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Deoxynivalenol interferes with intestinal motility via injuring the contractility of enteric smooth muscle cells: A novel hazard to the gastrointestinal tract by environmental toxins

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ABSTRACT

Deoxynivalenol (DON) is a prevalent *Fusarium* mycotoxin, occurs predominantly in the global environment, especially in cereals, animal feed and food commodities. The widespread contamination causes a serious risk to human and animal health. DON usually impairs weight gain, which is presumably from its capacity to reduce feed intake by interfering with intestinal motility. To clarify the role of smooth muscle cells (SMCs) contractility in intestinal motility and growth inhibition caused by DON, twelve weaned piglets were firstly divided into two groups to feed control or *Fusarium* mycotoxin-contaminated (MC) diet. Results showed that the final body weight, average daily gain and average daily feed intake were significantly reduced in piglets fed the MC diet. Exposure to the MC diet also significantly decreased the thickness of smooth muscle layer and SMCs contractile markers expression (myosin heavy chain 11, smooth muscle actin gamma 2, transgelin, calponin 1) in jejunum and ileum of piglets. Furthermore, oral DON supplementation (3 mg/kg body weight) to mice in six consecutive days could significantly inhibit the upper intestinal transit, impede normal defecation and downregulate SMCs contractile markers expression in small intestine. Finally, we generated a porcine enteric smooth muscle cell line (PISMC), and found that DON could depress its contractility by decreasing PISMC proliferation, migration and contractile markers expression. In conclusion, these findings in vivo and in vitro suggest that DON, as a common environmental toxin, can not only reduce proliferative and motile phenotype, but also decrease contractile apparatus components (contractile markers expression) in SMCs, which in turn influences SMCs contractility and then interferes with intestinal motility and growth performance.

1. Introduction

Fusarium mycotoxins are structurally diverse toxic metabolites produced by *Fusarium* species of fungi in the natural environment (Schelstraete et al., 2020). The typical *Fusarium* mycotoxins such as deoxynivalenol (DON), zearalenone (ZEN) and fumonisin are easily detected in a considerable percentage of cereals, animal feed and finished food annually (Pinton and Oswald, 2014; Shi et al., 2018). With ongoing global warming and other changes of environmental factors, it tends to increase food and animal feed contamination by these mycotoxins worldwide. Among them, DON is considered the most frequently detected *Fusarium* mycotoxin (Mishra et al., 2020). A previous survey

Abbreviations: ACTG, Gamma smooth muscle actin; ADFI, average daily feed intake; ADG, average daily gain; BCA, Bicinchoninic acid; CALM, Calmodulin; CNN, Calponin; CON, Control; DMSO, Dimethyl sulfoxide; DON, Deoxynivalenol; FBS, Fetal bovine serum; GI, Gastrointestinal; H&E, Hematoxylin & eosin; HISMC, Human intestinal smooth muscle cell; IFN-γ, Interferon-gamma; MC, *Fusarium* mycotoxin-contaminated; MLC, Myosin light chain; MYH, Myosin heavy chain; MYLK, Myosin light chain kinase; NC, Negative control; PISMC, Porcine enteric smooth muscle cell line; RT-qPCR, Reverse transcription quantitative PCR; SEM, Standard error of mean; SM, Smooth muscle; SMA, Smooth muscle actin; SMCs, Smooth muscle cells; TAGLN, Transgelin; ZEN, Zearalenone; β-actin, Actin beta.

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also reported that DON was the most prevalent contaminant than ZEN and aflatoxins B1 in cereals and feed samples from different provinces of China between 2016 and 2017 (Ma et al., 2018). Hence, there is a potential health threat to humans and animals via the consumption of contaminated food, indicating extremely necessary to focus on toxicities of DON to humans and animals.

Following ingestion of DON-contaminated diets, usually impairs weight gain, results in emesis and anorexia that probably impact nutritional efficiency (Pestka, 2007). Among various animal species, pigs are known to be especially susceptible to DON and could serve as a model for human sensitivity to this mycotoxin (Holanda and Kim, 2021). The gastrointestinal (GI) tract is the primary target organ of DON as well as the first barrier against environmental toxins, often exposed to a high concentration of DON (Waskiewicz et al., 2014). Previous studies indicated that a fast and efficient absorption of DON was through the proximal small intestine (mainly in the jejunum) after oral intoxication of pigs (Prelusky et al., 1988; Dänicke et al., 2004). Besides, the concentration of DON was higher in jejunum and ileum tissues than the duodenum tissue of pigs, when pigs were fed a DON-contaminated diet for 28 days (Waskiewicz et al., 2014). Our previous studies also observed that intestinal epithelium damage was notably induced in jejunum and ileum of piglets that fed a *Fusarium* mycotoxins contaminated diet (contained 1097 μg/kg DON and 501.56 μg/kg ZEN) (Ji et al., 2019).

GI motility is an important function of the GI tract which guarantees nutrient digestion (Olsson and Holmgren, 2001). Exposure to DON may be associated with intestinal motility disorder, subsequently, reduce food intake and induce lower weight gain (Pestka, 2010). However, only there few reports focus on DON-induced intestinal motility damage until now, and the specific manners and mechanisms are still blurred. Only Fioramonti et al. (1993) demonstrated that DON could disrupt gastric emptying and intestinal motility with the involvement of serotonin-3 receptors in mice. Moreover, DON exposure also increased the levels of plasma glucagon-like peptide-1 and gastric inhibitory peptide in mice, which are the hormones related to inhibiting gastric emptying and motility (Jia et al., 2017).

Smooth muscle cells (SMCs) in the gut wall are a crucial component of the GI tract (Olsson and Holmgren, 2001). Intestinal motility is mainly supported by the contractile activity of SMCs (Gays et al., 2017). Under normal physiological conditions, the smooth muscle (SM) layer may contract spontaneously or be stimulated by interstitial cells of Cajal to produce rhythmic contractions (Sanders et al., 2012). Besides, the contraction can be influenced by several physiochemical agents (e.g., hormones, drugs, neurotransmitters and toxins) (Mittal et al., 2017; Kitazawa and Kaiya, 2019; Zhong et al., 2020). Scirocco et al. (2010) reported that bacterial lipopolysaccharide impaired human colonic smooth muscle cell contractile function. Additionally, the mature SMCs have a contractile phenotype. These contractile cells are commonly characterized by the expression of contractile markers (contractile apparatus components), such as myosin heavy chain (MYH), smooth muscle actin (SMA), calponin (CNN) and transgelin (TAGLN), thereby maintaining smooth muscle contractile function (Ha et al., 2015). It has been revealed that Beauvericin, as a *Fusarium* mycotoxin produced by *F. subglutinans*, could reduce the contractility of isolated terminal ileum from guinea pigs, suggesting the inhibition of SM contraction happened in the intestine (Lemmens-Gruber et al., 2000). To date, nevertheless, only limited data about the toxicities of mycotoxins on enteric SMCs contraction are published.

In this experience, we investigated that whether DON interfered with intestinal motility via injuring the contractility of enteric SMCs. Our study contributes to uncovering novel perspectives about DON toxicities on the GI tract and further clues for its potential protective strategies on human and animal health.

2. Materials and methods

2.1. Chemical reagents

Purified DON was purchased from Pribolab (51481–10–8, Singapore) for the mice study. Besides, DON treated with cell lines was purchased from Sigma-Aldrich (D0156, St. Louis, MO, USA). They were diluted in dimethyl sulfoxide (DMSO) before use. The antibodies used in this article are presented in Table S1.

2.2. Experimental model and subject details

2.2.1. Piglets study

The preparation of experimental diets and experimental design were detailed described in our previous study (Ji et al., 2019). Briefly, we used the moldy maize contaminated by *Fusarium* graminearum strain 2021 to manufacture the MC diet, so that mimicking DON production by *Fusarium* species in the natural environment. Twelve weaned piglets (Landrace × Large × White) were used in this study with an initial average body weight of 7.77 ± 0.29 kg. Piglets were evenly assigned to 2 groups: negative control (NC) group and *Fusarium* mycotoxin-contaminated (MC) feed group, and individually housed in pens (1.2 m × 2.0 m) with provided feed and water ad libitum. The animal trial consisted of a 6-day adaption period and a 25-day experimental period. During the experimental period, the daily feed intake and final body weight of each piglet were recorded. Piglets in the NC group were fed an uncontaminated control diet that included 226.16 μg/kg DON and 9.61 μg/kg ZEN, while piglets in the MC group were fed MC diet, which contained 1097 μg/kg DON and 501.56 μg/kg ZEN. Due to poor health status, there was one piglet removed from two groups in the experimental period. Piglet experiments were performed and approved by the Nanjing Agricultural University Institutional Animal Care and Use Committee (Certification No.: SYXK (Su) 2011–0036, 11 August 2015).

2.2.2. Mice study

Six-week-old male C57BL/6 J mice (n = 32) with an average weight of 23.34 ± 0.11 g were obtained from Jiangsu GemPharmatech, Co., Ltd. (Nanjing, China). All mice were housed in a temperature- and humidity-controlled room under a 12 h light/dark cycle. After acclimatization for a week, the mice were randomly assigned to 2 groups (n = 16): control (CON) and deoxynivalenol (DON). The mice in the DON group received a daily oral gavage of DON (3 mg/kg body weight) and the mice in the CON group were treated with 200 μL of sterile water (vehicle only). Following the 6 days gavage, ten mice from each group were used for upper intestinal transit and fecal output frequency. Six mice from each group were euthanized and gastrointestinal tissues were collected for further analyses. Mouse experiments were also performed and approved by the Nanjing Agricultural University Institutional Animal Care and Use Committee (Certification No.: SYXK (Su) 2017–0007, 15 August 2020).

2.2.3. PISMC cell line

PISMC, a smooth muscle cell line of the pig ileum, was generated in our previous study (Ji et al., 2020). Normal PISMC was grown using Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA), 2 mM L-glutamine (Thermo Fisher Scientific), and 1% antibiotic-antimycotic. Cells were then maintained at 37 °C and 5% CO_2 in a humidified incubator, changing the medium every two days. All experiments were performed on PISMC between passages 7 and 11.

2.3. Histological analysis

The duodenum (5 cm from the gastric cardia), jejunum (8 cm before the end of jejunal Peyer's patches) and ileum (8 cm from the ileal-caecal

junction) segments were collected separately and then were fixed with 4% paraformaldehyde, embedded in paraffin and sectioned (5 μm) onto glass slides, five slices of each sample were made for measurement. Then, the sections were stained with hematoxylin and eosin (H&E) stain and examined under a light microscope (Olympus, Tokyo, Japan). Photomicrographs were taken with an Olympus CKX31 microscope (Olympus, Tokyo, Japan). The thickness of the SM layer was assessed at six predetermined intestine sites (12, 2, 4, 6, 8 and 10 o'clock) in each slice using ImageJ software (NIH, Bethesda, MD, USA) (Zhang et al., 2010). Additionally, in the whole experiment, the sample collection and thickness measurement were carried out by investigators who were completely blinded to the experimental data and all other variables.

2.4. Upper intestinal transit and fecal pellet output assay

To evaluate gastrointestinal motility in response to DON exposure, upper intestinal transit and fecal pellet output assay were performed. The upper intestinal transit test was performed according to the previously described method with slight modifications (Xu et al., 2020). Briefly, mice were fasted individually for 16 h in wire-bottom cages to prevent coprophagy with free access to water before the experiment, and then received 200 μL charcoal meal (a solution containing 5% w/v charcoal and 10% w/v gum arabic) by intragastrical administration. After 30 min, mice were sacrificed and the gastrointestinal tract from the pylorus sphincter to the cecum was removed. The length of the small intestine was measured (L1), and the distance traveled by the charcoal meal was determined (L2). The results were calculated by this formula: upper intestinal transit (%) = (L2/L1) × 100.

Fecal pellet output assay, which is a simple and non-invasive means to evaluate gastrointestinal motility, was also performed based on a previous report (Khuituan et al., 2019). All mice were removed from their home cages and individually placed in clean cages for observation. Fecal pellets were counted every 1 h, cumulative over 6 h. The accumulated number and weight of fecal pellets output from each mouse were recorded and measured, respectively.

2.5. Collagen gel contraction assay

The collagen gel contraction assay was performed using the 3D Culture Matrix Rat CollagenI(R&D Systems, Minneapolis, MN, USA) as described previously (Colgan, 2006). Briefly, the rat collagenIwas mixed with 10 ×PBS and 1 N NaOH and then added PISMC suspension (5×10^5 cells/mL). 0.5 mL aliquots (including 1.75×10^5 cells and 1 mg/mL rat collagenI) were placed in a 24-well plate and incubated for 1 h at 37 °C with 5% CO_2. After collagen polymerization, 0.5 mL cell culture medium was added onto the collagen gel lattice in each well. Subsequently, dissociate the gel from the bottom wall by gently running the tip of a 200 μL pipet tip along gel edges. Finally, we added 0 ng/mL DON (control), 10 ng/mL, 100 ng/mL, and 1000 ng/mL DON to the wells, incubated at 37 °C with 5% CO_2, and imaged gel lattices using a camera (Sony, Tokyo, Japan) at 24 h. The area of each PISMC/collagen gel lattice was measured by ImageJ software (NIH, Bethesda, MD, USA). Gel contraction at each time point was expressed as % of original area (contraction index). All experiments were performed in triplicate and repeated four times.

2.6. Cell proliferation assay

To explore cell proliferation in response to DON treatments, PISMC was seeded in 96 well plates, which contained 2000 cells in each well and allowed to attach to the wells overnight. The next day, the medium was replaced with a 0.2 mL fresh medium including no DON (control), 10 ng/mL, 100 ng/mL, and 1000 ng/mL DON for 24 h, respectively. Experiments were performed by the MTT Cell Proliferation Assay Kit (Promega, MD, USA) following the manufacturer's instructions. Briefly, after incubated for 24 h at 37 °C with 5% CO_2, each well added 15 μL Dye Solution and continually incubated for 4 h. After that, the MTT solution was removed and added 100 μL stopping solution to be continually incubated at 37 °C for 1 h. The absorbance was determined at 570 nm using a 96-well plate reader. Each group had five parallel and the experiment was repeated four times.

2.7. Migration/ wound healing assay

To study migration/ wound healing in response to DON exposure, PISMC (5×10^5 cells/well) was seeded in 6 well plates. After grown overnight, the confluence may reach approximately 100% and wounded with a p200 pipet tip, then incubated in a cell culture medium (2% FBS) containing 0 ng/mL DON (control), 10 ng/mL, 100 ng/mL, and 1000 ng/mL for 24 h. Photomicrographs of the wound area were taken at 0 h and 24 h using a microscope (OLYMPUS, USA). The wound area was measured by NIH ImageJ software and percent of wound closure at 24 h was expressed as % of the original area. These experiments were performed in duplicate and repeated four times.

2.8. Total RNA isolation and reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from the small intestinal tissues of piglets and mice, and PISMC (treated with 0 ng/mL DON, 100 ng/mL, or 1000 ng/mL) with TRIzol reagent following the manufacturer's instruction (Thermo Fisher Scientific). RNA-reverse transcription and quantitative PCR were performed as described in our previous publications. Sequences of genes-special primers in different species are shown in Table S2. The relative expression abundance of target genes was analyzed in this study using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The C_t values for target genes were normalized to the geometric mean of C_t values for β-actin and GAPDH, which were found to be stably expressed in different conditions.

2.9. Western blot analysis

Piglets and mice intestinal tissue samples were minced and homogenized in ice-cold RIPA lysis buffer containing the protease inhibitor cocktail (Roche, Shanghai, China). Protein concentrations in samples were determined using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Nantong, China). Protein extracts were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane (Boster, Wuhan, China). Membranes were blocked for 2 h at room temperature in 5% nonfat dry milk and subsequently incubated with primary antibodies overnight at 4 °C. Then membranes were incubated with secondary antibodies for 2 h at room temperature. These membranes were visualized by enhanced chemiluminescence using the Tanon™ High-sig ECL Western Blotting Substrate (Tanon, Shanghai, China). Signals were recorded using a Versa DocTM imaging system and analyzed using Quantity One software (Bio-Rad, USA).

2.10. Statistical analysis

In our study, power calculations identified the sample sizes for both pigs and mice. Our study sizes are enough to detect the effect size of 2.02 SD and 1.02 SD for the data, respectively, which are based on G*Power Data Analysis with 80% power and a type I error of 5% (Faul et al., 2007). Statistical analysis was performed using SPSS software (Version 23.0, SPSS Inc., Chicago, IL, USA) and all data were expressed as means ± standard error of mean (SEM). A two-tailed unpaired independent *t*-test was performed to compare two groups. When comparing one indicator among three or more groups, one-way ANOVA was used followed by the Turkey-Kramer test for multiple comparisons. Comparisons were considered a statistically significant difference with a P-value < 0.05. All figures were made by GraphPad Prism (Version 9.0,

Graph Pad Software Inc., San Diego, CA, USA) and Adobe Photoshop (Adobe Photoshop CC 2018, Inc., San Jose, California, USA).

3. Results

3.1. Exposure to MC diet influences the growth performance and small intestinal SM layer development in piglets

The experimental procedure of the piglets study is shown in Fig. 1A. Firstly, we observed the effects of MC diet exposure on the final body weight, average daily weight (ADG) and average daily intake (ADFI). The initial body weight in the two groups showed no difference. Conversely, exposure to the MC diet significantly reduced the final body weight of piglets ($P < 0.05$) (Fig. 1B). The ADG and ADFI of piglets (Fig. 1C and D) in the MC group were also significantly lower than the NC group ($P < 0.05$). Representative morphological observations of the SM layer in the small intestine from the NC and MC group are shown in Fig. 1E. The results that measured the thickness of the intestinal SM layer suggested that no difference was found in the duodenum between the NC and MC groups (Fig. 1F). However, in the jejunum (Fig. 1G), the MC group had a lower thickness of the SM layer than the NC group ($P < 0.05$). In the ileum (Fig. 1H), the thickness of the SM layer was also significantly reduced in the MC group compared to the NC group ($P < 0.05$).

3.2. Exposure to the MC diet downregulates the contractile phenotype-related gene and protein expression in the small intestinal tissues of piglets

We next determined the mRNA expression levels of contractile phenotype-related genes in the small intestinal tissues of piglets. As shown in Table 1, in the duodenum, there was no difference in mRNA expression levels of MYH11, smooth muscle actin gamma 2 (ACTG2), TAGLN and CNN1 between NC and MC groups. In the jejunum, the mRNA expression levels of MYH11, ACTG2, TAGLN and CNN1 were all strongly downregulated in the MC group compared to the NC group ($P < 0.05$). Additionally, the MC group also showed significantly lower mRNA expression levels of MYH11, ACTG2, TAGLN and CNN1 than the NC group in the ileum ($P < 0.05$).

Western blot technique was used to verify that the gene changes resulted in protein level modulation. The MYH11, ACTG2, TAGLN and

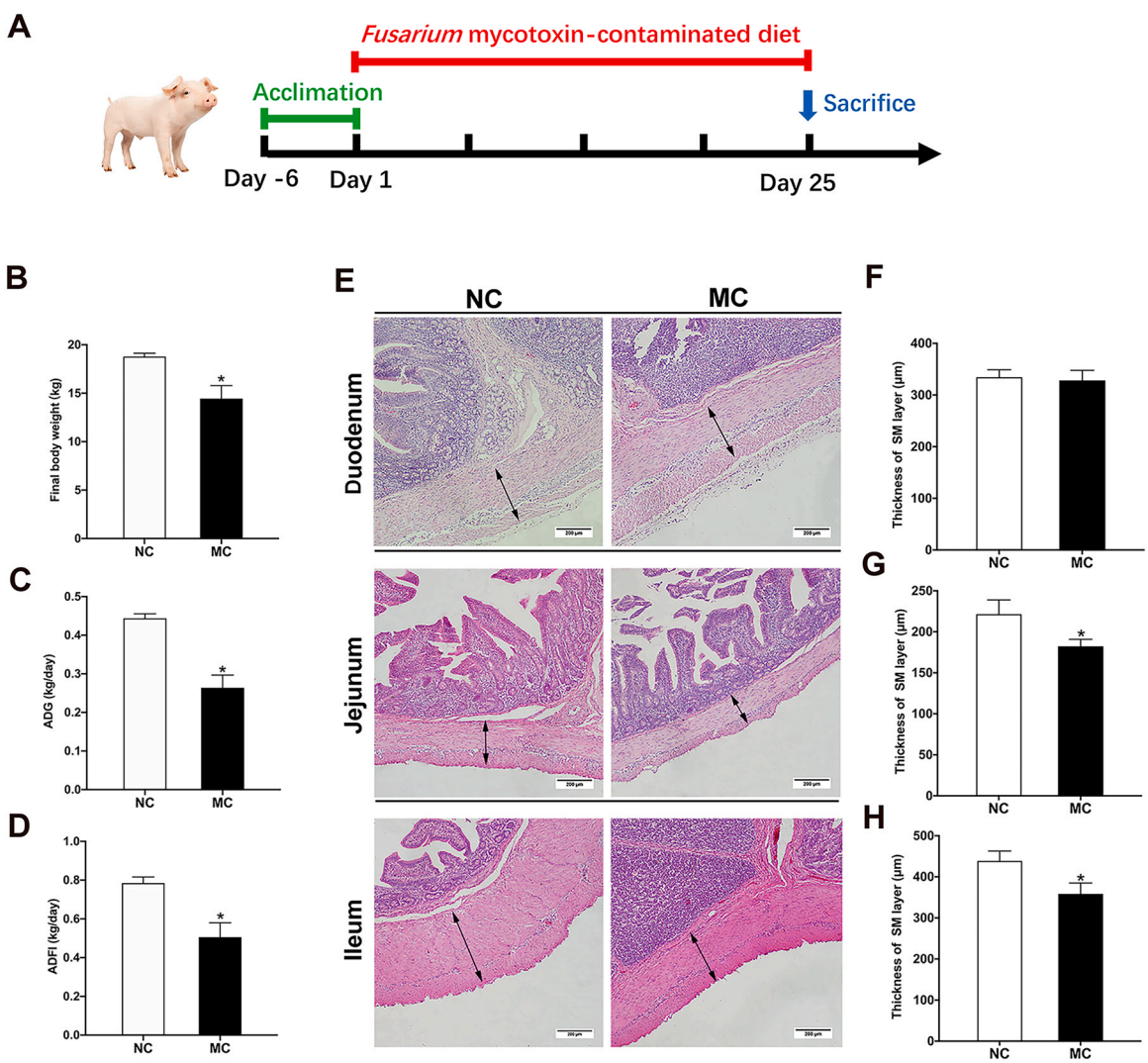

Fig. 1. Effects of MC diet exposure on the growth performance and development of smooth muscle (SM) layer in the small intestine of piglets. (A) Schematic illustration of piglet studies. (B) The final weight, (C) Average daily weight (ADG) and (D) average daily intake (ADFI) of piglets in the two groups. (E) Representative hematoxylin & eosin (H&E) staining images of SM layer in the small intestine. Black double-headed arrows show the thickness of the SM layer. Magnification, 100 × . The thickness of the SM layer in the duodenum (F), jejunum (G) and ileum (H) was quantified using Image J software. Each bar represents the mean ± standard error of mean (SEM) (n = 5) and * indicates that there were significant differences between the two groups ($P < 0.05$). NC (negative control), basal diet; MC, *Fusarium* mycotoxin-contaminated diet.

Table 1
The mRNA gene expression levels related to contractile phenotype in the duodenum, jejunum and ileum of piglets fed either the NC diet or MC diet.

Genes	NC	MC	*P*-value
Duodenum			
MYH11	1.16 ± 0.37	0.64 ± 0.14	0.239
ACTG2	1.03 ± 0.12	0.67 ± 0.11	0.070
TAGLN	1.02 ± 0.12	0.86 ± 0.17	0.469
CNN1	1.02 ± 0.10	0.64 ± 0.13	0.059
Jejunum			
MYH11	1.01 ± 0.07	0.03 ± 0.01	< 0.001
ACTG2	1.00 ± 0.04	0.45 ± 0.04	< 0.001
TAGLN	1.05 ± 0.14	0.04 ± 0.01	0.002
CNN1	1.01 ± 0.07	0.03 ± 0.01	< 0.001
Ileum			
MYH11	1.02 ± 0.10	0.67 ± 0.06	0.026
ACTG2	1.00 ± 0.05	0.69 ± 0.10	0.018
TAGLN	1.03 ± 0.15	0.51 ± 0.07	0.036
CNN1	1.01 ± 0.09	0.71 ± 0.07	0.039

The presented values are the means ± SEM (n = 5); NC (negative control), basal diet; MC, *Fusarium* mycotoxin-contaminated diet; MYH11, myosin heavy chain 11; ACTG2, smooth muscle actin gamma 2; TAGLN, transgelin, CNN1, calponin 1.

CNN1 proteins expression by Western blot in the small intestine of NC and MC group are shown in Fig. 2. In the duodenum (Fig. 2A and B), protein expression levels of MYH11, ACTG2, TAGLN and CNN1 showed no significant differences between the NC and MC groups. Strikingly, in the jejunum (Fig. 2C and D), protein expression levels of MYH11, ACTG2, CNN1 and TAGLN were significantly downregulated in the MC group compared to those in the NC group ($P < 0.05$). Moreover, the MC group also had significantly lower ACTG2, CNN1 and TAGLN protein expression levels ($P < 0.05$) than the NC group in the ileum (Fig. 2E and F).

3.3. DON exposure affects gastrointestinal motility in mice

To confirm the effects of DON exposure on gastrointestinal motility, the mice study was performed and the experimental procedure is shown in Fig. 3A. For the upper intestinal transit assay, the transit index in the DON group was significantly lower ($P < 0.05$) than the CON group (Fig. 3B and C). For fecal pellet output assay, the accumulated number of fecal pellets in the DON group were significantly declined in the first three hours ($P < 0.05$) compared to the CON group (Fig. 3D). Furthermore, the accumulated weight of fecal pellets produced by DON-treated mice significantly decreased ($P < 0.05$) compared to control mice during 2 h, 3 h, and 5 h, respectively (Fig. 3E).

3.4. DON exposure decreases the contractile phenotype-related gene and protein expression in the small intestinal tissues of mice

We determined the mRNA expression levels of contractile phenotype-related genes in the small intestinal tissues of mice. As shown in Table 2, there was no difference in mRNA expression levels of MYH11, ACTG2, TAGLN and CNN1 between the CON and DON groups in the duodenum. In the jejunum, the mRNA expression levels of ACTG2 and TAGLN were found to be downregulated in the DON group relative to those in the CON group ($P < 0.05$). The mRNA levels of MYH11 and CNN1 had no difference between the two groups. In the ileum, transcript levels of ACTG2, TAGLN and CNN1 were downregulated in the DON group ($P < 0.05$) compared to the CON group, however, no significant change was found for MYH11 gene expression level between the two groups.

The protein expression levels of MYH11, ACTG2, TAGLN and CNN1 in the small intestine of the CON and DON group were also determined (Fig. 4). In the duodenum, the protein expression level of TAGLN in the DON group was reduced compared to the CON group ($P < 0.05$). However, there was no significant difference in MYH11, ACTG2 and CNN1 protein expression between the two groups (Fig. 4A and B). Simultaneously, MYH11, ACTG2, TAGLN and CNN1 protein expression levels in the jejunum were all significantly lower in the DON group ($P < 0.05$) compared to the CON group (Fig. 4C and D). Furthermore, the DON group also had significantly lower MYH11, ACTG2, TAGLN and CNN1 protein expression levels ($P < 0.05$) than the CON group in the ileum (Fig. 4E and F).

3.5. DON reduces the magnitude of collagen gel contraction containing PISMC

PISMC was cultured in 3D Culture Matrix Rat CollagenIto assess the effects of DON on collagen gel contraction. We observed the gel contraction at 24 h after treatments with DON. Representative photographs of PISMC embedded in collagen gel disks treated with increasing concentrations of DON were shown in Fig. 5A. It revealed that the DON (10 ng/mL, 100 ng/mL and 1000 ng/mL) had a potent dose-dependent inhibitory effect on gel contraction. The contraction index that was expressed as a percentage of the original gel area was measured from photographs obtained at 24 h during gel contraction (Fig. 5B). It showed that 1000 ng/mL DON significantly inhibited gel contraction containing PISMC compared to other DON treatments ($P < 0.05$). However, there was no difference among 0 ng/mL (control), 10 ng/mL and 100 ng/mL DON treatments. Therefore, these results suggested that DON can directly impair PISMC contractility in vitro.

3.6. DON affects PISMC cellular morphology and proliferation measured (MTT assay)

To further explore how DON might inhibit PISMC contractility, we next observed its effect on PISMC cellular morphology by microscope. The results showed that PISMC was significantly sparser after 1000 ng/mL DON treatment. However, there were no significant changes in other treatments (Fig. 5C). The effects of PISMC proliferation induced by DON were evaluated using the MTT proliferation assay. Fig. 5D showed that a concentration-dependent suppression of PISMC proliferation by DON (10 ng/mL, 100 ng/mL and 1000 ng/mL). The progressive reductions in MTT signal suggested that DON dose-dependently inhibits the growth of PISMC in vitro. In addition, 1000 ng/mL DON significantly inhibited cell proliferation when compared to other DON treatments ($P < 0.05$).

3.7. DON inhibits PISMC wound healing

The influence of DON on PISMC migration was evaluated by wound healing assay. PISMC was grown to reach approximately 100% confluency and cells in the center of each well were scraped and then treated with different concentrations of DON (10 ng/mL, 100 ng/mL and 1000 ng/mL). Photomicrographs taken 24 h after wounding showed delayed wound closure by PISMC in a concentration-dependent fashion (Fig. 5E). The measurement of the wound healing percentage in various treatments revealed that 1000 ng/mL DON had a significant inhibitory effect ($P < 0.05$) compared to other DON treatments (Fig. 5F).

3.8. DON alters gene expression involved in PISMC contractile function

Because DON could notably inhibit PISMC contractility, we also evaluated the effects of DON on gene expression involved in PISMC contractile function by RT-qPCR. The genes for MYH11, ACTG2, TAGLN, CNN1, Myosin light chain kinase (MYLK), Calmodulin (CALM) 1, CALM2 and CALM3 in PISMC were determined after 24 h of culture. It was observed that DON dose-dependently downregulated the gene expression levels of MYH11, ACTG2, TAGLN and CNN1 in PISMC (Fig. 5G). Furthermore, 1000 ng/mL DON could significantly downregulate these genes expression in PISMC compared to other DON treatments ($P < 0.05$). Interestingly, the gene expression levels of MYLK

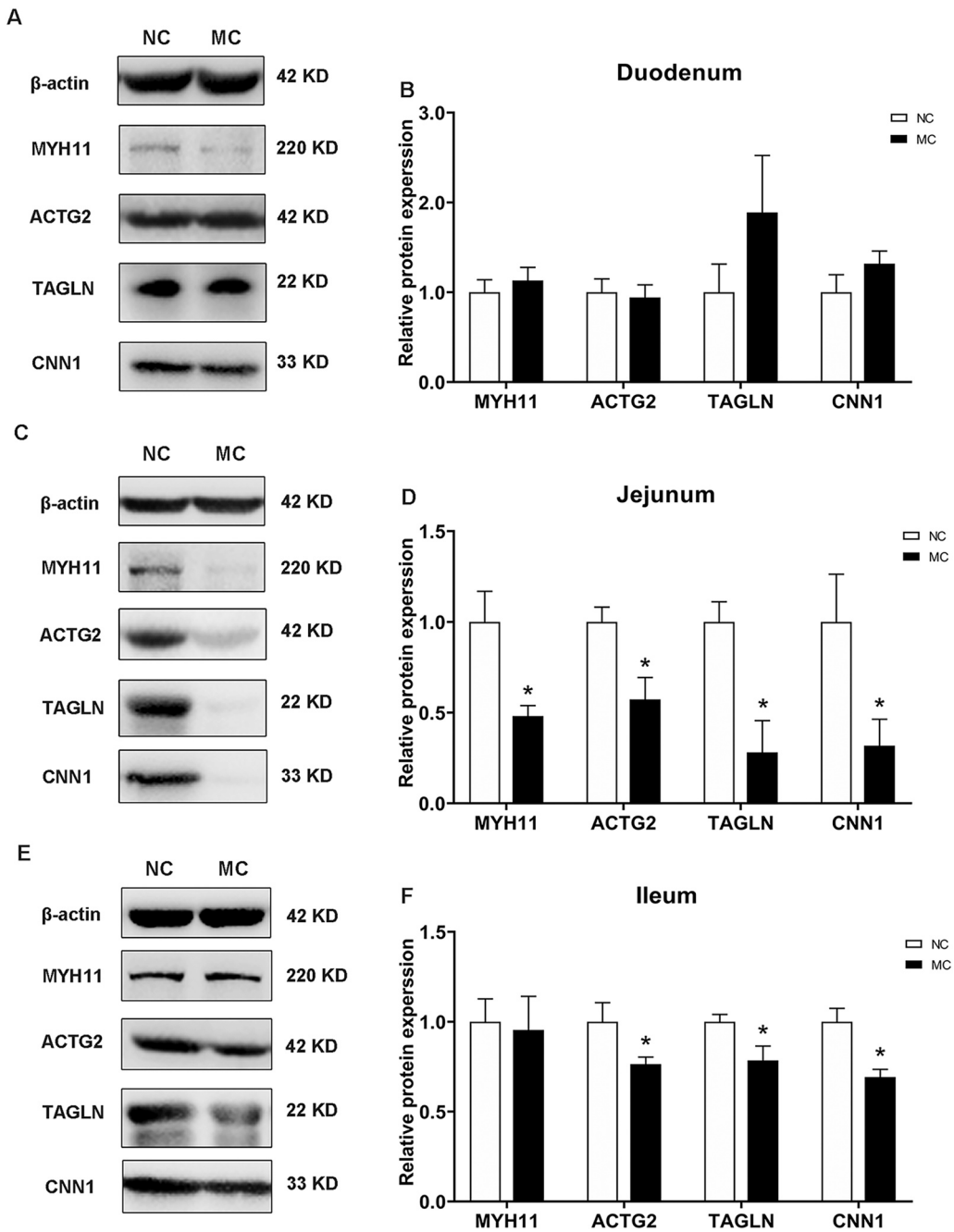

Fig. 2. Effects of MC diet exposure on the contractile phenotype-related protein expression in the small intestine tissues of piglets. Representative Western blot photographs of contractile phenotype-related proteins in the duodenum (A), jejunum (C) and ileum (E). The Western blot data of MYH11, ACTG2, TAGLN and CNN1 was semi-quantified using Quantity One software and protein expression data was calculated based on the ratio of β-actin in the duodenum (B), jejunum (D) and ileum (F). Each bar represents mean ± SEM (n = 5) and * indicates that there were significant differences between the two groups ($P < 0.05$). NC (negative control), basal diet; MC, *Fusarium* mycotoxin-contaminated diet; β-actin, actin beta; MYH11, myosin heavy chain 11; ACTG2, smooth muscle actin gamma 2; TAGLN, transgelin, CNN1, calponin 1.

and CLAM2 were significantly increased in PISMC treated with 1000 ng/mL DON ($P < 0.05$), but no change was observed for CALM1 and CALM3 (Fig. 5H).

4. Discussion

DON, as a *Fusarium* mycotoxin, occurs mostly in food commodities and animal feed (Mishra et al., 2020). Following the intake of DON-contaminated diets, a refusal to diets, low weight gain and GI tract dysfunctions will be induced (Yang et al., 2020; Holanda and Kim, 2021). DON induced lower weight gain partly via reducing food intake by interfering with intestinal motility (Pestka, 2010). However, the specific manners and mechanisms associated with DON impairment of GI motility are still unclear. GI motility is mainly through the contractile activity of SMCs of the gut wall (Olsson and Holmgren, 2001; Luo et al., 2018). The contraction of SMCs could also be stimulated to influence intestinal motility by other substances in the intestine, such as hormones, drugs, neurotransmitters and toxins (Scirocco et al., 2010; Mittal et al., 2017; Kitazawa and Kaiya, 2019; Zhong et al., 2020). Therefore, in the present study, we hypothesize that DON may interfere with intestinal motility via injuring the contractility of SMCs.

Firstly, we observed that the final body weight, ADG and ADFI were declined in weaned piglets fed the MC diet. These indicators are the most common clinical signs following intake of *Fusarium* mycotoxins contaminated diets in pig models. A previous study also showed that piglets had lower body weight, ADG and daily feed intake with feeding *Fusarium* mycotoxins contaminated diet (contained 1.0 mg/kg DON and 250 μg/kg ZEN) (Cheng et al., 2006). Additionally, our original design was to produce DON by using *Fusarium* graminearum strain 2021 contaminated maize. However, it contained 1097 μg/kg DON and 501.56 μg/kg ZEN. This may be because that DON and ZEN are often simultaneously produced by some isolates of *Fusarium* species in the

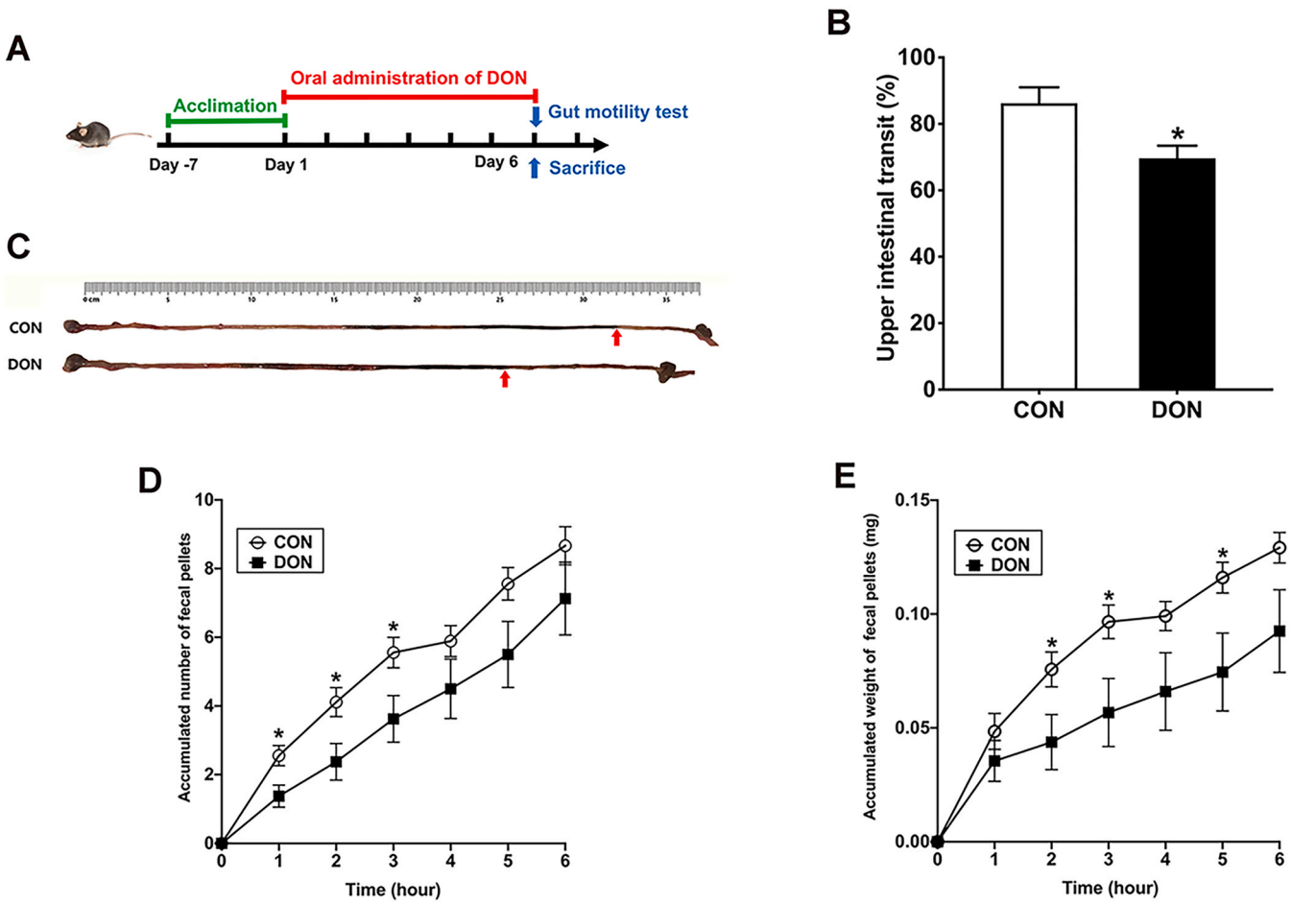

Fig. 3. Effects of DON exposure on gastrointestinal motility in mice. (A) Schematic illustration of mouse studies. (B) Upper intestinal transit index in two groups of mice. (C) Representative images of mice upper intestinal after oral administration with charcoal meal, red arrows indicate the distal end of a charcoal meal. (D and E) The accumulated number and weight of fecal pellets of each mouse were measured every 1 h, cumulative over 6 h. Each bar represents mean ± SEM (n = 10) and * indicates that there were significant differences between the two groups ($P < 0.05$). CON, control group; DON, deoxynivalenol group.

Table 2
The mRNA gene expression levels related to contractile phenotype in the duodenum, jejunum and ileum of mice.

Genes	CON	DON	*P*-value
Duodenum			
MYH11	1.05 ± 0.12	1.26 ± 0.12	0.244
ACTG2	1.02 ± 0.05	1.01 ± 0.12	0.979
TAGLN	1.08 ± 0.34	0.45 ± 0.07	0.101
CNN1	1.02 ± 0.09	1.13 ± 0.12	0.501
Jejunum			
MYH11	1.01 ± 0.06	0.80 ± 0.13	0.181
ACTG2	1.01 ± 0.05	0.81 ± 0.06	0.032
TAGLN	1.00 ± 0.05	0.72 ± 0.08	0.010
CNN1	1.00 ± 0.04	0.93 ± 0.14	0.614
Ileum			
MYH11	1.00 ± 0.05	1.11 ± 0.03	0.086
ACTG2	1.06 ± 0.07	0.87 ± 0.03	0.040
TAGLN	1.01 ± 0.07	0.69 ± 0.07	0.010
CNN1	1.01 ± 0.06	0.79 ± 0.04	0.023

The presented values are the means ± SEM (n = 6); CON, control group; DON, deoxynivalenol exposure group; MYH11, myosin heavy chain 11; ACTG2, smooth muscle actin gamma 2; TAGLN, transgelin, CNN1, calponin 1.

natural environment (Lee and Ryu, 2017).

The development of the SM layer in the small intestine is critical for SM contraction and intestinal motility (Sanders et al., 2012). A previous study reported that intestinal motility dysfunction was induced along with a significant decrease of the jejunum and ileum muscle layer thickness in a mouse model of gastric cancer peritoneal metastasis (Kong et al., 2015). Besides, it has been shown that hydrolyzed casein supplementation could accelerate gastrointestinal transit along with the increasing muscular layer thickness of gastric corpus in piglets (Shen et al., 2020). In our study, *Fusarium* mycotoxins strikingly decreased the thickness of the SM layer in the jejunum and ileum of piglets, indicating the possibility of intestinal motility dysfunction.

Phenotypic modulation of intestinal SMCs also plays an essential role in regulating intestinal motility (Sobue et al., 1999). SMCs have some special genes, such as MYH11, ACTG2, CNN1 and TAGLN, which regulated by similar transcription mechanisms and encoded SMCs contractile apparatus component proteins (Martire et al., 2021). Specifically, MYH11 functions as a major contractile protein that converting chemical energy into mechanical energy through the hydrolysis of ATP (Miano et al., 1994). ACTG2 is known as enteric-smooth muscle actin, which is mainly expressed in enteric smooth muscle (Arnoldi et al., 2013). CNN1 and TAGLN play roles in the regulation and modulation of smooth muscle contraction and involved in calcium-independent smooth muscle contraction, respectively (Sobue et al., 1999; Lee et al., 2015). All the genes discussed above are contractile markers for evaluating the SMCs' contractility. Moreover, Yu et al. (2017) have demonstrated that both mRNA and protein expression of MYH, smooth muscle α-actin and CNN were decreased in the gastric antral muscle layer in advanced glycation end products-induced gastric dysmotility of rats. Moore-Olufemi et al. (2015) also reported that transforming growth

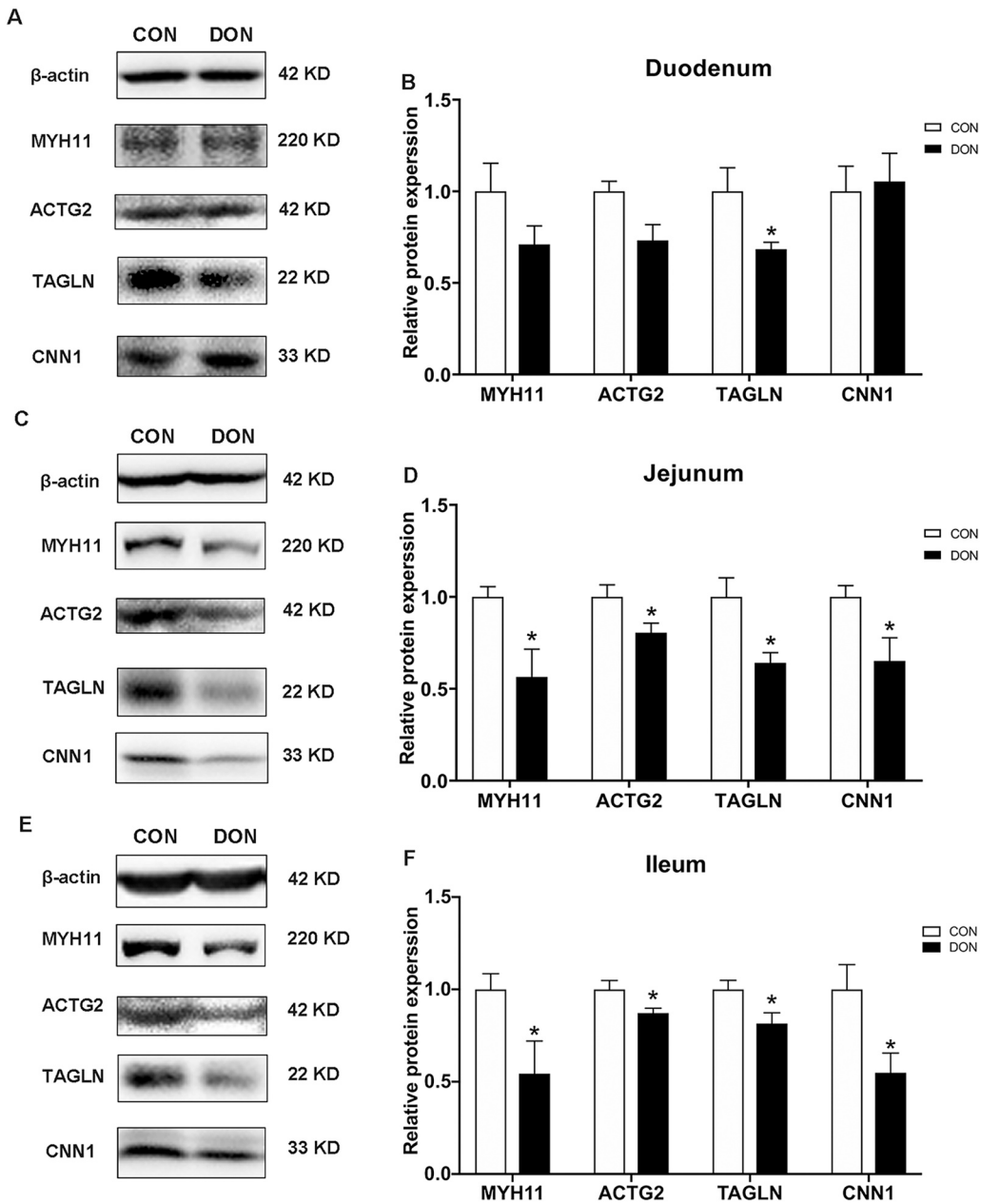

Fig. 4. Effects of DON exposure on the contractile phenotype-related protein expression in the small intestine tissues of mice. Representative Western blot photographs of contractile phenotype-related proteins in the duodenum (A), jejunum (C) and ileum (E). The Western blot data of MYH11, ACTG2, TAGLN and CNN1 was semi-quantified using Quantity One software and protein expression data was calculated based on the ratio of β-actin in the duodenum (B), jejunum (D) and ileum (F). Each bar represents mean ± SEM (n = 6) and * indicates that there were significant differences between the two groups ($P < 0.05$). CON, control group; DON, deoxynivalenol exposure group. CON, control group; DON, deoxynivalenol group; β-actin, actin beta; MYH11, myosin heavy chain 11; ACTG2, smooth muscle actin gamma 2; TAGLN, transgelin, CNN1, calponin 1.

factor-beta 3 increased human intestinal SMCs contraction with the higher expression levels of contractile genes (ACTG2, CNN1, MYH11 and TAGLN). In our study, both mRNA and protein expression levels of SMCs contractile markers (MYH11, ACTG2, TAGLN, CNN1) were all downregulated in the jejunum and ileum SM layer of piglets fed with the MC diet. Therefore, we speculate the growth inhibition of piglets fed with the MC diet in this study is partially ascribed to the intestinal transit dysfunction induced by *Fusarium* mycotoxins that mainly contain DON, based on our results that affecting the development of the SM layer and decreasing the expression of SMCs contractile markers in the small intestine.

Furthermore, we performed a mouse experiment to elucidate the direct effects of DON exposure on intestinal motility. The upper intestinal transit test and fecal pellet output assay were used to access gastrointestinal motility. Xu et al. (2020) proved that the nonapeptide DN-9 slowed the gastrointestinal transit in mice using the upper intestinal transit test and colonic bead expulsion assay. Khuituan et al. (2019) indicated that prebiotic oligosaccharides from dragon fruits altered gut motility in mice via evaluating the fecal pellets output assay over six hours. In the present research, we also found that DON induced slower upper intestinal transit and decreased the accumulated number and weight of fecal pellets. Moreover, the accumulated quantities and weight of fecal pellets dramatically declined in the first three hours after treated with DON. We speculate that it should be associated with the DON bioavailability in organs and tissues of mice. Following acute oral DON exposure, its concentrations in intestine, liver, spleen and kidney reached maximal within 0.5–1 h and declined by 50–90% after 2–4 h (Azconaolivera et al., 1995; Pestka and Amuzie, 2008).

In the mice study, we also examined the effects of DON exposure on final body weight and small intestinal SM layer. Conversely, following six gavages with DON (3 mg/kg body weight) in the six consecutive days, no significant variations were observed (data not shown here). This different observation should be related to many factors, such as the species and age of mice, exposure time and DON concentration. Sun et al. (2014) also implicated that oral administration of DON (5 mg/kg body weight) for two weeks could induce liver injury, however, a

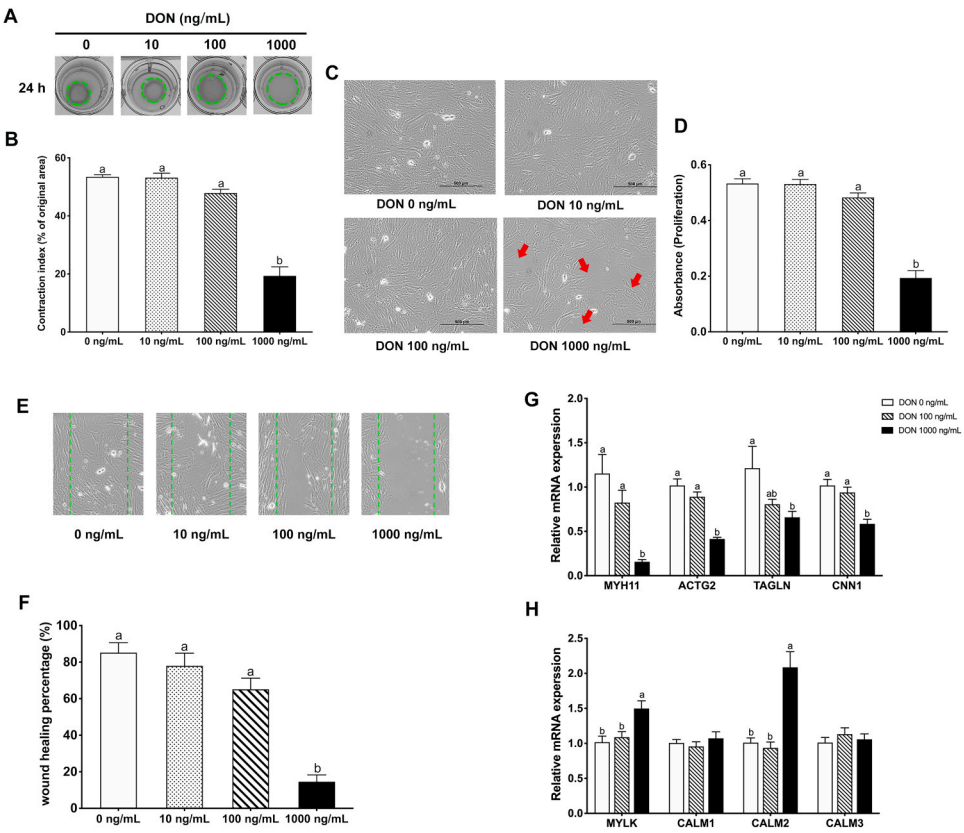

Fig. 5. Effects of different DON concentrations on PISMC contractility, proliferation, migration and gene expression involved in contractile function. (A) Photographs of PISMC embedded in collagen gel disks treated with increasing concentrations of DON (0–1000 ng/mL). (B) The percentage of the original area was measured from photographs obtained at 24 h during gel contraction. (C) Representative micrographs of PISMC monolayer cultures 24 h after treated with DON, red arrows show cell shedding. Magnification, 100 ×. (D) PISMC proliferation (MTT) treated with DON after 24 h. (E) Micrographs of PISMC monolayer cultures 24 h after scrape wounding with a pipette tip. The original wound area is indicated by the dotted white lines. Magnification, × 100. (F) Percent wound closure by PISMC treated DON (0, 100, 1000 ng/mL) was measured at 24 h after injury. (G and H) PISMC gene expression involved in contractile function after 24 h of stimulus with DON. Each bar represents 4 independent experiments performed in triplicates ± SEM. Different letters on top of the bars denote a significant difference within groups ($P < 0.05$). MYH11, myosin heavy chain 11; ACTG2, smooth muscle actin gamma 2; TAGLN, transgelin, CNN1, calponin 1; MYLK, myosin light chain kinase; CALM, calmodulin.

non-significant change in the final body weight was detected as well. Intriguingly, the mRNA and protein expression of SM contractile markers (MYH11, ACTG2, TAGLN, CNN1) were all downregulated in the small intestine of mice, which is consistent with our results in piglets study. Thus, these findings in our study reveal that DON indeed induces intestinal dysmotility, in which the SMCs contractile markers expression play a central role as well.

Finally, we investigated the effects of DON on enteric SMCs contractility in vitro. In our previous study, we have established a porcine smooth muscle cell line (PISMC) from the pig ileum (Ji et al., 2020), which is a suitable model for exploring DON impacts on the cellular and molecular physiology of pig and human intestinal SMCs in vitro. Our PISMC collagen gel contraction assay demonstrated that DON dose-independently inhibited the contractility of PISMC. The collagen gel contraction assay is a classic and useful method to observe the contraction exerted collectively by a population of SMCs in vitro (Ngo et al., 2006). Ford et al. (2019) showed that interferon-gamma (IFN-γ) inhibited human intestinal smooth muscle cell (HISMC) collagen gel contraction, suggesting that the depression of HISMC contractility.

To determine the underlying mechanisms of DON-induced inhibition of PISMC contractility, we observed the effects of DON on PISMC proliferation and migration. It has been reported the proliferation and migration of SMCs are closely related to their contractility. For instance, IFN-γ could depress HISMC contractility with the decline of cell proliferation and migration (Ford et al., 2019). Franco et al. (2006) also illustrated that doxycycline inhibited the proliferation, migration as well as contraction of vascular SMCs from rats in vitro. To our knowledge, DON has not been implicated in inhibiting PISMC proliferation and migration. Interestingly, we found that DON reduced PISMC proliferation and migration in a concentration-dependent manner in our study, which corresponded to the alterations of PISMC collagen gel contraction.

Our study also indicated that DON decreased the gene expression levels (MYH11, ACTG2, TAGLN, CNN1) involved in PISMC contractile function in a dose-dependent manner. These findings are consistent with our results in piglets and mice studies, suggesting that DON may disturb the expression of contractile apparatus components directly. Moreover, the gene expressions of MYLK and CLAM were also detected. These two genes are involved in calcium-dependent signaling pathways which is a principal regulatory mechanism for SMCs contraction (Sanders et al., 2012). It can be briefly described that intracellular sufficient Ca^{2+} binds to CLAM and the resulting complex stimulates MYLK. Then phosphorylated myosin light chain (MLC) by MYLK promotes SMCs contraction (Kuo and Ehrlich, 2015). Sun et al. (2015) reported that the concentration of MYLK was decreased in traumatic brain injury-mediated inhibition of contractility in intestinal SM in mice. Unexpectedly, our results showed that 1000 ng/mL DON markedly increased the gene expression of MYLK and CLAM2. The unpredicted results are probably due to the cellular compensatory effects for DON-induced inhibition of SMCs contractility. Recently, mitochondria and endoplasmic reticulum are reported to be the molecular targets of DON, which are the central organelles to control intracellular Ca^{2+} distribution and concentration. DON can disrupt intracellular calcium homeostasis, such as altering the Ca^{2+} activity and reducing its levels (Del Regno et al., 2015; Wang et al., 2018). Therefore, more concentrations of CALM are needed to bind Ca^{2+} and MYLK to phosphorylate MLC for keeping SMCs contraction homeostasis.

Taken together, our PISMC study demonstrates that DON presumably provokes a de-differentiation that transforming cells from a contractile type to a non-contractile type (i.e. less proliferative and motile phenotype), with less intestinal motility. These functional changes with the deficiency of contractile apparatus components may be the crucial potential causes that the DON impeded PISMC contractility and intestinal motility. More details about DON toxicity on PISMC contractility should be investigated in the further study. Additionally, the DON impacts on the co-culture of PISMC and intestinal epithelial cells also need to be further explored, to mimic the more realistic effects of DON on PISMC contractility in vivo intestinal microenvironment.

5. Conclusions

To the best of our knowledge, this is the first study that evaluated the toxicological effects of DON on intestinal motility via the compromised contractility of enteric SMCs. The present results demonstrate that DON may induce growth inhibition and intestinal dysmotility by blocking the SM layer development and SMCs contractile markers expression. Additionally, our study also indicates that DON suppressed PISMC contractility with the lessening of cell proliferation, migration and contractile markers expression. Collectively, these results both in vivo and in vitro suggest that DON can interfere with intestinal motility via injuring the contractility of enteric SMCs. Our findings may provide a novel explanation for DON-induced intestinal damage and growth suppression in humans and animals. Looking to the future, our results may also supply new insights for the potential therapeutic strategies that can reduce toxicity and pollution of these environmental toxins.

CRediT authorship contribution statement

Xu Ji: Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Yu Qiao:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Weijiang Zheng:** Methodology, Resources, Supervision. **Honglin Jiang:** Methodology, Resources, Supervision. **Wen Yao:** Methodology, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112656.

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