Zearalenone (ZEA)-induced intestinal inflammation is mediated by the NLRP3 inflammasome

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HIGHLIGHTS

- ZEA increased NLRP3 inflammasome expression and cytokines release in cells.
- Elevated cytokines induced severe intestinal inflammation in ZEA-treated mice.
- ZEA induced colitis by activating ROS mediated NLRP3 inflammasome.

ARTICLE INFO

ABSTRACT

To ascertain whether zearalenone (ZEA) could induce intestinal inflammation and investigate its possible mechanism, we investigated inflammatory cytokine release and the activation of the NLRP3 inflammasome after ZEA treatment both *in vitro* or *in vivo*. First, intestinal porcine enterocyte cell line (IPEC-J2) cells and mouse peritoneal macrophages were treated with ZEA to detect NLRP3 inflammasome activation, and the role of reactive oxygen species (ROS) in ZEA-induced inflammation was investigated. Then, Balb/c mice were fed a gavage of ZEA, and the disease activity indices (DAIs) and histological analysis were used to assess intestinal inflammation. Our study showed that the mRNA expression of NLRP3 inflammasome, pro-interleukin-1β (pro-IL-1β), and pro-interleukin-18 (pro-IL-18) was up-regulated 0.5- to 1-fold and that the release of IL-1β and IL-18 increased from 48 pg mL^{-1} to 55 pg mL^{-1} and 110 pg mL^{-1} to 145 pg mL^{-1}, respectively. However, ROS inhibitor N-acetyl-L-cysteine (NAC) reduced IL-1β and IL-18 release to 45 pg mL^{-1} and 108 pg mL^{-1}. Moreover, the same phenomenon was observed in intestinal tissues of ZEA-treated mice. In addition, clinical parameters of treated mice showed stools became loose and contained mucous. In addition, the presence of gross blood stool was found in the last 2 d. Histological analysis showed obvious inflammatory cell infiltration and tissue damage in the colon. These findings uncovered a possible mechanism of intestinal mucosal innate immunity in response to mycotoxin ZEA that ZEA could activate the ROS-mediated NLRP3 inflammasome and, in turn, contribute to the caspase-1-dependent activation of the inflammatory cytokines IL-1β and IL-18.

1. Introduction

Intestinal inflammation is a prominent feature of protective reactions in animal immunity against invading parasites, bacteria and other foreign substances. The host immune system can prevent overt inflammation against constant antigenic stimulation by regulating innate and adaptive mechanisms. When inadequately regulated, intestinal inflammasome activation leads to an inflammatory disorder of the intestinal tract that is characterized by malnutrition, abdominal pain, diarrhoea, and rectal bleeding (Zmora et al., 2017). The inflammasome is a cytosolic multi-protein system that senses a variety of environmental and endogenous

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stimuli. It contains NOD-like receptors (NLRs), apoptosis-associated adaptor protein (ASC), and the precursor form of caspase-1 (Lamkanfi and Dixit, 2012; Lu et al., 2014). The NLRP3 inflammasome is unique in its ability to recognize the molecular patterns associated with host-derived metabolites, such as saturated fatty acids or glucose (Sutterwala et al., 2006; Jin and Flavell, 2010; Masters et al., 2010), and exogenous crystalline particles, including silica, alum, nanomaterials, asbestos, and nigericin (Kanneganti and Lamkanfi, 2013). The NLRP3 inflammasome is responsible for the maturation and secretion of pro-inflammatory cytokines IL-1β and IL-18, which are central players in the pathogenesis of inflammatory bowel disease (IBD) (Bauer et al., 2010).

ZEA is one of the most frequently occurring toxins in the world. It has been reported that ZEA is associated with many mycotoxicosis diseases in farm animals (Finkgremmels and Malekinejad, 2007). Notably, as the first physical barrier against foreign materials, the small intestine absorbs ZEA first, so it is exposed to high concentrations of the toxin, which will certainly influence intestinal tract health. There is an increasing awareness of the deleterious effects attributed to mycotoxins within the gut (Pinton and Oswald, 2014). Evidence indicates that a disruption of the epithelial cell integrity and functions induced by ZEA is well established (Robert et al., 2017). However, as far as we know, studies of the inflammatory response of the intestinal tract to ZEA are still limited. Kostro et al. reported that ZEA participates in the development of inflammatory reactions in sheep intestines, but the mechanism of their activation has not been determined (Kostro et al., 2012). Previously, we proved that ZEA, a kind of common mycotoxin, could induce an accumulation of reactive oxygen species (ROS) in mitochondria (Fan et al., 2017) and that elevated ROS levels can act as signalling molecules in pathological conditions (Schieber and Chandel, 2014). Moreover, some studies have demonstrated that increased mitochondrial ROS production and the release of mitochondrial DNA into the cytosol are critical events associated with NLRP3 inflammasome activation (Tschopp and Schroder, 2010; Zhou et al., 2010; Kiichi et al., 2011). Therefore, we speculate that NLRs may mediate intestinal innate immunity in response to ZEA and thus studied the possible mechanism for ZEA induced releasing of IL-1β and IL-18. Moreover, the role of the ROS-mediated NLRP3 inflammasome in ZEA-induced intestinal inflammation was investigated.

2. Material and methods

2.1. Reagents, cell culture and animals

ZEA was purchased from Fermentek (Lot: 151012). Dextran sulphate sodium (DSS) reagent (30KW-50KW) was from MP Biomedicals (Lot: 60316es25). TRIzol reagent was from Vazyme (Lot: 7E102B7). NAC and Lysis buffer (RIPA:PMSF, 1:9) were from Beyotime (Lot: S0077, P0013B, ST506). All reagents used were of analytical grade. A reverse transcription kit and PCRMix were purchased from Thermo (Lot: 00393084). ELSIA kits for IL-18, IL-1β and myeloperoxidase (MPO) were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Lot: 04/2017). Polyvinylidene fluoride (PVDF) membranes (0.45 μm) were purchased from GE Healthcare (Lot: A10190850). Rabbit NLRP3 was provided by Abcam (Lot: AB210491-40UL). β-actin antibodies (antimouse) were provided by Absin (Lot: ABS830031SS). Goat anti-rabbit HRP-linked antibodies were purchased from CST (Lot: 7074S).

IPEC-J2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and high glucose supplemented with 10%foetal calf serum (GIBCO) and 1%penicillin—streptomycin (1 mg mL^{-1}). The cells were maintained in 25 cm^2 flasks at 37 °C in a humidified incubator with a 5%CO$_2$ atmosphere. After reaching 70%confluence,

the IPEC-J2 monolayers were treated with different doses of ZEA with or without NAC. The dose of ZEA used here was that determined in our previous study (Fan et al., 2017). The results showed that the ZEA did not induce a cytotoxic effect from 3.125 to 6.25 μg mL^{-1}, whereas exposure to 12.5 μg mL^{-1} ZEA decreased the cell vitality by half. Therefore, we chose 8 μg mL^{-1} ZEA to study the cell ROS levels and NLRP3 inflammasome changes. At this concentration, the ROS levels were 5- fold higher than those of the control group, but fewer cells died. ZEA6 and ZEA8 indicate that the cells were treated with 6 μg mL^{-1} or 8 μg mL^{-1} of ZEA, respectively. The treated cells were incubated for 24 h.

Peritoneal macrophages were isolated with the following method. Mice were sacrificed by cervical dislocation under 5%isoflurane anaesthesia (Hankenson et al., 2011) and were then intraperitoneally injected with 10 mL of PBS. After shaking, peritoneal lavage fluid was collected and centrifuged for 15 min (1000 r/min). The remaining cells were plated into cell plates overnight, and adherent cells were used for NLRP3 inflammasome expression and cytokine assays. Additionally, 8 μg mL^{-1} ZEA (ZEA8) was used to treat macrophages.

Twenty-four Balb/c mice at the age of 6—8 weeks were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were fed standard mice chow pellets, had access to tap water supplied in bottles, and were acclimatized at least 7 days before the experiments. Then, the mice were randomly divided into three groups: I (control group), II (DSS group), and III (ZEA group). The I group mice were given 1 mL of distilled water once a day by gavage. The II and III groups were administered 1 mL of 3%DSS or ZEA by gavage at a dosage of 4.5 mg kg^{-1} body weight once daily for 1 week. All mice were bred at the University of Nanjing Agricultural University (Permission Number: SYXK(Su)2017-0007). All experiments were approved by the regional animal study committee and are in agreement with the guidelines for the proper use of animals in biomedical research.

2.2. RNA extraction and reverse transcriptional PCR (RT-PCR) analysis

Total cellular RNA was extracted from the treated IPEC-J2 cells and mouse peritoneal macrophages using TRIzol reagent, respectively. Moreover, the tissue RNA was extracted from mice colon tissue. For reverse transcriptase (RT) reactions, 1 μL of total RNA, 1 μL of oligo-(dT) and 10 μL of DEPC water were denatured at 65 °C for 5 min, and then 5 × Reaction Buffer (4 μL), Ribolock RNase Inhibitor (1 μL), 10 mM dNTP Mix (2 μL), and ReverAid RTase (1 μL) were added. The RT reactions were performed at 42 °C for 1 h at 70 °C for 5 min. Then, 1 μL of the cDNA sample was extracted in sextuplicate. Subsequently, PCRMix (10 μL), primers (2 μL) of each NLRP3, ASC, caspase-1, pro-IL-1β, pro-IL-18, and β-actin primers (see Table S1) and dd H$_2$O (7 μL) were added. The optimal PCR conditions were 35 cycles of 92 °C for 30 s, 50—58 °C for 60 s, and 72 °C for 30 s.

To semiquantitate these PCR products, the intensity of the amplified bands was analysed using the AlphaImager software. The band intensities were normalized with the corresponding β-actin signal, and the relations with NLRP3/β-actin, ASC/β-actin, caspase-1/β-actin, pro-IL-1β/β-actin, and pro-IL-18/β-actin were calculated. The PCR results were repeated three times.

2.3. SDS-PAGE and western blotting (WB)

First, 100 mg of colon tissues was homogenized in 1 mL of lysis buffer and centrifuged with 12000 r/min for 5 min. The supernatants were dissolved in Laemmli buffer and separated using a 10% acrylamide-bisacrylamide gel. Proteins were blotted onto a 0.45 μm

PVDF membrane. Primary anti-NLRP3 (rabbit, antimouse) were applied at 1:500, and anti-β-actin (loading control) was used at 1:2000. Immunoglobulin G (IgG) anti-rabbit-HRP was diluted 1:8000, and an Amersham Imager 600 (GE Healthcare) was used to visualize chemo-luminescence.

2.4. Clinical score and histological analysis

The DAI were determined by two investigators blinded to the treatment groups. A scoring system was applied to assess body weight, diarrhoea and the presence of occult or overt blood in the stool (Table S2) (Walsh et al., 2014). Changes in body weight indicated the loss of baseline body weight as a percentage. After a postmortem, the colon was removed, and pieces of colonic tissue were used for PCR and ELISA analyses. For histology analysis, rings of the transverse part of the colon were fixed in 4% buffered formalin and then embedded in paraffin. Sections were stained with H&E according to the standard protocols. Histological analysis was performed by a pathologist. For tissue damage, discrete epithelial lesions were scored as 1, mucosal erosions as 2, and extensive mucosal damage as 3. For inflammatory cells, infiltration that focally increased in the lamina propria is scored as 1, infiltration extending into the submucosa was scored as 2 and transmural extension of the infiltrate was scored as 3. The two subscores (tissue damage and cell infiltration) were added to evaluate the histological colitis severity.

2.5. ELISA

Primary mouse macrophages seeded into 96-well plates at a density of 2×10^5 cells per well. After LPS priming for 2 h, cells were stimulated with ZEA, NAC, DSS for 24 h. Cell culture supernatant or colon homogenates were used for ELISAs, which were performed according to the manufacturer's protocol.

2.6. Statistical analysis

Data are expressed as the mean ± SEM. The statistical significance of the differences between treatment groups and the control group was determined using Student's t-test. Differences were considered statistically significant at $^*P < 0.05$ and $^{***}P < 0.001$.

3. Results

3.1. ZEA induces NLRP3 inflammasome activation

NLRP3 can form an inflammasome complex with the adaptor molecules ASC and caspase-1 in response to various stimuli. We speculated that caspase-1 activation by ZEA was mediated by the NLRP3 inflammasome and that the caspase-1 then enhanced IL-1β and IL-18 synthesis. After incubation with ZEA, we collected RNA from different groups of IPEC-J2 cells and then detected the mRNA expression of NLRP3, ASC, caspase-1, pro-IL-1β, and pro-IL-18. The results showed that the NLRP3 inflammasome and pro-IL-1β and pro-IL-18 mRNA expression of the ZEA-treated groups were enhanced (Fig. 1A). Significantly, the enhancement of ASC, caspase-1, pro-IL-1β and pro-IL-18 was more obvious in the ZEA$_8$ group than in the other groups (Fig. 1B). However, the increase in NLRP3 activation in the ZEA-treated group was not significant. We speculated that NLRP3 is a crucial regulator of intestinal homeostasis and that the NLRP3 inflammasome plays an important role in promoting the proliferation of epithelial stem cells to maintain the epithelial barrier (Zaki et al., 2011). Therefore, the NLRP3 activation was a relatively active control of IPEC-J2 cells. These findings indicated that high-dose ZEA could activate the NLRP3-ASC complex, leading

to the activation of caspase-1 and the subsequent cleavage of pro-IL-1β and pro-IL-18 into a mature secreted form of IPEC-J2 cells.

### 3.2. ZEA induced IL-1β and IL-18 processing relies on the NLRP3 inflammasome in murine macrophages

To confirm that ZEA induced the release of IL-1β and IL-18, we performed an ELISA of IL-1β and IL-18, which were both present in the supernatants of ZEA-stimulated macrophages. IL-1β increased from 48 pg mL^{-1} to 55 pg mL^{-1} in the ZEA group (8 μg mL^{-1}). The elevated IL-1β release in the ZEA group was limited. This may have occurred because we used 8 μg mL^{-1} ZEA to avoid decreasing the cell viability. The dose cannot induce a significant change in IL-1β. This result was similar to that of KWON et al. (Kwon et al., 2004). Compared to IL-1β, IL-18 increased significantly from 110 pg mL^{-1} to 145 pg mL^{-1} (Fig. 2A–B). The study also showed that mRNA expression of the NLRP3 inflammasome was enhanced in different mouse peritoneal macrophages (Fig. 2C). Similarly, the enhancements of NLRP3 and ASC expression were more obvious in the ZEA group than in the other groups (Fig. 2D). Macrophages play a pivotal role in the defence against environmental and endogenous stimuli and are important mediators in acute inflammatory responses. A series of immediate effector functions and the expression of pro-inflammatory genes enable macrophages to initiate an immune response against the injurious agent. Thus, macrophages have higher levels of NLRP3 induction. These findings indicated that ZEA could activate the NLRP3-ASC complex, leading to the pro-IL-1β and pro-IL-18 maturation and release in macrophages. NLRP3 inflammasome activation has been linked to ROS generation in response to various stimuli. ZEA was proven to stimulate ROS production in our previous research. To investigate the role of ROS in ZEA-induced IL-1β and IL-18 release, we treated macrophages with the ROS inhibitor NAC. The inhibitor reduced IL-1β and IL-18 secretion and NLRP3-ASC complex expression (Fig. 2C–D). These data suggest that ROS contribute to ZEA-induced NLRP3 inflammasome activation.

3.3. Colitis and inflammatory response in DSS and ZEA treated mice

We next investigated the role that the NLRP3 inflammasome plays in ZEA-mediated intestinal inflammation in vivo. Disease activity indices (DAIs), including the mental state, body weight, stool consistency, diarrhoea, and presence of occult or gross blood per rectum, were determined daily. 3%DSS significantly induced mice colitis, which led to a poor state of mind, serious loss of body weight, and diarrhoea. In particular, the stool consistency changed markedly. A loose stool was 2–3 mm larger than normal (Fig. S1A-1, 2, 3, 4). Moreover, bloody stool and haematochezia were found in the treated mice (Figs. S1B-a and S1C-b). Although the disease activity indices (DAIs) of the ZEA group were lower than those of the 3%DSS group (Fig. S1D), the body weight loss, stool consistency changes, and bloody stool were serious compared with the control group. It is worth noting that 2 of the 8 DSS mice died due to colitis by day 7, whereas all 8 ZEA mice survived. 3%DSS group mice colonic histology showed a large number of infiltrating inflammatory cells (Fig. 3B–a), distorted crypt (Fig. 3B–b), and extensive damage in the lamina propria and submucosa (Fig. 3B–c). Histological analysis of colonic tissue obtained in the ZEA group mice also revealed severe mucosal infiltration by inflammatory cells (Fig. 3C–d) as well as lamina propria and submucosa damage (Fig. 3C–e) compared to the control group (Fig. 3A). However, the severe intestinal inflammation with disruption of the epithelial layer was not as serious as 3%DSS-induced colitis, but the histological score was significantly enhanced (Fig. 3D).
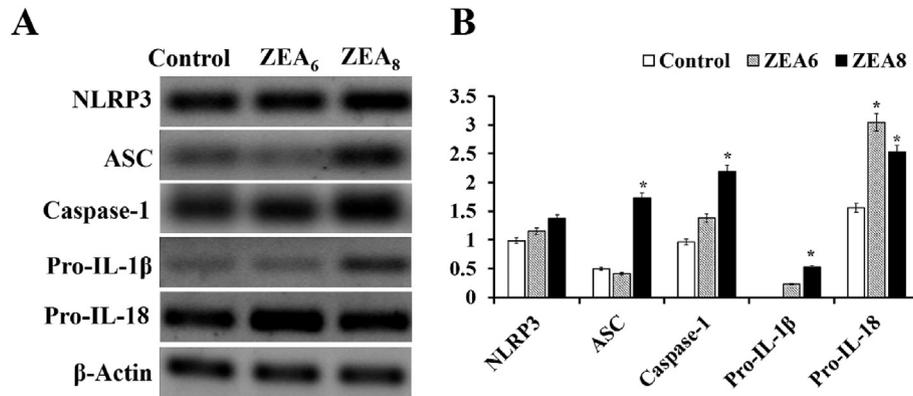
Fig. 1. mRNA expression of the NLRP3 inflammasome complex and pro-inflammatory cytokines in IPEC-J2 cells. IPEC-J2 cells were treated with 6 or 8 μg mL^{-1} of zearalenone (ZEA). Total RNA was extracted from treated cells. (A) Semiquantitative PCR results for NLRP3, apoptosis-associated adaptor protein (ASC), caspase-1, pro-interleukin-1β (pro-IL-1β), and pro-interleukin-18 (pro-IL-18). (B) Intensity ratios of each amplified band to β-actin. Data represent the mean ± SEM; n = 3/group; *P < 0.05, vs control group. ZEA6: ZEA concentration was 6 μg mL^{-1}, ZEA8: the ZEA concentration was 8 μg mL^{-1}.
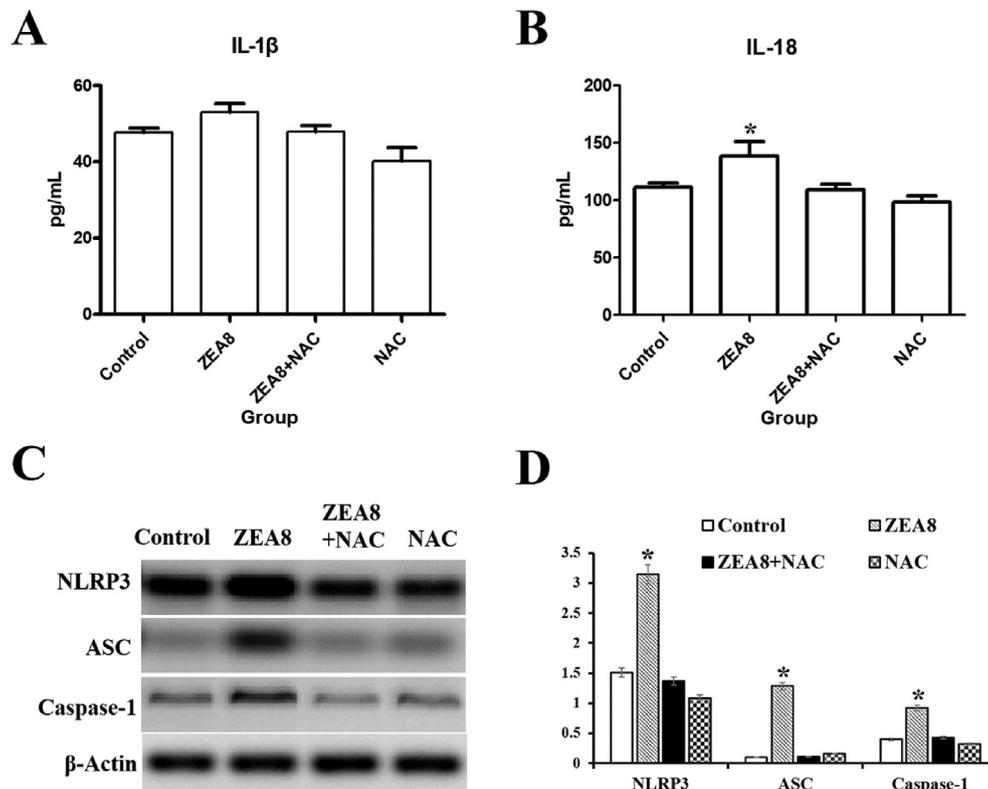
Fig. 2. Zearalenone (ZEA) induces interleukin-1β (IL-1β) and interleukin-18 (IL-18) release, which relies on the NLRP3 inflammasome from murine macrophages. Macrophages were treated with 8 μg mL^{-1} ZEA in the absence or presence of N-acetyl-ʟ-cysteine (NAC). The cellular supernatant was collected to detect cytokines using an ELISA. The total RNA was also extracted from treated cells. (A) Results of IL-1β release. (B) Results of IL-18 release. (C) Semiquantitative PCR results of NLRP3, apoptosis-associated adaptor protein (ASC), and caspase-1. D: Intensity ratios of each amplified band to β-actin. Data represent the mean ± SEM; n = 3/group; *P < 0.05, vs control group. ZEA8: the ZEA concentration was 8 μg mL^{-1}.

3.4. ZEA upregulated inflammatory cytokines and myeloperoxidase in the colonic tissue of ZEA-treated mice

The levels of IL-1β, IL-18 and myeloperoxidase (MPO) in mouse colon tissues were analysed to investigate the effects of ZEA on the inflammatory response. The results showed that DSS induction led to 50 pg mL^{-1}, 75 pg mL^{-1} and 80 pg mL^{-1} increases in the IL-1β, IL-18 and MPO levels, respectively. Moreover, ZEA in the colon significantly enhanced the levels of these inflammatory factors. The IL-1β, IL-18 and MPO release reached 50 pg mL^{-1}, 150 pg mL^{-1} and

145 pg mL^{-1}, respectively (Fig. 4; P < 0.05, P < 0.001, P < 0.001). These increased IL-1β, IL-18 and MPO levels indicated that ZEA induced inflammatory responses in the intestine.

3.5. ZEA induced intestine inflammatory in mice depends on the NLRP3 signalling pathway

We observed that the ZEA-treated groups exhibited increased NLRP3, ASC, caspase-1, pro-IL-1β and pro-IL-18 mRNA levels compared to the control group. However, the levels of the mRNA
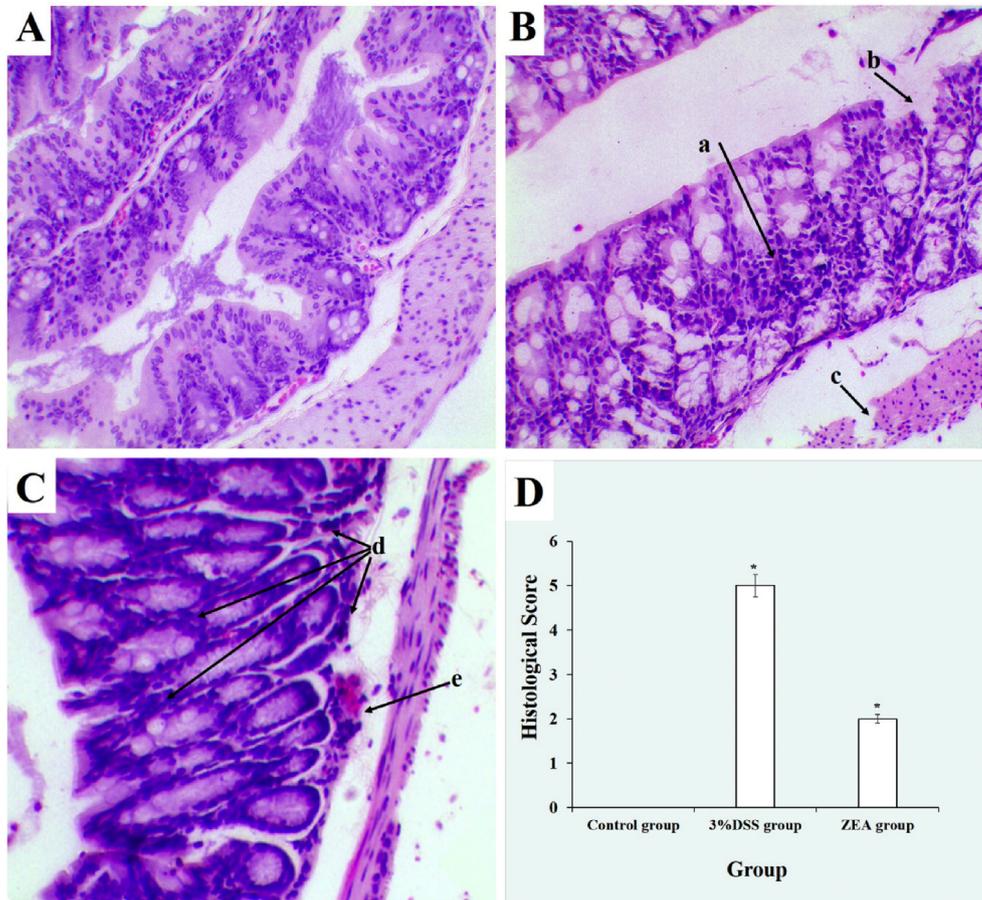
Fig. 3. Histopathological changes of zearalenone (ZEA) induced intestinal inflammation in mice. Mice colons were collected at day 8 after 3%destran sulphate sodium (DSS) or ZEA (4.5 mg kg^{-1}) treatment. (A) Histological section of control mice. (B) Histological section of 3%DSS-treated mice. (C) Histological section of ZEA-treated mice. HE staining was used to evaluate the morphological changes in the colon tissues. The arrows (a, b, c, d, e) highlight areas of tissue lesions and inflammatory cell infiltration (original magnification $\times$ 20). (D) Histological score of colon sections of controls, 3%DSS-, and ZEA-treated mice at day 8. Data represent the mean $\pm$ SEM; n = 8/group; *$P < 0.05$ vs the control group.
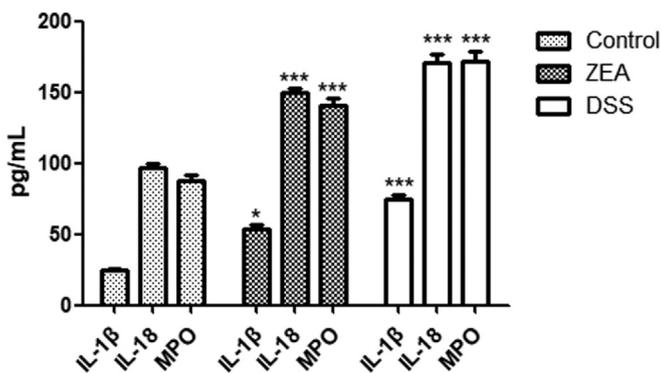
**Fig. 4.** Increased production of interleukin-1β (IL-1β), interleukin-18, and myeloperoxidase (MPO) in treated mice colons. Mice colons were collected at day 8 after 3% dextran sulphate sodium (DSS) and zearalenone (ZEA, 4.5 mg kg^{-1}) treatment, and homogenates were used to determine IL-1β and IL-18 and MPO levels with an ELISA. Data represent the mean $\pm$ SEM; n = 8/group; *$P < 0.05$, ***$P < 0.001$, vs the control group.

expression of the control colitis group (3%DSS) were higher than those of the ZEA group (Fig. 5A). Significantly, the enhancements of NLRP3, ASC, and caspase-1 expression were obvious in the ZEA group (Fig. 5B). There was not much activation of pro-IL-1β in our research. We postulated that one possible reason for this finding is that IL-1β is active solely as an extracellular secreted product,

whereas its precursor is inactive. And we used 8 μg/mL ZEA to avoid decreasing the cell viability. This dose may not induce a significant change in pro-IL-1β. Furthermore, the changes in the protein expression of NLRP3 were analysed. The NLRP3 levels were up-regulated in DSS- and ZEA-treated colon tissue, in agreement with the PCR results (Fig. 5C). The western blot analyses were quantified by densitometry (Fig. 5D). These findings indicated that ZEA could activate the NLRP3-ASC complex, leading to the maturation and release of pro-IL-1β and pro-IL-18 *in vivo*.

4. Discussion

The contamination of foods or feeds by mycotoxins is a serious threat to animals and humans around the world, especially in many developing countries, and can lead to foodborne ailments or diseases and many health problems. These include a variety of fusariotoxicoses such as leukoencephalomalacia in equines, vulvovaginitis and pulmonary edema in porcines, alimentary toxic aleukia and oesophageal carcinoma in humans, various kinds of aflatoxicoses, toxic hepatitis in both humans and dogs, porcine/chicken nephropathy, and many other conditions (Reddy and Raghavender, 2007; Stoev, 2015). The intestinal epithelial cell layer is the first barrier that prevents mycotoxins from entering the underlying tissues. Underneath the epithelium, immunocompetent cells are found the lamina propria. Macrophages are largely responsible for the innate immune response in the mucosa.
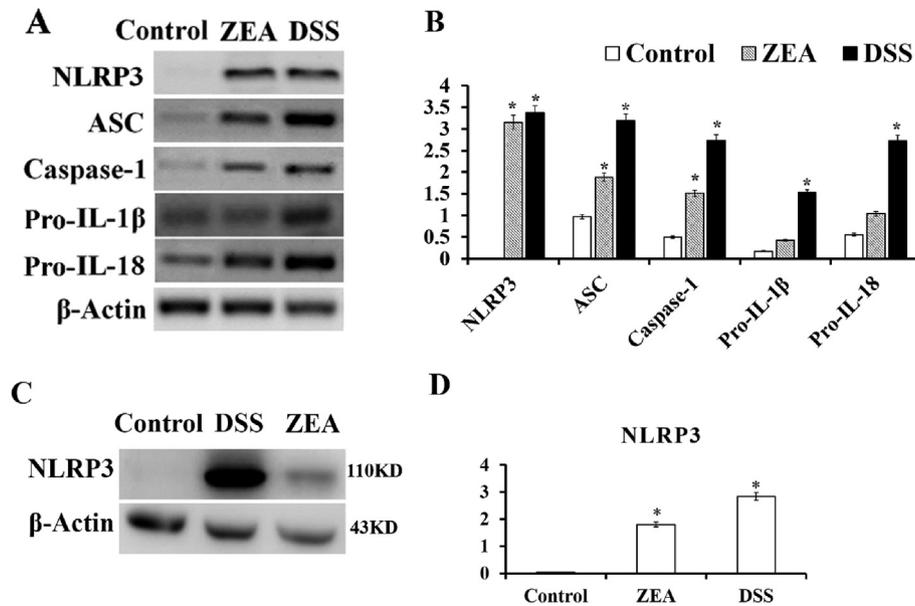
Fig. 5. mRNA and protein expressions of the NLRP3 inflammasome complex and pro-inflammatory cytokines in mice colonic tissue. Mice colons were collected at day 8 after 3% dextran sulphate sodium (DSS) or zearalenone ZEA (4.5 mg kg^{-1}) treatment. The total RNA or protein was extracted for PCR or western blotting detection. (A) Semiquantitative PCR results for NLRP3, apoptosis-associated adaptor protein (ASC), caspase-1, pro-interleukin-1β (pro-IL-1β), and pro-interleukin-18 (pro-IL-18). (B) Intensity ratios of each of the amplified bands to β-actin. (C) Analysis of NLRP3 protein expression. (D) Intensity ratios of NLRP3 to β-actin. Data represent the mean ± SEM; n = 3/group; *$P < 0.05$, vs the control group.

Epithelial cells and immune cells produce various cytokines, which are mainly innate components of immunity (Fig. 6A). Recently, researchers have demonstrated the effects of individual and mixtures of *Fusarium* toxins on the release of pro-inflammatory cytokines (Wan et al., 2013) and the modulation of secretory mucins as well as total mucin-like glycoprotein secretion (Wan et al., 2014), all of which are essential components of host mucosal immunity. Our data also showed that ZEA leads to an enhancement of cytokines, such as IL-1β and IL-18, in IPEC-J2 cells, mouse peritoneal macrophages, and colon tissue. Pistol reported that ZEA increased the expression of pro-inflammatory pro-TNF-α, IL-1β with 133.67% and 139.91% in pig spleens (Pistol et al., 2015). The excessive enhancement of these cytokines results in intestinal inflammatory responses to ZEA. This was also evidenced by histological analysis. Focally increased numbers of inflammatory cells were observed in the lamina propria, submucosa and transmural extension of the infiltrate. Tissue damage included distortion of crypts, mucosal erosions, and extensive mucosal damage (Bauer et al., 2010). Furthermore, measuring MPO activity is a feasible method for evaluating granulocyte infiltration in colonic tissues following the induction of ZEA (Camuesco et al., 2005). There was increased MPO activity in the ZEA-induced mouse intestinal inflammation, implying that leukocytes had recruited in mouse colonic tissue after ZEA induction.

IL-1β participates in the generation of local and systemic responses to injury, immunological challenges, and infection by generating fever, promoting leukocyte infiltration, and activating lymphocytes at sites of injury or infection (Dinarello, 1996). IL-18 is involved in the induction of several secondary pro-inflammatory cytokines, cell adhesion molecules, chemokines, and nitric oxide synthesis (Horwood et al., 1998). Data from human specimens and murine colitis models indicate that the excessive expression of IL-1β and IL-18 plays a key role in the pathogenesis of IBD (Siegmund et al., 2001; Kwon et al., 2005). Furthermore, caspase-1, which regulates the secretion of biologically active cytokines IL-1β and IL-18, has been identified as a central mediator of DSS-induced colitis

(Bauer et al., 2010). In addition, some studies have identified and characterized the inflammasomes and their molecular mechanisms on controlling caspase-1 activity. Inflammasomes are proteins of the NLR family. Their assembly and activation can recruit and activate caspases-1 or caspases-5 (Okamoto et al., 2010). In our study, we provide evidence that ZEA can induce caspase-1 activation in IPEC-J2 cells or mouse peritoneal macrophages, which is regulated by the NLRP3 inflammasome. IPEC-J2 cells can express and produce cytokines, toll-like receptors, defensins, and mucins, and they mimic porcine physiology more closely than any other cell line does; thus, they can be used to study epithelial transport and interactions with enteric bacteria, probiotic microorganisms, nutrients, and other feedstuffs (Vergauwen, 2015). Thus, our results for IPEC-J2 cells provide one explanation for why diarrhoea outbreaks occurred frequently in pigs with concomitant ZEA ingestion and porcine circovirus-2 infection (Duarte et al., 2013). However, ZEA-treated mice developed a weaker clinical and histological colitis severity and lower levels of pro-inflammatory cytokines in the colonic tissue than observed in the DSS-induced mice colitis model. We speculated that ZEA can bind to oestrogen receptors β (ERβ), which is the predominant ER form in both normal and malignant colon tissue (Ascenzi et al., 2006). Harris et al. suggested that one function of ERβ may be to modulate the immune response and that ERβ selective ligands may be therapeutically useful agents for treating joint and chronic intestinal inflammation (Harris et al., 2003).

The precise mechanism of NLRP3 inflammasome activation is not fully understood. The data obtained in present study demonstrate that ROS production is required for the release of cytokines IL-1β and IL-18 by macrophages in response to ZEA. Studies have reported that mycotoxin exposure may lead to various health effects and that this is mainly due to the induction of oxidative stress (Ferrer et al., 2009; Mary et al., 2012; Prosperini et al., 2013). Moreover, an abnormal increase of ROS and oxidative stress can trigger a cascade of subsequent toxic pathways associated with the development of many diseases. For example, studies found that
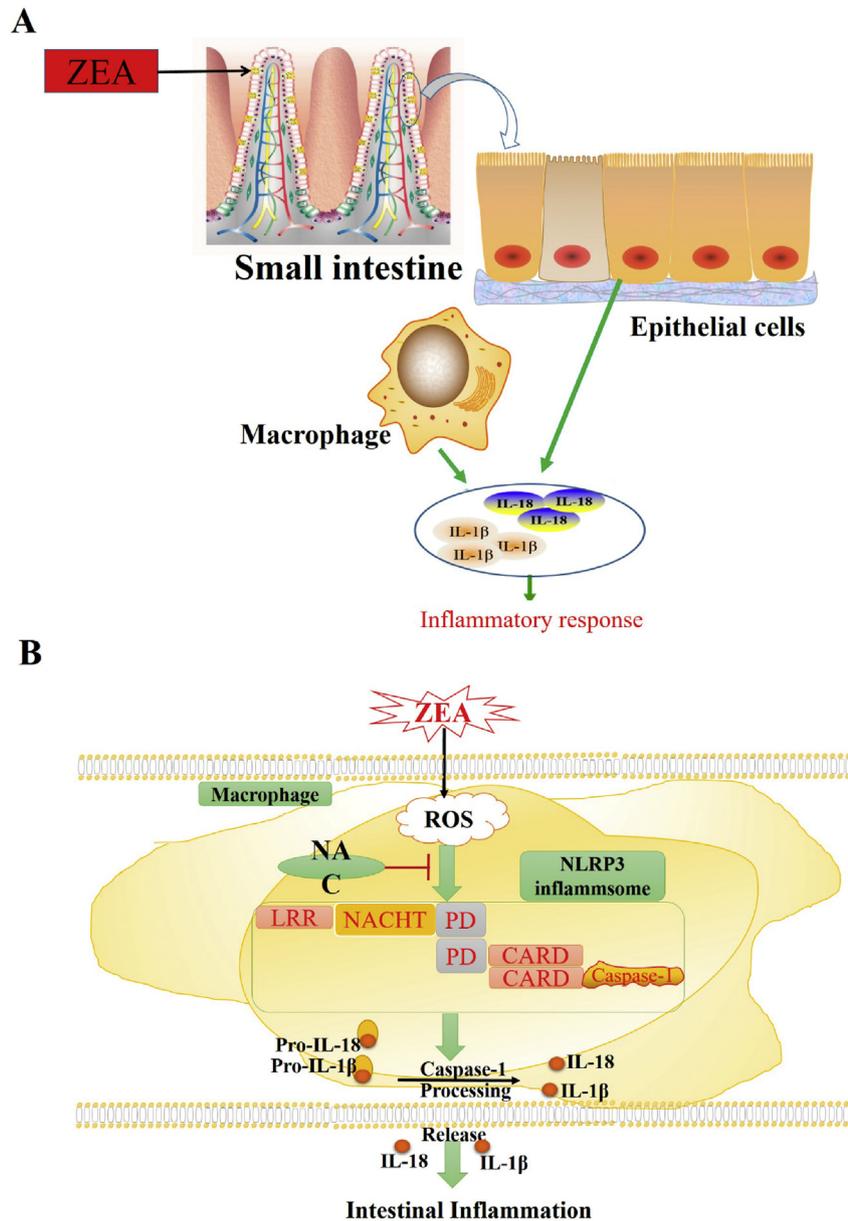
Fig. 6. Schematic depiction of the inflammatory response in the small intestine to Zearalenone (A) and potential mechanisms of Zearalenone-induced intestinal inflammation (B).

oxidants play a direct role in the chronic inflammatory process by increasing the number of macrophages and neutrophils in tissue that induce a self-sustaining phlogogenic loop (Yao et al., 2010). In addition, Yao et al. validated the important role of oxidative stress involved in the inflammatory process and pathogenesis of ulcerative colitis (UC). In our research, the ROS inhibitor N-acetylcysteine (NAC) reduced the secretion of IL-1β and IL-18 and the expression of the NLRP3-ASC complex in mouse peritoneal macrophages. These data suggested that ROS contribute to ZEA-induced NLRP3 inflammasome activation and intestinal inflammation in mice. This is consistent with previous findings showing that increased lipid peroxidation products contributed to the inflammatory process in Crohn's disease (CD) and UC (Kruidenier et al., 2003). Wang et al. also proved that NAC inhibits amyloid β-induced NLRP3 inflammasome activation in retinal pigment epithelial cells from 452.33 ± 53.24 to 242.17 ± 21.35 of controls (Ke et al., 2017). Thus, ROS reduction could be used as an indicator for the high-throughput screening of new anti-inflammatory drugs targeting

the NLRP3 inflammasome to treat mycotoxin-induced inflammation. In conclusion, we explored a potential mechanism of ZEA-induced intestinal inflammation. Initially, ZEA exerts a direct toxic effect on the epithelial barrier and enhances the accumulation of ROS. Then, macrophages are stimulated by the ROS, which accordingly enhance the transcription of pro-IL-1β and pro-IL-18. In a second step, ZEA induces caspase-1 activation via the NLRP3 inflammasome complex, cleaving pro-IL-1β and pro-IL-18 into their biologically active forms, which initiates an intestinal inflammatory cascade (Fig. 6B).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2017.09.145.

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