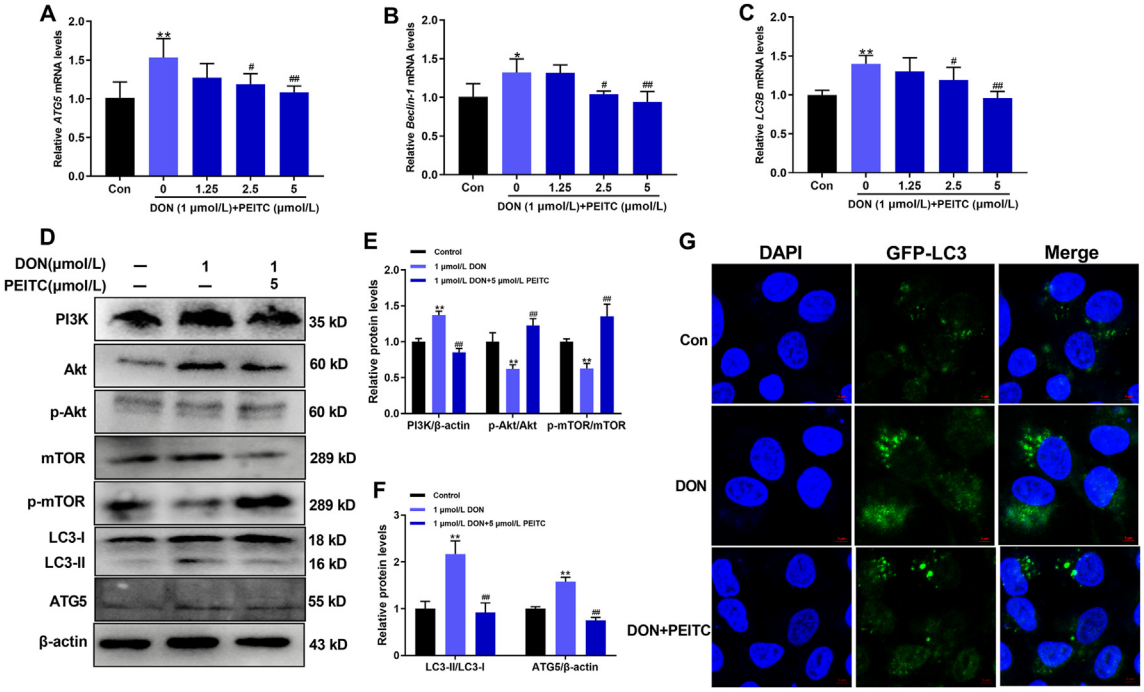


**Fig. 5. Effect of PEITC on DON-induced autophagy in IPEC-J2 cells.** (A–C) The mRNA levels of *ATG5*, *beclin-1* and *LC3B* were measured by qRT-PCR. (D–F) The expressions of PI3K, Akt, p-Akt, mTOR, p-mTOR, ATG5, LC3-I, LC3-II and β-actin were analyzed by Western blot. (G) IPEC-J2 cells were transfected with GFP-LC3 plasmid. After 12 h, the cells were treated with DON (1 μmol/L) or PEITC (5 μmol/L) for 24 h. The fluorescence signal was visualized by confocal immunofluorescence microscopy. Date are presented as mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the DON alone group. *ATG5* = autophagy-related protein 5; *LC3B* = microtubule-associated protein 1 light chain 3B; GFP-LC3 = green fluorescent protein-microtubule-associated protein 1 light-chain 3.

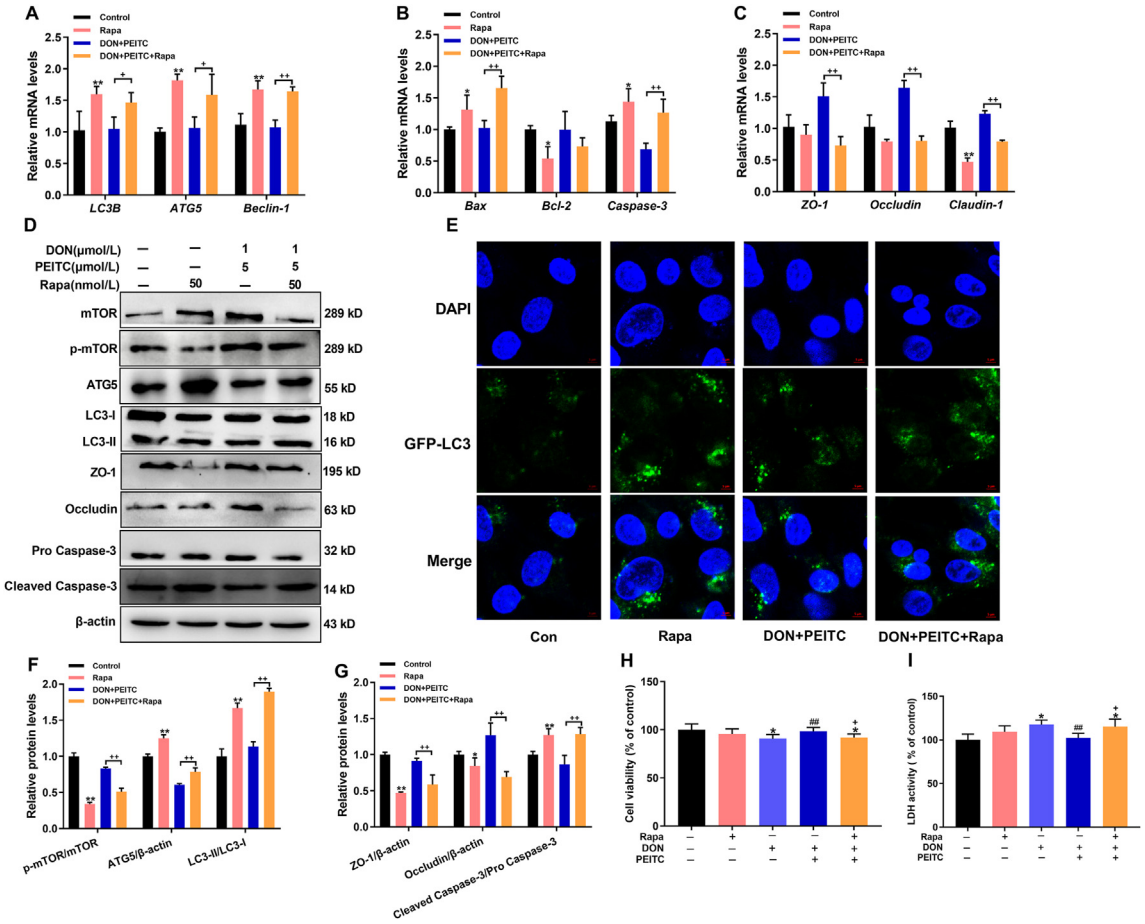


**Fig. 6. Activating of autophagy by rapamycin reversed the protective effect of PEITC on DON-induced intestinal porcine epithelial cell injury in IPEC-J2 cells.** IPEC-J2 cells were pretreatment with rapamycin (50 nmol/L) for 2 h before exposure to DON and PEITC combined for 24 h. The autophagy-related mRNA levels (*ATG5*, *beclin-1* and *LC3B*) (A), apoptosis-related mRNA levels (*Bcl-2*, *Bax*, *caspase-9*) (B) and the intestinal tight junction related mRNA levels (*ZO-1*, *occludin*, and *claudin-1*) (C) were measured by qRT-PCR. (D, F and G) The protein expressions of mTOR, p-mTOR, ATG5, LC3-I, LC3-II, ZO-1, occludin, cleaved caspase-3, pro caspase-3 and β-actin were analyzed by Western blot. (E) IPEC-J2 cells were transfected with GFP-LC3 plasmid. After 12 h, the cells were pretreated with rapamycin (50 nmol/L) for 2 h, and then cells were treated with DON (1 μmol/L) or PEITC (5 μmol/L) for 24 h. The fluorescence signal was visualized by confocal immunofluorescence microscopy. The cell viability (H) and LDH activity (I) were assayed. Date are presented as mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the DON alone group. $^{+}P < 0.05$ and $^{++}P < 0.01$ versus the DON and PEITC combination group.

that PEITC could reduce the production of intracellular ROS. Next, we measured the mRNA/protein expression of ZO-1, occludin, and claudin-1 by qRT-PCR and Western blotting. Our results indicated that a decrease in *ZO-1*, *occludin*, and *claudin-1* mRNA levels, and ZO-1 and occludin protein expression by DON exposure in IPEC-J2 cells for 24 h, which might demonstrate that DON brings about intestinal barrier function and allows toxic substances contained in feed or food to pass through the intestinal cells (Ge et al., 2020; Ying et al., 2019). In our study, we observed that treatment with PEITC before exposure to DON restored intestinal barrier function via upregulating the mRNA/protein expression of tight junction. Therefore, we further confirmed that PEITC may enhance intestinal barrier function by scavenging intracellular ROS induced by DON exposure. But the accurate mechanism of PEITC on the effects of intestinal barrier function is not fully understood, so more studies are needed to determine this.

Autophagy, called “self-eating”, is a fundamental cellular process in eukaryotes that is involved in removing or reusing the damaged organelles and protein aggregates via lysosomes to maintain cellular homeostasis and function, thus dysregulated autophagy can lead to various pathological condition (Cadwell, 2016; Kroemer et al., 2010). Autophagy reveals a dual role in regulating cell death. On the one hand, at the basal level of autophagy, it provides cells with nutrients and defends cells against harmful conditions to promote cell survival (Jing and Lim, 2012). On the other hand, excessive autophagy induces cell apoptosis and accelerates cell death, which is called as autophagy-mediated cell death (Gu et al., 2019; Jing and Lim, 2012). Numerous studies have indicated that the mammalian target of rapamycin (mTOR) signaling pathway is a significant way to regulate autophagy, and activation of autophagy is correlated to the suppression of mTOR, which can inhibit autophagy (He and Klionsky, 2009; Zhou et al., 2013). Also, it has been reported that DON exposure induced cell apoptosis and autophagy by inhibiting Akt/mTOR signaling pathway (Gu et al., 2019; Tang et al., 2015). Similar to these results, the current study results indicated that DON could significantly suppress Akt and mTOR protein expression, and increased ATG5 and LC3-II protein expression and the number of GFP-LC3 puncta to activate autophagy, while PEITC treatment reversed this process via activating Akt/mTOR signaling pathway, and down-regulating ATG5 and LC3-II protein expression and the number of GFP-LC3 puncta. Hence, the protective effects of PEITC on DON-induced intestinal cell injury may be associated with repression of autophagy by activating Akt/mTOR signaling pathway.

Previous research has demonstrated that autophagy is related to the intestinal mucosal barrier (Cadwell, 2016), but the role of autophagy in PEITC alleviation of DON-induced intestinal barrier function remains unclear. Hence, we further testified that the protective effect of PEITC on the intestinal epithelial barrier was related to autophagy using rapamycin that was an autophagy agonist. We found that ATG5, beclin-1 and LC-3 II mRNA/protein expression, and the number of GFP-LC3 puncta were significantly increased by rapamycin-pretreatment. Further, PEITC didn't attenuate DON-induced apoptosis and intestinal tight junction disorder when the IPEC-J2 cells were pretreated with rapamycin. Thus, these findings indicated that autophagy played a crucial role in the effects of PEITC on the DON-induced intestinal epithelial barrier function, and PEITC protected against DON-induced intestinal tight junction disorder through inhibiting autophagy via activation of mTOR in IPEC-J2 cells.

## 5. Conclusions

In summary, we demonstrate that PEITC has an apparent cytoprotective effect on DON-induced damage in IPEC-J2 cells. Treatment with PEITC alleviated DON-induced oxidative damage, apoptosis and intestinal tight junction disorder, which may be related to suppressing autophagy via ROS-mediated mTOR signaling pathway. Hence, the novel findings may provide insights for broadening the application of PEITC to alleviate the toxicity of mycotoxins in animals or humans.

## Author contributions

**S. Liu** and **X. Mao** designed the research; **S. Liu**, **X. Mao**, and **L. Ge** performed the experiments; **S. Liu**, **L. Hou** and **G. Le** analyzed date; **S. Liu** wrote the paper; **F. Gan**, **L. Wen** and **K. Huang** reviewed the paper. All authors read and approved the final manuscript.

## Declaration of competing interest

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

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