Fumonisin B_1 induces chicken heterophil extracellular traps mediated by PAD4 enzyme and P2 × 1 receptor

Zhikai Wu,[1] Xingyi Zhu,[1] Peixuan Li, Xia Wang, Youpeng Sun, Yiwu Fu, Jingjing Wang, Zhengtao Yang, and Ershun Zhou[2]

College of Life Sciences and Engineering, Foshan University, Foshan 528225, Guangdong Province, PR China

ABSTRACT Fumonisin B_1 (**FB_1**) is a common mycotoxin contamination in agricultural commodities being considered as a significant risk to human and livestock health, while the mechanism of FB_1 immunotoxicity are less understood, especially in chicken. Given that extracellular traps as a novel defense mechanism of leukocytes play an important role against foreign matters, in this study we aimed to investigate the effects of FB_1 on chicken heterophil extracellular traps (**HETs**) formation. Our result showed that FB_1 induced HETs release in chicken heterophils observed via immunostaining, and it was concentration-dependent during 10 to 40 μM. Moreover, in 40 μM FB_1-exposed chicken heterophils, reactive oxygen species (**ROS**) level was increased, while catalase (**CAT**), superoxide dismutase (**SOD**), and glutathione peroxidase (**GSH-Px**) activity and glutathione (**GSH**) content were decreased. Simultaneously, FB_1 (40 μM) activated ERK and p38 MAPK signaling pathways via increasing the phosphorylation level of ERK and p38 proteins. However, pretreatment of SB202190, U0126, and diphenyleneiodonium chloride (**DPI**) did not change FB_1-triggered ROS production and HETs formation, suggesting FB_1-induced HETs was a nicotinamide adenine dinucleotide phosphate (**NADPH**) oxidase, p38, and extracellular regulated protein kinases (**ERK**) signaling pathways-independent process. Inhibition of peptidyl arginine deiminase 4 (**PAD4**) enzyme and P2 × 1 receptor showed their vital role in 40 μM FB_1-triggered HETs. This study reported for the first time that 40 μM FB_1 induced the release of HETs in heterophils, and it was related to ROS production, PAD4, and P2 × 1, but was independent of NADPH oxidase, p38 and ERK signaling pathways, which might provide a whole novel perspective of perceiving and understanding the role of FB_1 in immunotoxicity.

Key words: fumonisin B_1, chicken, heterophil extracellular traps, immunotoxicity

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INTRODUCTION

Fumonisins (**FBs**) are mycotoxins produced by fungi such as *Fusarium verticillioides* and *Fusarium proliferatum*, which are widely distributed in maize and other farm products (Wang et al., 2016). Until now, more than 15 fumonisin homologues has been characterized (Braun and Wink, 2018), among which fumonisin B_1(**FB_1**) is the most widely distributed, and the most abundant one, and has great potential health hazards to humans and animals (Schelstraete et al., 2020). FB_1 exhibits hepatotoxicity, nephrotoxicity and intestinal damage (Müller et al., 2012; Poersch et al., 2014; Gu et al., 2019; Chen, et al., 2020; Liu, et al., 2020). It has been reported that dietary FB_1 resulted in depressed weight gains, increased organ weights, diarrhea, thymic cortical atrophy, multifocal hepatic necrosis, biliary hyperplasia, and rickets in broiler chicks (Ledoux et al., 1992; Henry et al., 2000; Sousa et al., 2020).

Neutrophil extracellular traps (**NETs**), an entirely new type of defense mechanism of neutrophils, are network structures composed of decondensed chromatin as a scaffold decorated with various proteins (Brinkmann et al., 2004). With the deepening of research, researchers have discovered that NETs are not only related to the body's immune defense, but also implicated with various immune-related and other diseases such as systemic lupus erythematosus (Frangou et al., 2019), gout (Caution et al., 2019), rheumatoid arthritis (Carmona-Rivera et al., 2017), multiple types of vasculitis (Nakazawa, et al., 2019; Lu et al., 2020), thrombosis (Kimball et al., 2016), etc. Poultry heterophils similar to mammalian neutrophils can

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[1]These two authors contributed equally to this study.

[2]Corresponding author: zhouershun@fosu.edu.cn

phagocytose and kill pathogens, containing distinct acidophilic granules, but without myeloperoxidase (Harmon, 1998). It has been reported that FB_1 induced NETs release from bovine neutrophils (Wang et al., 2020), but the effects of FB_1 on chicken heterophils is still unclear. In this study, we first investigated the role of FB_1 on heterophil extracellular traps (**HETs**) formation, and further tried to examined the potential molecular mechanisms aiming to provide experimental reference for further research on the immunotoxicity of FB_1.

MATERIALS AND METHODS

Isolation of Chicken Heterophils

Chicken heterophils were isolated from the peripheral blood of healthy adult cock (n = 5) using Chicken Heterophils Isolation Kit (TianJin HaoYang Biological Manufacture Co., China) according to manufacturer's instructions. In brief, peripheral blood was diluted 1:1 in RPMI 1640 medium (Beijing solarbio science & technology co., ltd., China), and the diluted blood was gently layered on the separation solution in 50 mL centrifuge tube followed by centrifugation at 850 *g* for 40 min. The heterophil layer was collected, and then washed twice using red cell lysis solution. Finally, purified heterophils (≥90% of the cells) were then resuspended in RPMI 1640 medium without phenol red (Beijing solarbio science & technology co., ltd.), and placed in an incubator for further use. Approval for the use of Chicken was obtained from Institutional Animal Care and Use Committee of the Foshan University.

Cytotoxicity Assay of FB_1 on Chicken Heterophils

Chicken heterophils (2×10^5/well) (n = 5) were cultured in serum-free medium with different concentrations of FB_1 (0, 10, 20, 40, or 80 μM, Sigma-Aldrich, Saint Louis, MO) in white, clear-bottomed 96-well plate for 4 h at 37°C with 5% CO_2. Cytotoxicity of FB_1 on chicken heterophils (n = 5) was measured by using CCK-8 (2-(2-methoxyl-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfonyl benzene)-2H tetrazolium monosodium salt according to CCK-8 Cell Proliferation and Cytotoxicity Assay Kit (Beijing solarbio science & technology co.,ltd.) following the manufacturer's operating instructions. Briefly, 10 μL CCK-8 solution was added into each well, and the plate was incubated in an incubator for 1 to 4 h at 37°C until the color turned orange. The absorbance of CCK-8 at 45 nm was measured using microplate reader (TECAN, Austria).

Fluorescence Microscopy Analyses on FB_1-Induced NETs

Heterophils were seeded on clean glass coverslips and incubated with FB_1 (10, 20, or 40 μM) for 1.5 h at 37°C with 5% CO_2. The cells on coverslips (n = 3) were then fixed with 4% paraformaldehyde for 20 min, blocked in 3% goat serum for 1.5 h, and incubated with antibodies of H_3 (Abcam, UK) or NE (Abcam, UK) at 4°C overnight. After thrice washing with PBS, the samples were incubated with the second conjugated antibody (goat anti-rabbit IgG, FITC, Absin Bioscience Inc., China) for 1.5 h, followed by being incubated with 5 μM Sytox Orange (Invitrogen, Eugene, OR) for 20 min. Images were taken by fluorescence microscope.

HETs Quantitation in Chicken Heterophils

Isolated Chicken heterophils (2×10^5/well) (n = 5) were stimulated with FB_1 (10, 20, 40, or 80μM) and zymosan (1 mg/mL, Beijing solarbio science & technology co., ltd.) for 1.5 h. Zymosan is one of the frequently reported inducers of NETs and HETs (Muñoz Caro, et al., 2014; Boonlaos, et al., 2021; Jiang, et al., 2021), so zymosan stimulation was used as positive group. No Stimulation was used as negative group. In the time-kinetics experiments, heterophils (2×10^5/well) were stimulated with 40 μM FB_1 for 0.5 h, 1 h, 1.5 h, 2 h, 3 h.

In the experiments using inhibitors, heterophils were pretreated with the inhibitors (10 μM SB202190, 50 mM U0126, 10 μM DPI, 6 μM cl-amidine, 100 μM NF449) for 30 min before FB_1 (40 μM) stimulation. HETs (n = 5) in the plate were detected and quantified using an Infniti M200 fluorometric plate reader (TECAN) based on Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR). The detecting condition was excitation wavelength of 485 nm and emission wavelength of 535 nm.

ROS Detection in Chicken Heterophils

Heterophils (2×10^5/well) (n = 5) were stimulated with FB_1 (40 μM) for 1.5 h as FB_1 group and were excited with zymosan (1 mg/mL) for 1.5 h as positive group. To investigate the relationship between FB_1-induced ROS and NADPH oxidase, ERK and p38 MAPK signaling pathways, we used the NADPH oxidase inhibitor (DPI, 10 μM), ERK signaling pathway inhibitor (U0126, 50 mM), and p38 MAPK pathway inhibitor (SB202190, 10 μM) for inhibitor experiments. After 30 min of pretreatment with inhibitors, heterophils were treated with 40 μM FB_1 for 1.5 h. For ROS detection, 2,7 dichlorofluorescein diacetate (1 μM, DCFH-DA, Sigma-Aldrich, Saint Louis, MO) was added into each well, and the plate was read using an Infniti M200 fluorometric plate reader at excitation wavelength of 485 nm and emission wavelength of 535 nm.

Detection of Antioxidant Enzymes Activity in Chicken Heterophils

Heterophils (n = 5) were treated with 40 μM FB_1 in white, clear-bottomed 6-well plates for 1.5 h at 37°C

with 5% CO_2. Cells were then lysed by M-PER Mammalian Protein Extraction Reagent, and protein concentrations were measured with Pierce BCA Protein Assay Kit. The activity of antioxidant enzymes SOD, CAT, and GSH-Px and the content of GSH in samples were detected by the corresponding assay kits (NanJing Jian-Cheng Bioengineering Institute, China) according to Manufacturer's product usage guide. Briefly, protein extraction samples and relative solutions were added into a 96-well plate, and mixed. After incubation at 37°C in an incubator, the plate was read by a microplate reader.

Western Blot Analysis

For protein extraction, heterophils were incubated with 40 μM FB_1 in white, clear-bottomed 6-well plates for 1.5 h at 37°C with 5% CO_2. Then cells were lysed by M-PER^{TM} Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL) for 7 min at 37°C. The lysates (n = 3) were collected into clean 1.5 mL centrifuge tubes and centrifuged at 12,000 rpm for 10 min at 4°C, and protein concentrations were measured with Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Each sample with protein (40 μg) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and moved onto polyvinylidene difluoride membrane for 45 min at 85V in blotting system (Bio-Rad, Singapore). Membranes subsequently were blocked with 5% Blotting Grade (dissolved in TBST) and incubated with polyclonal antibody to β-actin (YT0099, Immunoway Biotechnology Company, Plano, TX), p38 (8690S, Cell Signaling Technology, Danvers, MA), p-p38 (4511S, Cell Signaling Technology, Danvers, MA), ERK (BM4326, BOSTER, China), or p-ERK (bs-3016R, BIOSS, China) at 4°C overnight. Next, the membranes were incubated with the corresponding HRP-linked secondary antibodies. Finally, the blots were detected by the ECL Plus Western Blotting Detection System (Amersham Life Science, UK).

Statistical Analysis

All data were expressed as mean ± SEM. Differences among multiple groups were assessed by one-way analysis of variance (**ANOVA**) followed by Dunnett or Turkey's multiple comparison tests as applicable. The results of the analysis are considered statistically significant at the level of P-values less than 0.05 ($P < 0.05$). The GraphPad 7.0 software was used to analyze data.

RESULTS

FB_1 Had no Cytotoxicity to Heterophils

Seen in Figure 1, compared to that of control group, FB_1 (0, 10, 20, 40, or 80 μM) stimulation had no influence on heterophil viability ($P > 0.05$) indicating that FB_1 had no cytotoxicity to chicken heterophils.

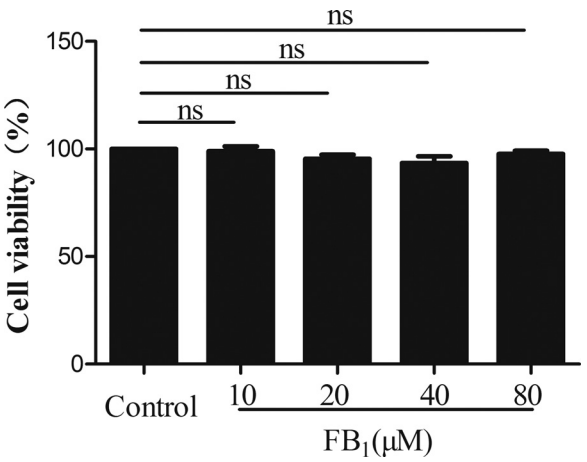

Figure 1. Cytotoxicity of FB_1 on chicken heterophils. Chicken heterophils were incubated with FB_1 (10, 20, 40, or 80 μM) for 4 h and using CCK-8 assay detected the cytotoxicity. Data were presented as mean ± SEM (technical replicates "n" = 5, this applies to all the assays), and P-values < 0.05 were considered significant ("ns" means not significant, $P > 0.05$). Abbreviation: FB_1, Fumonisin B_1.

FB_1 Induced HETs Formation

As shown in Figure 2, we observed the formation of HETs-like clearly induced by FB_1 compared to the control group, which contained a piece of decondensed chromatin decorated with H_3 and NE. This finding illustrated that FB_1 is a potent HETs inducer.

FB_1 Triggered HETs in a Concentration-Dependent Way

In the Figure 3, we could see that HETs were increasing with increasing FB_1 exposure (10−40 μM) showing a concentration-dependent manner, and the maximum release was observed with 40 μM FB_1, which increased 210% compared with control group, indicating 40 μM FB_1 robustly stimulated HETs release. HETs induced by 40 μM FB_1 and 80 μM FB_1 had no significant difference, although there was a slight decrease with 80 μM FB_1.

The Time-Kinetics of FB_1−Triggered HETs

To investigate the time-kinetics of FB_1−triggered HETs, heterophils were treated with 40 μM FB_1 for 0.5 h, 1 h, 1.5 h, 2 h, 3 h. Figures 4A and 4B showed that compared with the control group, FB_1 significantly increased the release of HETs at 0.5 h, 1 h,1.5 h, 2 h, and 3 h ($P < 0.001$). We can see in the Figure 4C that the maximum release of HETs induced by FB_1 was at 1.5 h.

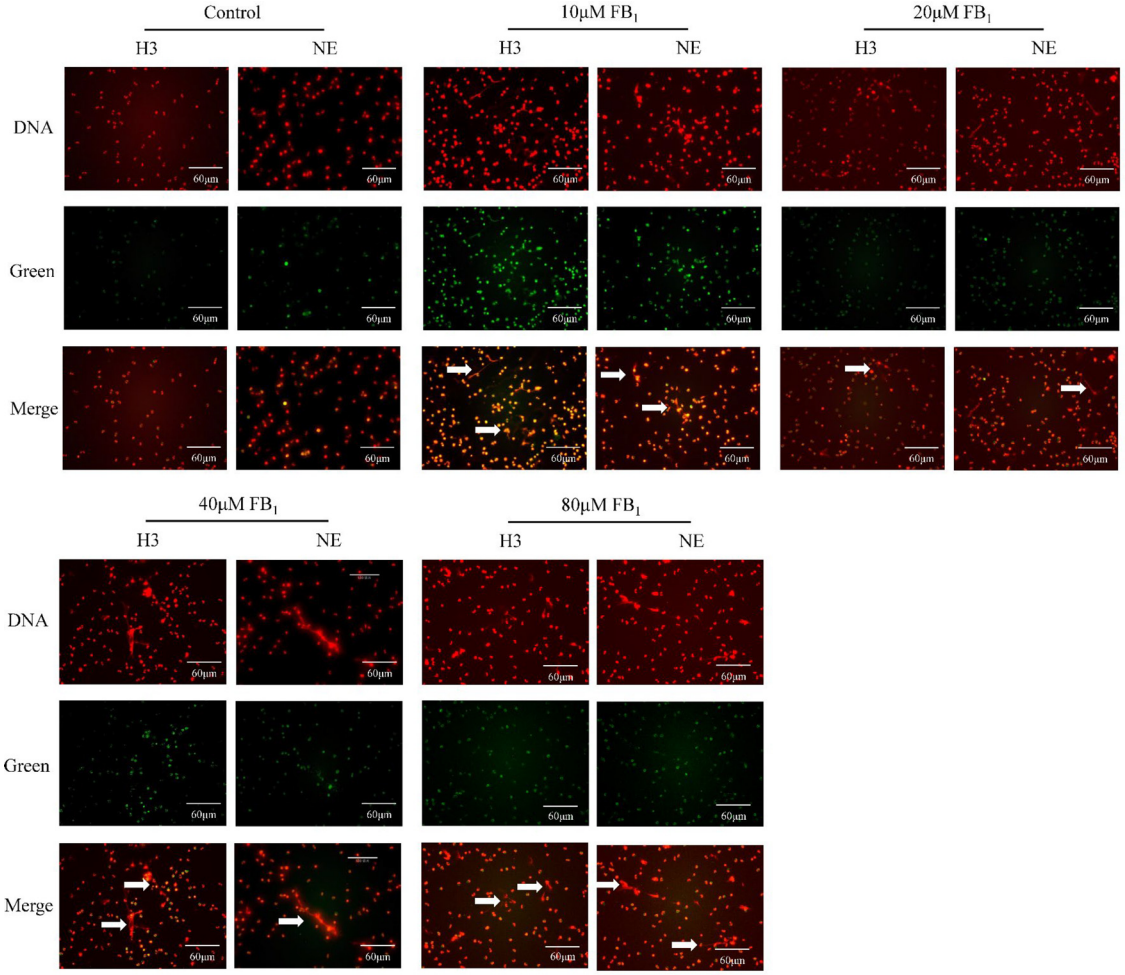

Figure 2. FB_1 triggered HETs formation. Heterophils were seeded on glass coverslips and stimulated with FB_1 for 1.5 h, fixed with 4% paraformaldehyde and stained with SYTOX Orange and fluorescent antibody labeled H3 and NE. Green represents H3 or NE. The white arrow represents HETs. Abbreviations: FB_1, Fumonisin B_1; HETs, heterophil extracellular traps.

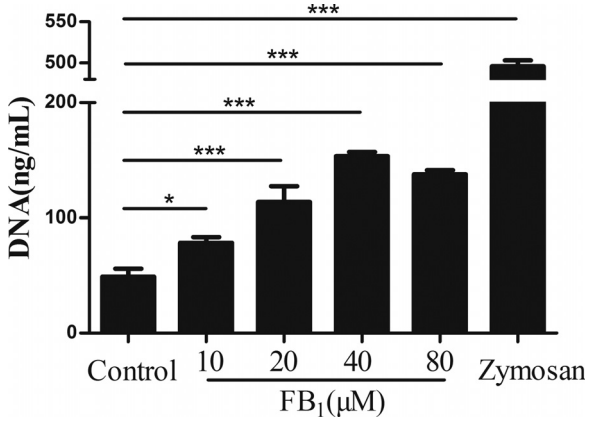

Figure 3. HETs quantification. Heterophils were treated with FB_1 (10, 20, 40, or 80 μM), and HETs were quantified using PicoGreen by a microplate reader. Data were presented as the mean ± SEM. ($^*P < 0.05$,$^{***}P < 0.001$). Abbreviations: FB_1, Fumonisin B_1; HETs, heterophil extracellular traps.

FB_1 Increased ROS Production in Heterophils

Given that ROS play an important role in NETs release, we measured ROS production using DCFH-DA in heterophils exposure to FB_1. As shown in our data (Figure 5), FB_1 exposure increased ROS production significantly ($P < 0.001$), indicating FB_1-induced HETs was related to ROS production.

FB_1 Reduced the Antioxidant Capacity of Heterophils

To detect the influence of FB_1 on the activity of antioxidant enzymes in chicken heterophils, the cells were co-incubated with 40 μM FB_1 for 1.5 h. Proteins were obtained by lysing cells, and the activity of SOD, CAT, and GSH-Px and the content of GSH were measured using the respective commercial kits. Seen in Figure 6, SOD, CAT, and GSH-Px activity and GSH content were decreased after FB_1 treatment. Compared with the control group, SOD activity in heterophils was reduced ($P < 0.05$), CAT activity was reduced ($P < 0.001$),

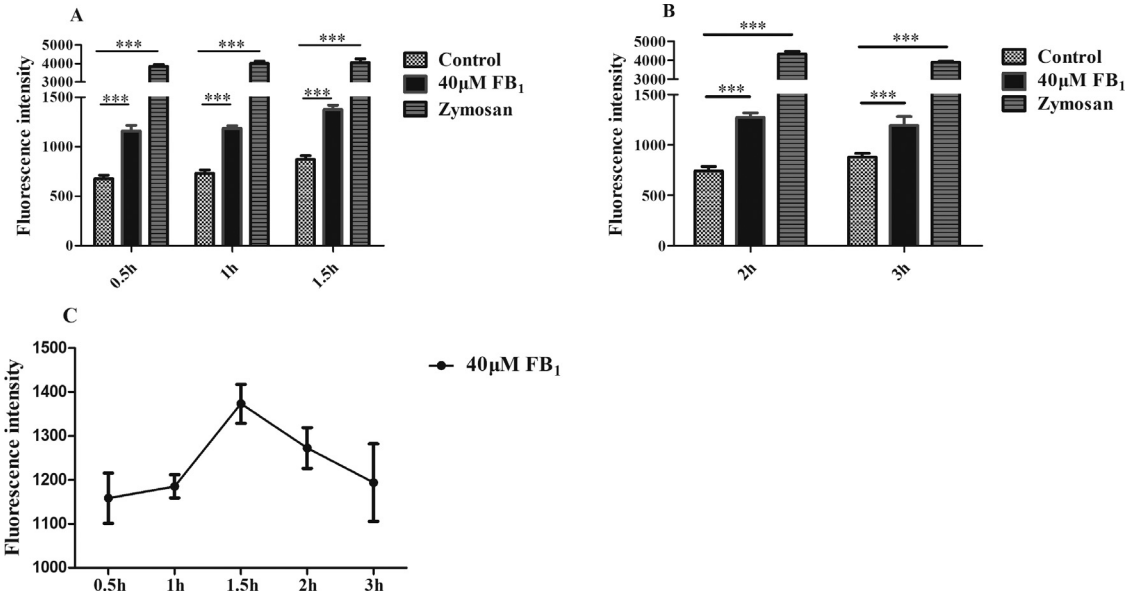

Figure 4. The time-kinetics of FB_1-triggered HETs. Heterophils were treated with 40 μM FB_1 for 0.5 h, 1 h, 1.5 h, 2 h, 3 h. Then, HETs were quantified by picogreen using fluorescence microplate reader. (A) FB_1 treated for 0.5 h, 1 h, and 1.5 h; (B) FB_1 treated for 2 h and 3 h; (C) comparison of different FB_1 treating times. Data were presented as the mean ± SEM. ("ns" means not significant, $^{***}P < 0.001$). Abbreviations: FB_1, Fumonisin B_1; HETs, heterophil extracellular traps.

GSH-Px activity was reduced ($P < 0.01$), GSH content was reduced ($P < 0.05$), indicating FB_1 could reduce the antioxidant capacity of chicken heterophil by decreaing the activity of antioxidant enzymes.

FB_1 Activated ERK and P38 Signaling Pathways

To further survey the role of ERK and p38 signaling pathways in FB_1-induced HETs release, we investigated the phosphorylation levels of ERK and p38 MAPK signaling pathways using western blot analysis. Our result (Figure 7) showed that FB_1 (40 μM) caused a significant increase in phosphorylated ERK and p38 proteins, suggesting that FB_1 activated ERK and p38 MAPK signaling pathways in heterophils.

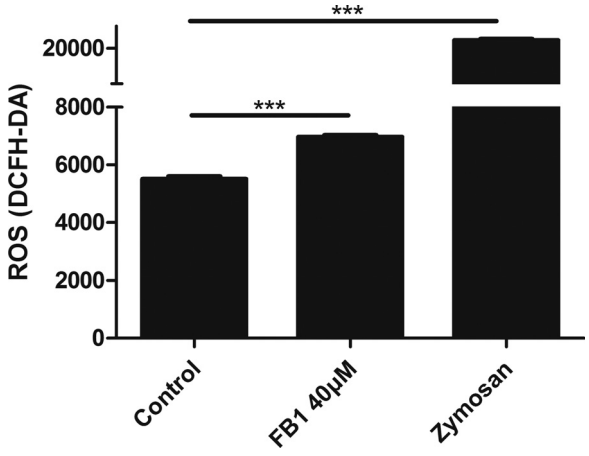

Figure 5. FB_1 increased ROS production in heterophils. Heterophils were incubated with 40 μM FB_1 for 1.5 h. Finally, ROS were quantified by DCFH-DA using fluorescence microplate reader. Data were presented as the mean ± SEM. ($^{***}P < 0.001$). Abbreviations: FB_1, Fumonisin B_1; ROS, reactive oxygen species.

FB_1-Induced ROS in Heterophils was Independent of NADPH Oxidase, ERK, and P38 MAPK Signaling Pathways

As shown in our data (Figure 8), FB_1 exposure increased ROS production significantly ($P < 0.001$), but inhibitors (SB202190, U0126, and DPI) pretreatment did not resulted significantly reduction in ROS production induced by FB_1 ($P > 0.05$).

FB_1-Induced HETs Release was Independent of NADPH Oxidase, ERK, and P38 MAPK Signaling Pathways

To further investigate the relationship between FB_1-induced HETs and NADPH oxidase, ERK, and p38 MAPK signaling pathways, after 30 min of pretreatment with inhibitors (SB202190, U0126, and DPI), heterophils were treated with 40 μM FB_1 for 1.5 h, HETs were quantified using PicoGreen by a microplate reader. The result (Figure 9) showed that FB_1 increased HETs release significantly ($P < 0.05$). Nevertheless, inhibitors (SB202190, U0126, and DPI) did not obviously reduce HETs release induced by FB_1 ($P > 0.05$), suggesting FB_1-induced HETs was a NADPH oxidase, p38 and ERK MAPK signaling pathways-independent process.

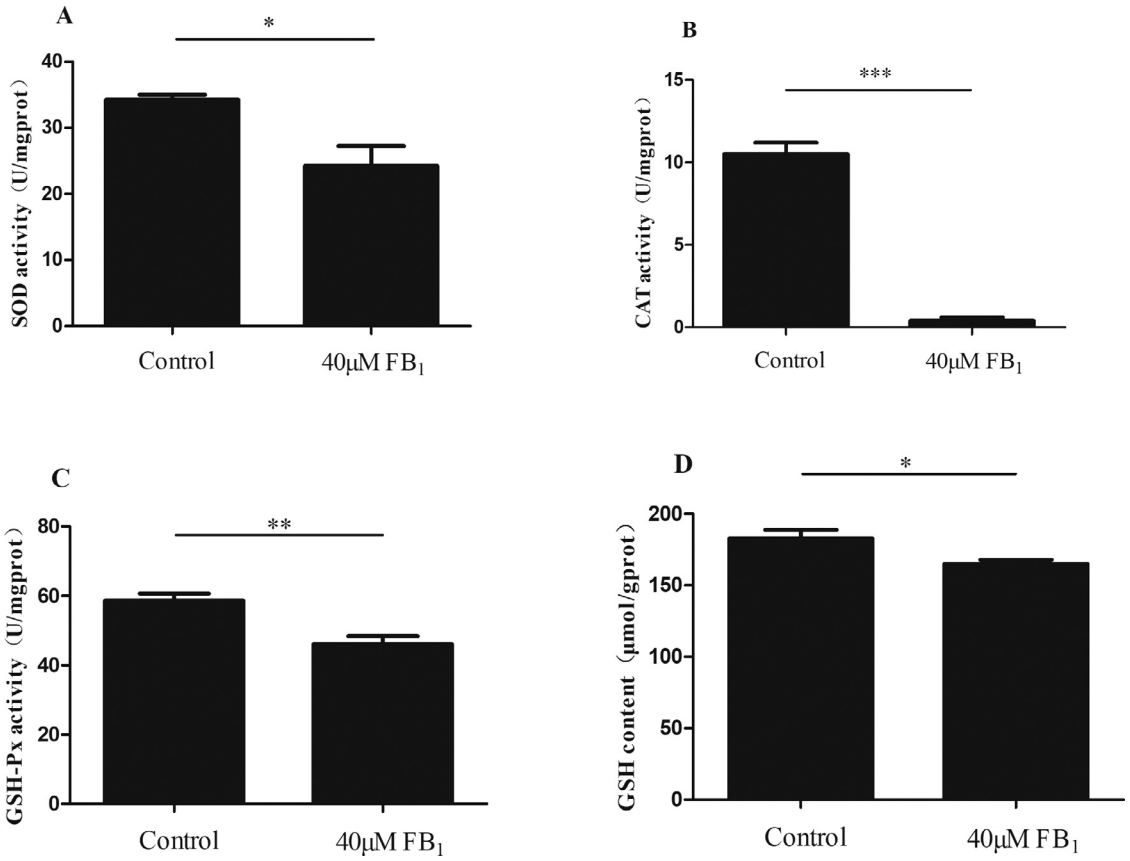

Figure 6. FB_1 decreased the activity of SOD, CAT, GSH-Px, and GSH content. Heterophils were stimulated with FB_1 (40 μM) for 1.5 h. SOD, CAT, and GSH-Px activities and GSH content were measured by corresponding detection kits. (A) SOD; (B) CAT; (C) GSH-Px; (D) GSH. Data were presented as the mean ± SEM. ("ns" means not significant, $^{*}P < 0.05$, $P^{**} < 0.01$, $^{***}P < 0.001$). Abbreviations: FB_1, Fumonisin B_1; CAT, catalase; GSH, glutathione; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase.

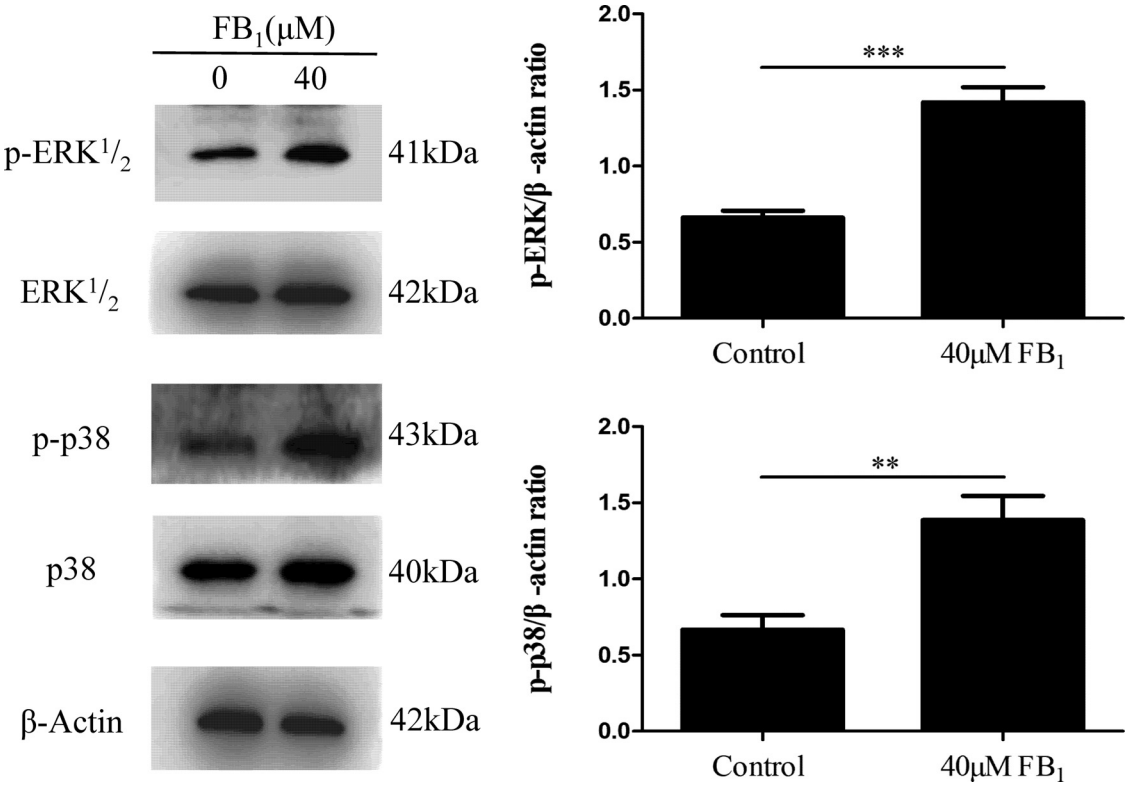

Figure 7. ERK and p38 MAPK signaling pathways were activated by FB_1 in heterophils. Chicken heterophils were stimulated with 40 μM FB_1 for 1.5 h. The cells were then lysed and the total protein was extracted. ERK and p38 signaling pathways were investigated by western blot. Data were presented as the mean ± SEM. ($^{**}P < 0.01$,$^{***}P < 0.001$). Abbreviations: FB_1, Fumonisin B_1; ERK, extracellular regulated protein kinases.

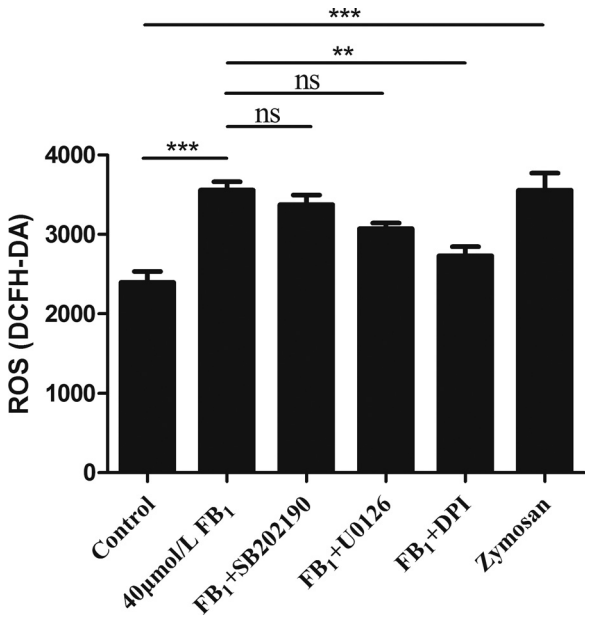

Figure 8. FB_1-induced ROS in heterophils was independent of NADPH oxidase, ERK, and p38 MAPK signaling pathways. Heterophils were pretreated with inhibitors (SB202190, U0126, or DPI) for 30 min and then co-incubated with 40 μM FB_1 for 1.5 h. Finally, ROS were quantified by DCFH-DA using fluorescence microplate reader. Data were presented as the mean ± SEM. ("ns" means not significant, $P > 0.05$, $^{**}P < 0.01$,$^{***}P < 0.001$). Abbreviations: FB_1, Fumonisin B_1; ERK, extracellular regulated protein kinases; NSDPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

FB_1-Induced HETs Release was Associated With PAD4 and P2 × 1 Receptor

Peptidylarginine deiminase 4 (**PAD4**) mediated histone hypercitrullination correlates with chromatin decondensation during NET formation. As shown in Figure.10, FB_1-induced HETs was markedly reduced with the pretreatment of cl-amidine indicating PAD4 plays an important role in the formation of FB_1-induced HETs. Moreover, P2 × 1 receptor is related with ATP signaling, and our result showed that the inhibition of P2 × 1 receptor with NF449 significantly decreased the release of FB_1-induced HETs, suggesting that ATP signaling has a crucial role in this process.

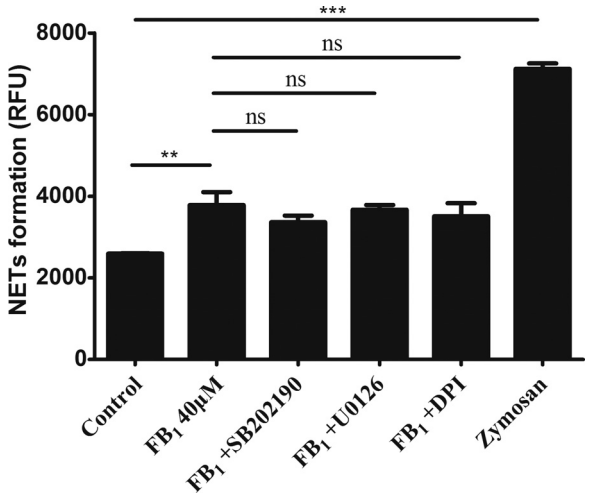

Figure 9. FB_1-induced HETs was independent of NADPH oxidase, p38, ERK MAPK signaling pathways. The cells were pretreated with inhibitors (SB202190, U0126, or DPI) for 30 min and then treated with 40 μM FB_1 for 1.5 h. Conclusively, HETs were measured by PicoGreen using fluorescence microplate reader. Data were presented as the mean ± SEM. ("ns" means not significant, $P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Abbreviations: DPI, diphenyleneiodonium chloride; FB_1, Fumonisin B_1; ERK, extracellular regulated protein kinases; HETs, heterophil extracellular traps; NSDPH, nicotinamide adenine dinucleotide phosphate.

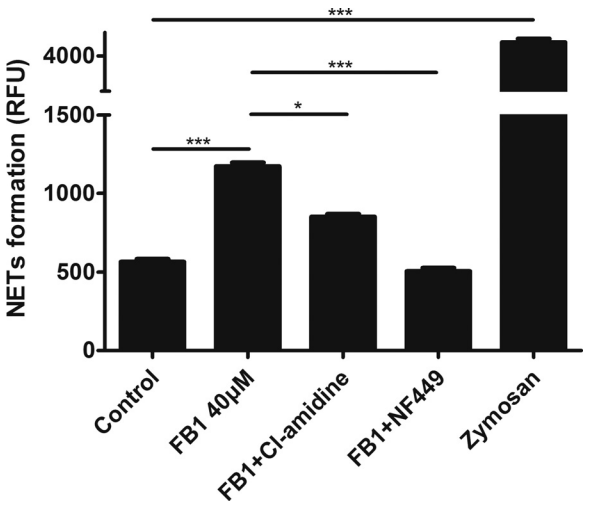

Figure 10. FB_1-induced HETs release was mediated by PAD4 and P2 × 1 receptor. Heterophils were pretreated with inhibitors (cl-amidine or NF449) for 30 min and then treated with 40 μM FB_1 for 1.5 h, and HETs release were measured by PicoGreen using fluorescence microplate reader. Data were presented as the mean ± SEM. ($P > 0.05$, $^{*}P < 0.05$, $^{***}P < 0.001$). Abbreviations: FB_1, Fumonisin B_1; HETs, heterophil extracellular traps.

DISCUSSION

FB1 as a common mycotoxin poses a huge threat to human and animal health. Despite a large number of studies showing that FB1 has immunosuppressive effects, the mechanisms of FB1 immunotoxicity remains unclear. In this present study, we revealed for the first time that 40 μM FB_1 induced HETs release, and the mechanism of HETs release was related to ROS production, PAD4 and P2 × 1, but did not depend on the activation of NADPH oxidase, ERK, and p38 MAPK signaling pathways. This study provides experimental reference for further research on the immunotoxicity of FB1.

We firstly examined the cytotoxicity of FB_1 to chicken heterophils. Our data showed that FB_1 did not significantly affect cell viability, which means that there are no cytotoxic effects to heterophils in the dose of FB_1 (10 −80 μM). Then, we observed the formation of HETs induced by FB_1 via immunofluorescence staining, which illustrated that FB_1 induced chicken heterophils to produce NETs-like structures. Moreover, HETs quantification showed that FB_1 induced HETs release was in a dose-dependent manner, which is consistent with ochratoxin A that is also a mycotoxin (Han et al., 2019a).

ROS include superoxide free radicals, hydrogen peroxide and its downstream products peroxides and hydroxylates, etc., and participate in cell growth and proliferation, development and differentiation, aging, and apoptosis as well as many physiological and pathological processes (Li et al., 2016; Yang and Lian, 2020). Evidence has accumulated that ETosis is related to ROS, including the role of NADPH oxidase-derived ROS, NADPH oxidase-independent ROS, extracellular ROS and ROS from sources other than NADPH oxidase (Kirchner et al., 2012; Stoiber et al., 2015). Our results displayed that FB_1 significantly reduced the activity of SOD, CAT, and GSH-Px and the content of GSH, implying FB_1 reduced the ability to scavenge free radicals by reducing the activity of antioxidant enzymes (SOD, CAT, and GSH-Px) and the content of GSH in heterophils, thereby leading to increased ROS production. Moreover, we detected the phosphorylation level of p38 and ERK, and found that FB_1 activated the p38 and ERK MAPK signaling pathway in heterophils, which further validated the above findings. In addition, it has reported that ROS-dependent activation of ERK and p38 MAPK is also involved in nanosilver-induced NETs release of mouse neutrophil granulocytes (Wang et al., 2019).

Whether FB_1-triggered HET formation is related with ROS production, and its molecular mechanism remains unclear. FB_1 strongly increased the content of ROS in chicken heterophils. Moreover, inhibitors (SB202190, U0126, or DPI) could not obviously decrease ROS generation induced by FB_1, suggesting ROS production induced by FB_1 was independent of NADPH oxidase, ERK, and p38 MAPK signaling pathways, which is similar to the HETs-related data for ochratoxin A (Han et al., 2019b). Finally, as quantification of HETs parallel experiment, DPI, U0126, and SB202190 were used to examine the relationship between FB_1-triggered HETs release from chicken heterophils and NADPH oxidase, ERK, and p38 MAPK signaling pathways. Our results showed that inhibitor (SB202190, U0126, or DPI) could not reduce formation of HETs, indicating the release of HETs induced by FB_1 did not depend on the activation of NADPH oxidase, p38, and ERK signaling pathways, which is similar to nickel (II) nitrate hexahydrate triggered canine neutrophil extracellular traps release (Wei et al., 2018).

As our result showed that FB_1-induced HETs was independent of NADPH oxidase, and PAD4 plays a vital role in NADPH oxidase-independent NETois (Leshner et al., 2012), we further investigated the role of PAD4 in FB_1-induced HETs. In addition, Purinergic receptors including P2 × 1 has been reported to be involved in PMNs' effector mechanisms such as oxidative burst and degranulation (Chen et al., 2010; Grassi, 2010), we also explored its role in the formation of FB_1-induced HETs. Indeed, our data found that both PAD4 and P2 × 1 exerted a key role in FB_1-induced HETs, which could give us some thoughts on FB_1-related diseases.

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DISCLOSURES

There is no conflict of interest.

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