



Zearalenone can relieve dextran sulfate sodium-induced inflammatory reaction

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Funding information

National Key R&D Program, Grant/Award Numbers: 2016YFD0501009, 2016YFD0501200; Natural Science Foundation of Jiangsu Province, Grant/Award Number: BK20161452; Fundamental Research Funds for the Central Universities, Grant/Award Number: KYZ201848; NAU International Cooperation and Cultivation Project, Grant/Award Number: 2018-AF-20; Key Program of Science and Technology Planning of Guangdong Province, Grant/Award Number: 2017B020202010; Priority Academic Program Development of Jiangsu Higher Education Institutions.

Abstract

In this study, we investigated the influence of zearalenone (ZEA) on the dextran sulfate sodium (DSS)-induced colitis model both in vitro and in vivo. Our results show that the mRNA levels of IL-1 β , IL-18, NLRP3, ASC, and caspase-1 in the DSS+ZEA-treated group are lower than those in either the DSS or ZEA group, and the protein expression trends are similar. Furthermore, colitis, which is characterized by body weight loss, stool consistency, and the presence of bloody feces, was significantly alleviated in the DSS+ZEA group when compared with that in the DSS group. In addition, histological analysis showed that inflammatory cell infiltration and tissue damage of the colon in the DSS+ZEA group were recovered compared with that in the DSS-treated group. These results suggest that, instead of aggravating DSS-induced colitis, ZEA relieves the inflammatory reaction in colon tissue, which may be related to its estrogenic activity.

KEYWORDS

dextran sulfate sodium (DSS), estrogen receptors (ERs), inflammation, NLRP3, zearalenone (ZEA)

1 | INTRODUCTION

NOD-like receptors (NLRs) are intracellular microbial and nonmicrobial sensors that respond to danger signals and function by forming large cytoplasmic complexes, termed inflammasomes, to activate proinflammatory cytokines.^[1] The small intestine is the first physical barrier against

pathogenic foreign substances. Normal intestinal inflammation is a protective reaction against these substances. Once the barrier is disrupted, NLRs are activated to protect the intestinal tract through the release of inflammasomes^[2]; among these, the NLRP3 inflammasome is closely associated with colitis.^[3] As one of the best characterized NLRs, NLRP3 can form the NLRP3 inflammasome together with

apoptosis-associated adaptor protein (ASC) and the precursor of caspase-1 and can convert pro-IL-1 β and IL-18 into their active forms under a variety of environmental and endogenous stimuli.^[4–6] However, over-activation of inflammasomes will result in an inflammatory disorder of the intestinal tract.^[2,7]

Zearalenone (ZEA) is a nonsteroidal estrogenic mycotoxin and is mainly produced by several *Fusarium* species. It can widely contaminate cereal crops, including corn, barley, wheat, oats, sorghum, and sesame grains.^[8] It has been reported that ZEA is associated with many mycotoxicoses in farm animals.^[9] ZEA accounts annually for millions of dollars in pig breeding losses worldwide.^[10] If an animal ingests ZEA-contaminated feed, the intestine will be exposed to high concentrations of ZEA. The adverse influences of ZEA on the gut have been confirmed.^[11–13] In our previous study, we proved that ZEA exposure could induce mitochondrial damage by reducing antioxidant enzyme activities, accumulation of reactive oxygen species (ROS), and decreasing mitochondrial membrane potential (MMP).^[14] The mitochondria-derived components could trigger NLRP3 inflammasome activation to convert the proinflammatory cytokines IL-1 β and IL-18 into their active forms, which were the central players in the pathogenesis of colitis and mediated ZEA-induced intestinal inflammation in mouse colon, which were characterized by significant inflammatory cell infiltration and tissue damage.^[7] Because ZEA can induce intestinal inflammation, we speculated that the ingestion of large amounts of ZEA-contaminated feed might aggravate inflammatory reactions in the gastrointestinal tract. To verify this notion, we investigated the influence of ZEA on the dextran sulfate sodium (DSS)-induced colitis model both in vitro and in vivo. DSS has been widely used to induce colitis in model mice.^[15] And this model is particularly useful for research into the role of innate immune mechanisms in intestinal inflammation.^[16] Surprisingly, the results indicated that, instead of aggravating DSS-induced colitis, ZEA relieved the inflammatory reaction in colon tissue.

2 | MATERIALS AND METHODS

2.1 | Chemicals

DSS (30-50KW; Lot: 60316es25) was purchased from MP Biomedical (CA), and ZEA (Lot: 151012) was obtained from Fermentek (Jerusalem, Israel). Lysis buffer (radio-immunoprecipitation assay [RIPA]: phenylmethanesulfonyl fluoride [PMSF], 1:9; Lot: P0013B, ST506) was obtained from Beyotime (Shanghai, China). TRIZOL reagent (Lot: 7E102B7) was purchased from Vazyme (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits (Lot: 04/2017) for IL-1 β , IL-18, and myeloperoxidase (MPO) were provided by Shanghai Enzyme-linked Biotechnology Co., Ltd (Shanghai, China). A reverse transcription kit and polymerase chain reaction (PCR) Mix were obtained from Thermo (MA), and ChamQ SYBR qPCR Master Mix (Lot: 7E14117) was obtained from Vazyme (Nanjing, China). Polyvinylidene fluoride (PVDF) membranes (0.45 mm) (Lot: A10190850) were acquired from GE Healthcare (Pittsburgh, PA). High glucose-supplemented Dulbecco modified Eagle medium (DMEM; Lot: 8118050) was obtained from Invitrogen Trading (Shanghai, China). β -Actin antibodies (Lot: ABS830031SS) were provided by Absin

(Shanghai, China). Rabbit NLRP3 polyclonal antibody (Lot: AB210491-40UL) was obtained from Abcam (Cambridge, United Kingdom). Rabbit anti-ASC polyclonal antibody (Lot: OM122624) and rabbit anti-caspase-1 P10 polyclonal antibody (Lot: OM122904) were provided by OmnimAbs (CA). Goat anti-rabbit HRP linked antibodies (Lot: 7074S) were purchased from CST (MA).

2.2 | Cell acquisition and culture

Peritoneal macrophages were isolated from mouse abdominal cavity. Briefly, mice were killed by cervical dislocation under 5% isoflurane anesthesia; then, 10 mL phosphate-buffered saline was injected into the abdominal cavity. After soft shaking, intraperitoneal fluids were collected and centrifuged for 15 minutes (1000g). Finally, peritoneal macrophages were acquired. Intestinal porcine enterocyte cell line (IPEC-J2) cells and peritoneal macrophages were cultured in high glucose-supplemented DMEM containing 1% penicillin-streptomycin and 10% fetal calf serum at 37°C under an atmosphere containing 5% CO₂. Both the macrophages and IPEC-J2 cells were incubated with 3% DSS, ZEA (8 μ g/mL), and 3% DSS+ZEA (8 μ g/mL) for 24 hours. The concentrations of ZEA used in this study had been determined in the previous studies, and the cell viability assay was also performed.^[7]

2.3 | Colitis model mice and experimental method

In total, 12 male Balb/c mice aged 6–8 weeks (20–22 g) were randomly divided into four groups: control, DSS, ZEA, and DSS+ZEA groups. The DSS, ZEA, and DSS+ZEA groups were administered 0.5 mL of 3% DSS, 0.5 mL of ZEA (4.5 mg/kg), and 0.5 mL of 3% DSS + 0.5 mL of ZEA (4.5 mg/kg), respectively, by gavage once a day for 9 days. The control mice were given 0.5 mL of distilled water each day. All experiments were approved by the regional animal study committee and were consistent with the guidelines for the proper use of animals in biomedical research.

2.4 | Disease activity index (DAI) and histological analysis

Body weight, the presence of occult or gross blood per rectum, and stool consistency were determined by two investigators who were blinded to the treatment groups. A scoring system was applied to assess diarrhea and the presence of occult or overt blood in the stool. The specific method used to calculate DAI was as follows: DAI = (Weight loss + Stool consistency + Occult/gross bleeding)/3. Changes in body weight are indicated as a percentage loss of baseline body weight: less than 5% weight loss was scored as 1, weight loss between 5% and 10% was scored as 2, and weight loss greater than 10% was scored as 3. For stool consistency, slightly loose stools were scored as 1, loose stools were scored as 2, and diarrhea was scored as 3. For occult/gross bleeding, occult bleeding was scored as 1, gross bleeding early stage was scored as 2, and gross bleeding was scored as 3. Post-mortem, the colons were removed, and pieces of colonic tissue were used for analysis. For histological analysis, rings of the transverse part of the colon were fixed in 4% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin staining (H&E) according to standard protocols.

2.5 | SDS-PAGE and western blot analysis

Colon tissue (100 mg) was ground and homogenized with 1 mL lysis buffer and then centrifuged for 5 minutes at 12 000g. Then, the supernatant was resolved on a 10% acrylamide-bisacrylamide gel (EpiZyme, Shanghai, China), and the proteins were transferred onto a 0.45- μ m PVDF membrane. Primary anti-NLRP3 was applied at 1:500 dilution, anti-ASC and anti-caspase-1 antibody were applied at 1:1000 dilution, and anti- β -actin (loading control) was used at 1:2000 dilution. Immunoglobulin G anti-rabbit horseradish peroxidase (HRP) was diluted at 1:8000. An Amersham Imager 600 (GE Healthcare) was used to visualize chemoluminescence.

2.6 | mRNA extraction, quantitative reverse transcription-PCR, and reverse transcription-PCR

Colonic tissues were snap-frozen in liquid nitrogen and stored at -80°C . Total colonic tissue RNA and cellular RNA were extracted as usual. Reverse transcription (RT) reactions were performed according to the manufacturer's recommendations. A Light Cycler instrument (Roche, Mannheim, Germany) and a Light Cycler Fast Start DNA Master SYBR Green I Kit (Roche, Mannheim, Germany) were used for real-time PCR analysis. For RT-PCR, the complementary DNA sample (1 μ L), PCR Mix (10 μ L), primers (2 μ L of each primer), and double-distilled H_2O (7 μ L) were mixed. The optimal PCR conditions were 35 cycles of 92°C for 30 s, 58°C for 60 s, and 72°C for 30 s. The PCR assays were repeated three times. The expression levels of mRNA were determined at different time points.

2.7 | Analysis of colonic cytokines

Colon tissues were ground with 200 μ L RIPA reagent, and the homogenate was then centrifuged at 12 000g at 4°C for 15 minutes. The amount of total extracted protein in the supernatant was determined using a Coomassie Brilliant Blue kit. The amount of IL-1 β , IL-18, and MPO in the colon homogenate was quantified by ELISA.

2.8 | Enzyme-linked immunosorbent assay

Primary macrophages and IPEC-J2 cells were seeded into 96-well plates at a density of 2×10^5 cells per well. After induction with lipopolysaccharide for 2 hours, the cells were stimulated with 3% DSS, ZEA (8 $\mu\text{g}/\text{mL}$), and 3% DSS+ZEA (8 $\mu\text{g}/\text{mL}$) for 24 hours, respectively. Cell culture supernatant or colon homogenates were used in the ELISAs, which were performed according to the manufacturer's protocol

2.9 | Statistical analysis

The results are expressed as the means of three independent experiments \pm the standard error of the means. The post hoc test was performed to investigate the significant differences between the DSS+ZEA group and the control, DSS, or ZEA groups for all parameters. The statistical analysis was carried out using GraphPad Prism

software 5.0. Differences were considered statistically significant at $*P < 0.05$, $^{\#}P < 0.05$, $^{\&}P < 0.05$, $^{***}P < 0.001$, and $^{###}P < 0.05$.

3 | RESULTS

3.1 | Influence of ZEA on NLRP3, ASC, caspase-1, pro-IL-1 β and pro-IL-18 mRNA levels, IL-1 β and IL-18 production and NLRP3, ASC, and caspase-1 expression in vitro

The total mRNA levels of IPEC-J2 for NLRP3, ASC, caspase-1, pro-IL-1 β , and pro-IL-18 were quantified by RT-PCR. As shown in Figure 1A, compared with the control, the mRNA levels of these factors in the DSS group reached 2.46-, 2.66-, 3.05-, 2.57-, and 4.10-fold, respectively, and those in the ZEA group reached 2.31-, 2.37-, 2.33-, 1.54-, and 3.44-fold, respectively; the data for the DSS+ZEA group were obviously different, with values of 1.84-, 2.25-, 2.13-, 1.21-, and 3.07-fold, respectively. Compared with the control group, all inflammatory factors in these three groups were upregulated. However, compared with levels in the DSS and ZEA groups, the total mRNA levels of these factors in the DSS+ZEA group were all downregulated.

The influence of ZEA on IL-1 β and IL-18 production in peritoneal macrophages quantified in the study. As shown in Figure 1B, compared with the control group, the concentrations of IL-1 β and IL-18 were significantly increased in the DSS, ZEA, and DSS+ZEA groups ($P < 0.01$). The concentrations of IL-1 β and IL-18 were 65.65 and 251.92 pg/mL in the control group, respectively. After treatment with DSS, ZEA, or DSS+ZEA, the amounts of these two cytokines reached 277.78 and 702.56 pg/mL, 240.83 and 690.13 pg/mL, and 228.06 and 622.82 pg/mL, respectively. However, the concentrations of these two inflammatory factors were lower in the DSS+ZEA group than in the other two experimental groups.

Furthermore, changes in the levels of NLRP3, ASC, and caspase-1 were also evaluated in the study. The results demonstrated that the expression of NLRP3 in the DSS, ZEA, and DSS+ZEA groups were all upregulated compared with that in the control group (Figure 1C and 1D). In agreement with previous results, the enhancement of NLRP3 in the DSS+ZEA group was lower than that in the other groups. However, different from NLRP3, the expression of ASC and caspase-1, the adaptor for the NLRP3 inflammasome, showed no difference among the DSS, ZEA, and DSS+ZEA groups.

3.2 | Effects of ZEA on DAI

For DAI, 3 days after drug administration, mice in the DSS group developed diarrhea, the feces became loose, and the weight was slightly reduced; the changes in both the ZEA and DSS+ZEA groups were not obvious. After 5 days of treatment, almost all the mice had mild or severe inflammatory reactions, except for the control group. It should be pointed out that one mouse in the DSS group had developed hematochezia. After 9 days, except for the control group, all mice showed severe inflammation. Of note, one mouse in the DSS group died on day 7 due to the severe colitis. As shown in the line chart (Figure 2),

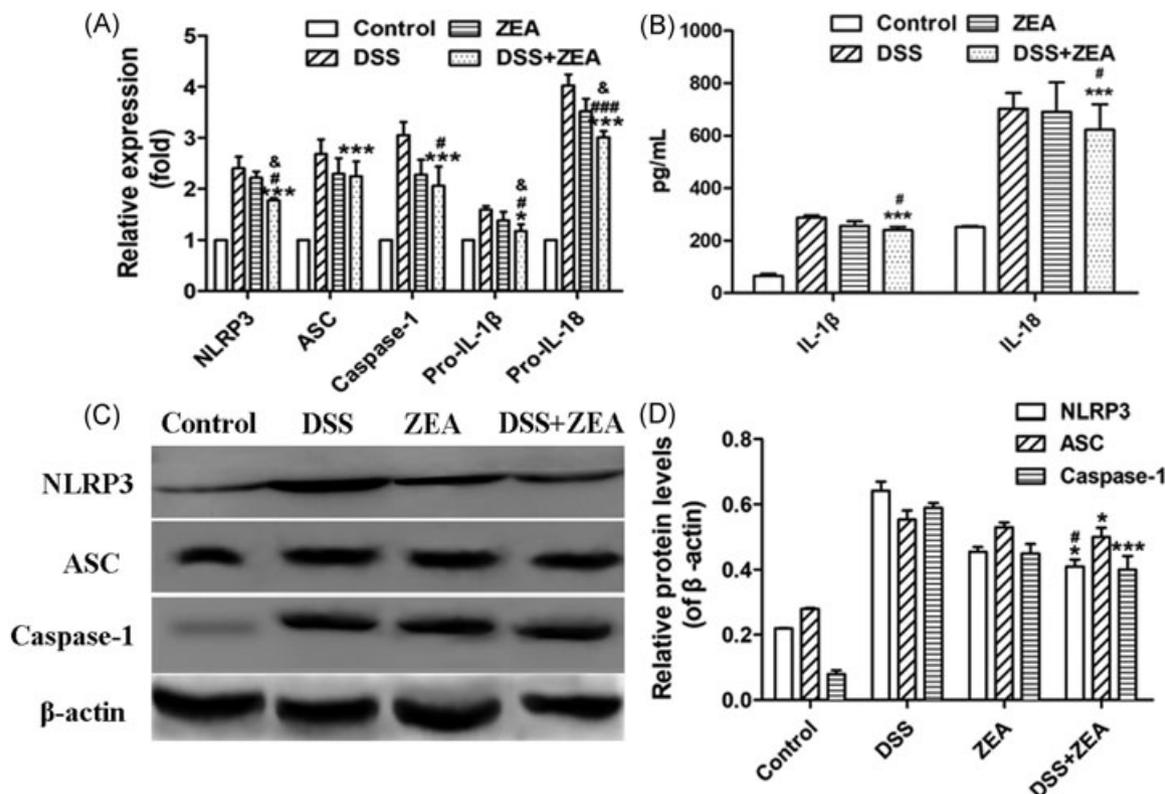


FIGURE 1 A, The total RNA levels of NLRP3, ASC, caspase-1, pro-IL-1 β , and pro-IL-18 in IPEC-J2 cells. B, The concentration of cytokines IL-1 β and IL-18 in the macrophage supernatant. C, NLRP3, ASC, and caspase-1 protein expression in IPEC-J2 cells. D, Intensity ratios of NLRP3, ASC, and caspase-1 protein to β -actin. IPEC-J2 cells and macrophages were treated with 3% DSS, ZEA, and 3% DSS+ZEA. The data are presented as the means \pm SEM (n = 3 independent experiments). * P < 0.05 for the DSS+ZEA group vs the control group, # P < 0.05 for the DSS+ZEA group vs the DSS group, $^{\&}$ P < 0.05 for the DSS+ZEA group vs the ZEA group, *** P < 0.01 for the DSS + ZEA group vs the control group, ### P < 0.01 for the DSS+ZEA group vs the DSS group. DSS, dextran sulfate sodium, IPEC-J2, intestinal porcine enterocyte cell line; SEM, standard error of mean; ZEA, zearalenone

the intestinal inflammation in the DSS+ZEA group was less severe than those in the other two groups.

3.3 | Effects of ZEA on histological changes

Histological analysis further demonstrated that after 9 days of gavage, large numbers of infiltrating inflammatory cells, distorted crypts, and extensive damage in the lamina propria and submucosa were observed in the DSS, ZEA, and DSS+ZEA groups (Figure 3). In particular, the colon crypts in the DSS mice disappeared completely (Figure 3B-1). In the ZEA group, the crypts were destroyed (Figure 3C-2) only to a small extent (Figure 3C-3). However, in the DSS+ZEA group, the majority of crypts were normal (Figure 3D-4), and only a small number of the crypts were destroyed (Figure 3D-5).

3.4 | Influence of ZEA on NLRP3, ASC, caspase-1, pro-IL-1 β , and pro-IL-18 mRNA levels, IL-1 β , IL-18, and MPO production and NLRP3, ASC, and caspase-1 expression in vivo

The change in mRNA levels of NLRP3, ASC, caspase-1, pro-IL-1 β , and pro-IL-18 in mouse colon tissues was similar to that observed in the in vitro experiments (Figure 4A). The total mRNA levels of mice treated with DSS +ZEA were significantly lower than those in the DSS group, with the

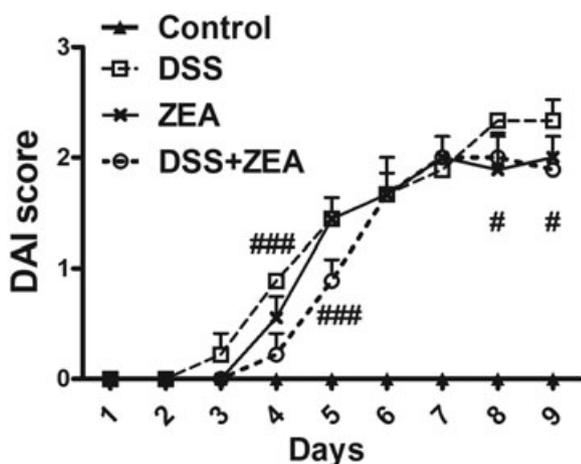


FIGURE 2 Colitis severity and inflammatory response in terms of disease activity index (DAI) in mice. The data are presented as the means \pm SEM (n = 3 independent experiments). # P < 0.05 for the DSS+ZEA group vs the DSS group, ### P < 0.01 for the DSS+ZEA group vs the DSS group. DSS, dextran sulfate sodium; SEM, standard error of mean; ZEA, zearalenone

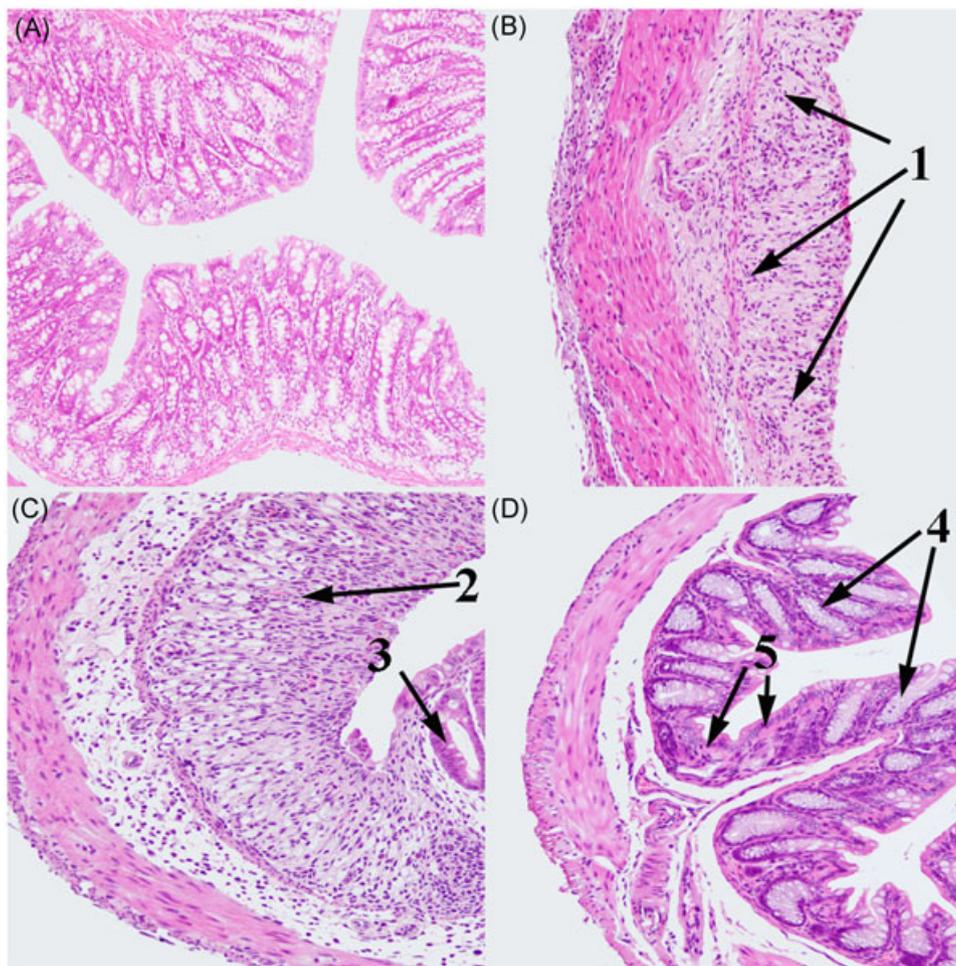


FIGURE 3 Histopathological changes of DSS- and ZEA-induced intestinal inflammation in mice. Mouse colons were collected at day 8 after 3% DSS, ZEA, and DSS+ZEA feeding. A, Histological section of control mice. B, Histological section of 3% DSS-treated mice. C, Histological section of ZEA-treated mice. D, Histological section of 3% DSS+ZEA-treated mice. HE staining was used to evaluate morphological changes in the colon tissues. The arrows (1, 2, 3, 4, and 5) highlight areas of tissue lesions and inflammatory cell infiltration (original magnification $\times 10$). DSS, dextran sulfate sodium; HE, hematoxylin-eosin staining; ZEA, zearalenone

relative expression of mRNA (fold, compared with the control group) of 3.76, 3.21, 2.96, 1.47 and 2.26, respectively, in the DSS group, and 2.89, 1.80, 1.02, 1.43, and 1.85, respectively, in the DSS+ZEA group.

After treatment with ZEA, DSS, or DSS+ZEA, the release of IL-1 β , IL-18, and MPO increased in these groups (Figure 4B); likewise, the extent of the improvement in the DSS+ZEA group was lower than that in the DSS group ($P < 0.05$). In particular, the levels of IL-1 β , IL-18, and MPO were 23.61, 66.54, and 242.51 pg/mL in the control group; 99.88, 245.58, and 575.71 pg/mL in the DSS treatment group; 88.75, 216.35, and 561.43 pg/mL in the ZEA group; and 73.61, 209.62, and 522.14 pg/mL in the DSS+ZEA group.

To further investigate the influence of ZEA, the expression levels of NLRP3, ASC, and caspase-1, the main factors in the NLRP3 signaling pathway, were measured in the present study. In agreement with the mRNA analysis, the expression levels of NLRP3, ASC, and caspase-1 in the mouse colon tissues were obviously upregulated in the three experimental groups compared with the control group (Figure 4C and 4D).

3.5 | Influence of ZEA on ER α and ER β mRNA levels in vivo

As shown in Figure 5, the mRNA levels of ER α in the control and DSS groups was obvious, while that in the ZEA group and DSS+ZEA group was quite low. Interestingly, the mRNA expression of ER β was just the opposite. In the control and DSS groups, no mRNA of ER β could be detected; however, in the ZEA and DSS+ZEA groups, the ER β mRNA levels were high. It is worth noting that compared with other groups, the amount of ER β mRNA was extremely high in the DSS+ZEA group.

4 | DISCUSSION

DSS has been widely used to establish a colitis model in mice that exhibits the characteristics of diarrhea and bloody stools. In our previous study, we proved that ZEA can induce intestinal inflammation in mouse colon, which is mediated by the NLRP3 inflammasome.^[7]

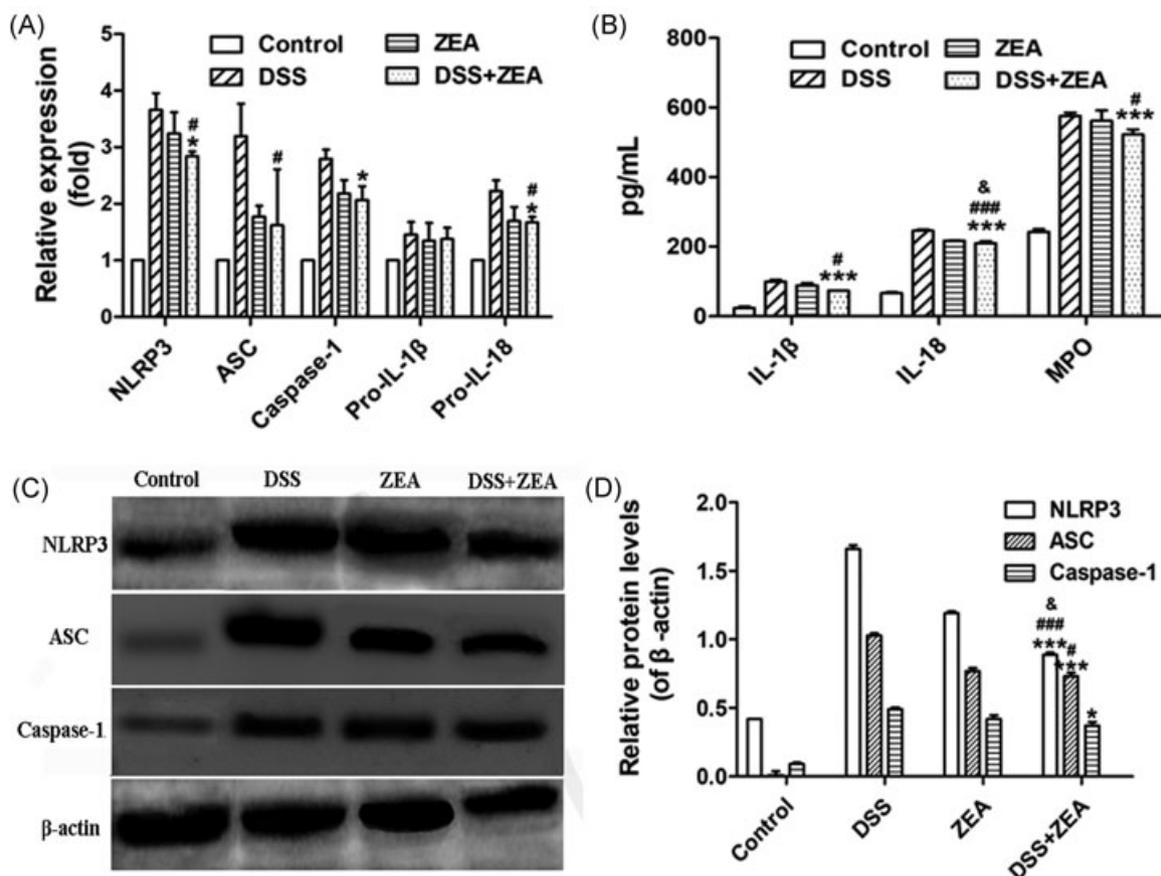


FIGURE 4 A, Total RNA levels of NLRP3, ASC, caspase-1, pro-IL-1 β , and pro-IL-18 from mouse colons assessed by quantitative reverse transcription PCR. B, IL-1 β , IL-18 and MPO release in mouse colons induced by DSS and ZEA. IL-1 β , IL-18, and MPO were analyzed by ELISA. C, Protein expression levels of NLRP3, ASC, and caspase-1 in mouse colon tissue. D, Intensity ratios of NLRP3, ASC, and caspase-1 to β -actin. The mice were treated with 3% DSS, ZEA (4.5 mg/kg), and 3% DSS+ZEA (4.5 mg/kg) for 9 days, respectively. Data are presented as the means \pm SEM (n = 3 independent experiments). * P < 0.05 for the DSS+ZEA group vs the control group, # P < 0.05 for the DSS group vs the DSS+ZEA group, & P < 0.05 for the DSS+ZEA group vs the ZEA group, *** P < 0.01 for the DSS+ZEA group vs. the control group, ### P < 0.01 for the DSS+ZEA group vs the DSS group. ACS, apoptosis-associated adaptor protein; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; MPO, myeloperoxidase; PCR, polymerase chain reaction; SEM, standard error of mean; ZEA, zearalenone

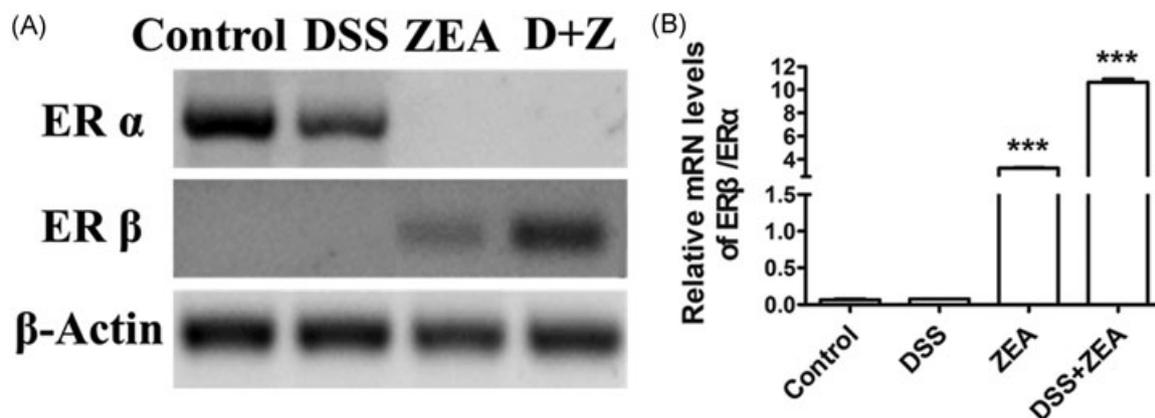


FIGURE 5 A, Total RNA levels of ER α and ER β from mouse colons assessed by reverse transcription PCR. B, Intensity ratio of ER β to ER α . The data are presented as the means \pm SEM (n = 3 independent experiments). *** P < 0.01 for the group vs the control group. DSS, dextran sulfate sodium; ER, estrogen receptor; PCR, polymerase chain reaction; SEM, standard error of mean; ZEA, zearalenone

Therefore, we speculated that the ingestion of ZEA-contaminated feed may aggravate inflammatory reactions in the gastrointestinal tract. To verify this hypothesis, *in vivo* and *in vitro* experiments were performed. The results showed that the total mRNA levels of NLRP3, ASC, caspase-1, pro-IL-1 β , and pro-IL-18 were significantly increased in IPEC-J2 cells after treatment by 3% DSS and ZEA, respectively, which is consistent with previous findings. Although the total mRNA levels of these inflammatory factors also increased in contrast to the control when the IPEC-J2 cells were treated with DSS+ZEA, when compared with those of the DSS and ZEA groups, the overall level showed a downward trend, particularly compared with those in the DSS group.

In this study, we found that the secretion of IL-1 β and IL-18 was significantly improved in the above three groups. Likewise, when compared with those in the DSS and ZEA groups, the secretion of IL-1 β and IL-18 revealed a significant downward trend. In addition, NLRP3 protein expression in the DSS+ZEA group was also decreased both *in vivo* and *in vitro* when compared with the DSS and ZEA groups. The expression of NLRP3 protein reflects the degree of inflammation; thus, when compared with the DSS and ZEA groups, the inflammation was relatively mild in the DSS+ZEA group. Therefore, we speculated that, to some extent, ZEA has the ability to slow the inflammatory response to 3% DSS. Changes in the DAI score as well as histological analyses of the mouse colon sections further suggested that ZEA might have a function in relieving colitis. These results are contrary to our speculation. It is worth noting that both *in vivo* and *in vitro*, different from the tendency of NLRP3, the other two parts of the inflammasome, ASC and procaspase-1, showed no change among the DSS, ZEA, and DSS+ZEA groups. The reason for this might be that these two adaptors can form inflammasomes with other NLRs besides NLRP3, such as NLRP1, NLRC4, and NLRP12.^[2,17–19]

ZEA is a nonsteroidal estrogenic mycotoxin that is produced by several *Fusarium* fungi. The best-known trait of ZEA is its estrogenic activity.^[20] It has been reported that the structure of ZEA is similar to that of estradiol-17 β (E2), which is the most potent endogenously synthesized and secreted ovarian estrogen, which exhibits anti-inflammatory properties, including the suppression of proinflammatory cytokines.^[21] The anti-inflammatory activities of E2 are mediated by the two classical estrogen receptors (ERs), ER α and ER β . Previous studies suggested that ER β might regulate the innate immune response through the inflammasome.^[22] ERs also inhibit the proinflammatory cytokines IL-1 β in serum and the lung.^[23] In combination with the obvious difference of mRNA levels of ER α and ER β among difference groups, we speculated that the function of ZEA in relieving inflammatory bowel disease might be related to the ER β . This provides a new direction for the treatment of inflammatory bowel disease.

ACKNOWLEDGMENTS

This research was funded by the National Key R&D Program (2016YFD0501009 and 2016YFD0501200), the Natural Science Foundation of Jiangsu Province (BK20161452), the Fundamental Research Funds for the Central Universities (KYZ201848), NAU International Cooperation and Cultivation Project (2018-AF-20), the

Key Program of Science and Technology Planning of Guangdong Province (2017B020202010), and Priority Academic Program Development of Jiangsu Higher Education Institutions.

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How to cite this article: Ding C, Fan W, Shen T, Huang K, Song S, Yan L. Zearalenone can relieve dextran sulfate sodium-induced inflammatory reaction. *J Biochem Mol Toxicol.* **2018**;e22236. <https://doi.org/10.1002/jbt.22236>