T-2 toxin inhibits osteoblastic differentiation and mineralization involving mutual regulation between Wnt signaling pathway and autophagy

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Abstract

Mycotoxins are most frequent contaminants in environment and agricultural production globally. The T-2 toxin of *Fusarium* species is the most toxic type of A trichothecene mycotoxins. T-2 toxin can accumulate in bone and cause bone development disorders. Osteoblast is the functional cell responsible for bone formation. Whereas, the mechanism of T-2 toxin toxicity on osteoblast remains unknown. In present study, MC3T3-E1 cells were used to investigate the effect of T-2 toxin on differentiation and mineralization and potential underlying mechanism. The results showed that T-2 toxin inhibited both osteoblastic differentiation and mineralization. T-2 toxin repressed Wnt signaling pathway both during osteoblastic differentiation and mineralization. T-2 toxin similarly promoted autophagy during osteoblastic differentiation, while inhibited it during osteoblastic mineralization. In addition, the activation of Wnt signaling pathway mitigated T-2 toxin-induced osteoblast impairment, while the inhibition of autophagy exacerbated it. Our results also indicated that there was a positive feedback loop between the Wnt signaling pathway and autophagy. This study can provide a better understanding for relieving T-2-related bone diseases.

1. Introduction

Mycotoxins are toxic metabolites produced by different kinds of fungi and widely exist in soil, water source and various agro-environmental matrices(Cao et al. 2022; Juraschek et al. 2022). T-2 toxin, a trichothecene mycotoxin produced by *Fusarium* species, is the most frequently-detected mycotoxin contaminant in cereals, livestock feed and other agricultural products worldwide. A study in the Czech Republic showed that the detection rate of T-2 toxin in spring barley samples was 88%(Pernica et al. 2022). In Croatia, 70% oats tested positive for sum T-2/HT-2 toxin(Kovac et al. 2022). According to a 6-year survey of mycotoxins in animal feed, T-2 toxin was detected in 98.9% and 96.4% of complete feeds and total mixed ration respectively, with the highest content of up to 131 µg/kg(Twaruzek et al. 2021). T-2 toxin can be enriched in food chains due to the stable physicochemical property, eventually harm human health and agricultural development(Li et al. 2011). T-2 toxin could easily be absorbed by the intestine and mainly accumulates in skeletal system. T-2 toxin causes bone dysplasia and degenerative joint changes(Wang et al. 2011; Yan et al. 2010) and is an environmental risk factor of many bone diseases represented by Kaschin-Beck disease. Nevertheless, the osteotoxic mechanism of T-2 toxin is not fully understood.

Skeletal growth and development depend upon the formation of growth plate cartilage by chondrocytes and the bone-forming function of osteoblast. Several studies have demonstrated that T-2 toxin causes oxidative stress, apoptosis, and inflammation in chondrocyte(Chang et al. 2017; Chen et al. 2012; Yang et al. 2017), involving abnormalities of Wnt/β-catenin, ROS/NF-κB, TGF-β1/Smad3 and other signaling pathway(Li et al. 2017; Shi et al. 2021; Xu et al. 2015). The toxicity mechanisms of T-2 toxin on chondrocyte have been extensively studied. As another important bone forming cell during bone development, osteoblast undergo differentiation and mineralization to eventually form bone. During differentiation, osteoblast secrete large amounts of alkaline phosphatase (ALP) and differentiate into
osteocyte and bone lining cell (Sawa et al. 2019). Mineralization is the formation of hydroxyapatite in extracellular matrix secreted by mature osteoblast. Abnormal differentiation and mineralization of osteoblast results in bone formation disorders which have been implicated in the nosogenesis of many bone disease, including bone dysplasia, fragility fracture, and osteomalacia (Delgado-Calle and Bellido 2022). However, the effects of T-2 toxin on osteoblast have still not yet been investigated so far.

The Wnt signaling pathway is mainly divided into two categories, namely the classic Wnt signaling pathway (Wnt/β-catenin signaling pathway) and the non-canonical Wnt signaling pathway. The classic Wnt signaling pathway (hereafter referred to as “Wnt signaling pathway”) is a critical pathway in regulating bone formation. Wnt signaling pathway regulates osteoblastic differentiation and mineralization by bone formation factors osterix (OSX), runt-related transcription factor-2 (RUNX-2), collagen types I (Col-I) and osteocalcin (OCN). Our previous research indicated that T-2 toxin induced femur formation disorders with down-regulated Wnt signaling pathway in mice (Zhang et al. 2022). More importantly, T-2 toxin repressed Wnt signaling pathway in chondrocyte in vitro. Additionally, up-regulated Wnt signaling pathway alleviates intestinal injury and reproductive dysfunction caused by trichothecene mycotoxin (Yang et al. 2018; Zhou et al. 2019a). Autophagy is a protecting mechanism to against cellular stress, dynamic process of degradation of cellular macromolecules and organelles by lysosomal hydrolases (Mizushima and Levine 2010). During development and differentiation, the drastic cellular and tissue remodeling is often accompanied by autophagy (Mizushima and Komatsu 2011). Studies have shown that suppression of autophagy impairs bone formation with osteoblastic differentiation disorders in mice (Cui et al. 2021; Liu et al. 2013). Autophagy is also closely associated with osteoblastic mineralization. Autophagic vesicles can serve as vehicles to secrete hydroxyapatite participating in osteoblastic mineralization (Nollet et al. 2014). Moreover, animal experiments showed that T-2 toxin promoted autophagy in mouse liver and femur (Yin et al. 2020; Zhang et al. 2022). Therefore, there is reason to believe that Wnt signaling pathway and autophagy are possible mechanisms and potential therapeutic targets of T-2 bone toxicity. The purpose of this investigation was to explore the effects of T-2 toxin on differentiation and mineralization of osteoblast and the regulatory mechanisms of Wnt signaling pathway and autophagy.

2. Materials And Methods

2.1 Preparation for reagents

T-2 toxin was purchased from Pribolab Technology (Qingdao, China). T-2 toxin was first dissolved in absolute ethanol and diluted with culture medium into different concentrations (Wang et al. 2018).

Wnt signaling pathway activator 1 was purchased from MCE (Shanghai, China). Wnt signaling pathway activator 1 was dissolved and diluted in culture medium with a final concentration of 10 nM according to the instructions.
Autophagy inhibitor, 3-Methyladenine (3-MA), was purchased from APExBIO (Houston, USA). 3-MA was dissolved by dimethyl sulfoxide (DMSO) in accordance with the instructions and diluted with culture medium. The final concentration of 3-MA in cell culture medium was 5 mM (Yang et al. 2014).

2.2 Cell culture and treatment

Murine osteoblastic cell line, MC3T3E1, was purchased from Kunming Cell Bank (Kunming, China). Cells were cultured in α-MEM (Thermo, Shanghai, China) containing 12% fetal bovine serum (FBS, Cellmax, Beijing, China), 1% penicillin-streptomycin (Biosharp, Hefei, China) at 37°C in a humidified 5% CO\textsubscript{2} incubator. Cell differentiation and mineralization were induced by supplementing with 10 mM β-glycerol phosphate, 50 µg/mL ascorbic acid and 100 nM dexamethasone and culture medium was replaced every 2–3 days. MC3T3-E1 cells were induced to differentiate and mineralize for 7 and 14 days, respectively. Then ALP staining and Alizarin red S staining were used for identification. Experimental grouping and processing are detailed in the Supplementary Table S1.

2.3 Cell vitality

The MC3T3-E1 cell vitality was assessed using CCK-8 kit (APExBIO Houston, USA). Briefly, cells were inoculated into 96-well plates and placed in the incubator. Then the cells were respectively treated with 0, 0.5, 1, 2, 4, 6, 8, 10, 20, 40, 60, 80 nM T-2 toxin for 12 h. After T-2 toxin treatment, the medium (90 µL) and CCK-8 reactant (10 µL) were mixed in each well. Afterwards, the plate was incubated at 37°C in the dark for 1.5 h. The absorbance was recorded at 490 nm by the microplate reader (Sanco instrument, Shanghai, China).

2.4 ALP staining

The cells were washed three times with PBS, then fixed in 4% formaldehyde at room temperature for 30 minutes. After washing the cells three times with distilled water, add the BCIP/NBT working solutions according to the instructions (Beyotime, Jiangsu, China). The plate was incubated for 30 minutes at 37°C without light. ALP staining was observed and photographed by the light microscope with a photographic machine (Olympus, Tokyo, Japan).

2.5 Alizarin red S staining

The cells were washed three times with phosphate buffered saline (PBS), then fixed in 95% ethanol at room temperature for 30 minutes. After washing the cells three times with distilled water, add the Alizarin Red S staining reagent (Solarbio, Beijing, China) for 20 minutes with gentle agitation. Alizarin red S staining was observed and photographed by the light microscope with a photographic machine (Olympus, Tokyo, Japan).

2.6 MDC staining

Monodansylcadaverine (MDC) staining was used to detect autophagic vacuoles. After washing the cells three times with distilled water, add the MDC working solutions according to the instructions (Beyotime, Jiangsu, China). The plate was incubated for 30 minutes at 37°C without light. After staining, the cells
were washed three times with Assay Buffer. Cells were observed and photographed by the fluorescent microscopy with a photographic machine (Niko, Tokyo, Japan).

2.7 Western blotting

The protein expressions were detected by western blotting. As described previously (Cao et al. 2019), the total cell protein was extracted according to instructions of protein extraction reagents kit (Biosharp, Hefei, China), and then protein concentration was determined with a BCA analysis kit (Biosharp, Hefei, China). Proteins were separated by SDS-PAGE and wet-transferred to PVDF membranes. Membranes were blocked with 5% nonfat milk for 2 h, then incubated with primary antibody overnight at 4°C. Membranes were washed three times in PBST and incubated with appropriate secondary antibodies for 2 h at room temperature. The enhanced ECL reagent (Biosharp, Hefei, China) was used to detect the target protein. Amersham TM Imager 600 (GE Healthcare, Pittsburgh, USA) was used for quantitative analysis. Densitometry analysis of specific bands was performed by Image J software. The antibody information is detailed in the Supplementary Table S2.

2.8 Data analysis

The data were presented as means ± SEM. One-way ANOVA was used followed by LSD (equal variances) or Dunnett's T3 (unequal variances) as a posttest to determine statistical significance. The data was plotted using Origin Pro 2021 (Origin, Northampton, USA). A p-value less than 0.05 was considered significant, while a value less than 0.01 was considered markedly significant.

3. Results

3.1 T-2 toxin inhibited osteoblast cell vitality

As shown in Fig.1, T-2 toxin treatment changed the cell morphology and decreased density of MC3T3-E1 cell. T-2 toxin in concentrations from 4 to 80 nM significantly suppressed viability of MC3T3-E1 cell in a dose-dependent effect. Less than 2 nM T-2 toxin treatment had no significant effect on cell viability ($P>0.05$), 4nM T-2 toxin treatment significantly decreased cell viability ($P<0.05$), 8nM T-2 toxin treatment extremely significantly decreased cell viability ($P<0.01$). Thus, these concentrations were selected for use in the subsequent series of experiments.

3.2 T-2 toxin induced osteoblastic differentiation disorders with the inhibition of Wnt signaling pathway and the activation of autophagy

As shown in Fig.2, the differentiation capacity of MC3T3-E1 cell was decreased after T-2 toxin treatment. The results obtained from ALP staining (Fig.2-A) and protein expressions of RUNX-2 and OSX (Fig.2-C) corroborated with each other. As shown in Fig.2-D, T-2 toxin treatment decreased the expressions of Wnt
and β-catenin and increased the expression of GSK-3β, indicating T-2 toxin repressed Wnt signaling pathway. Furthermore, MDC staining (Fig.2-B) and detections of autophagy-related protein (Fig.2-E) showed that T-2 toxin treatment enhanced autophagy.

3.3 Wnt signaling pathway and autophagy together protect against T-2 toxin-induced osteoblastic differentiation disorders

To further determine the role of Wnt signaling pathway and autophagy in T-2 toxin-induced osteoblastic differentiation disorders, we conducted intervention experiments. Treatment with Wnt activator promoted autophagy (Fig.3-B and E) and alleviated T-2 toxin-induced osteoblastic differentiation disorders (Fig.3-A). 3-MA (autophagy inhibitor) treatment blocked the osteogenic capacity of MC3T3-E1 cells, as indicated by ALP staining (Fig.3-A) and protein expressions of RUNX-2 and OSX (Fig.3-C). Moreover, 3-MA treatment further decreased the expressions of Wnt and β-catenin and increased the expression of GSK-3β (Fig.3-D). These results showed that Wnt signaling pathway and autophagy synergistically antagonized T-2 toxin-induced differentiation disorders.

3.4 T-2 toxin induced osteoblastic mineralization disorders with the inhibition of Wnt signaling pathway and autophagy

As shown in Fig.4, the mineralization capacity of MC3T3-E1 cell was decreased after T-2 toxin treatment. The results obtained from Alizarin red S staining (Fig.4-A) and protein expressions of Col-I and OCN (Fig.4-C) corroborated with each other. As shown in Fig.4-D, T-2 toxin treatment decreased the expressions of Wnt and β-catenin and increased the expression of GSK-3β, indicating T-2 toxin repressed Wnt signaling pathway. Unlike in differentiation, MDC staining (Fig.4-B) and detections of autophagy-related protein (Fig.4-E) showed that T-2 toxin treatment suppressed autophagy during mineralization process.

3.5 Wnt signaling pathway and autophagy together protect against T-2 toxin-induced osteoblastic mineralization disorders

To further determine the role of Wnt signaling pathway and autophagy in T-2 toxin-induced osteoblastic mineralization disorders, we also conducted intervention experiments. Treatment with Wnt activator promoted autophagy (Fig.4-B and E) and alleviated T-2 toxin-induced osteoblastic mineralization disorders (Fig.4-A). 3-MA treatment blocked the osteogenic capacity of MC3T3-E1 cells, as indicated by Alizarin red S staining (Fig.4-A) and protein expressions of Col-I and OCN (Fig.4-C). Furthermore, 3-MA treatment further decreased the expressions of Wnt and β-catenin and increased the expression of GSK-
3β (Fig.4-D). These results showed that Wnt signaling pathway and autophagy synergistically antagonized T-2 toxin-induced mineralization disorders.

4. Discussion

Mycotoxin contamination of environment is a serious health risk that is receiving increasing attention throughout the world (Cao et al. 2020; Juraschek et al. 2022). T-2 toxin, the most toxic mycotoxin of A. trichothecene mycotoxins, has attracted the most attention for the bone toxicity. However, the effect of T-2 toxin on osteoblast remains unclear. In this research, we investigated the effects of T-2 toxin on osteoblastic differentiation and mineralization, and the regulatory roles of Wnt signaling pathway and autophagy during this process. Results showed that T-2 toxin disturbed osteoblastic differentiation and mineralization. Meanwhile, T-2 toxin repressed Wnt signaling pathway during both differentiation and mineralization. T-2 toxin activated autophagy during differentiation and inhibited autophagy during mineralization. Intervention trials showed that up-regulated of Wnt signaling pathway mitigates T-2 toxin-induced osteoblast damage while inhibition of autophagy aggravates it. Furthermore, the mutual regulation between Wnt signaling pathway and autophagy participated in the T-2 toxin-induced osteoblast impairment.

Bone formation and reparation are complex, involving differentiation and mineralization of osteoblast. The dysfunction of osteoblast causes a variety of bone diseases such as osteoporosis. MC3T3-E1 cell line is a murine preosteoblast cell line with the ability to differentiate and mineralize and has been extensively used to assess osteoblast function in vitro (Jie et al. 2018). Our study indicated that the cell viability of MC3T3-E1 cell was reduced in a dose-dependent manner after 4–80 nM T-2 toxin treatment. To further investigate the impairment of T-2 toxin on osteoblast, the effects of T-2 toxin on differentiation and mineralization were subsequently detected.

ALP staining was used to detect the differentiation of osteoblast after T-2 toxin treatment. RUNX-2 is the transcription factor required for osteoblastic differentiation. OSX is the osteogenic differentiation marker downstream of RUNX-2. RUNX-2 is essential in formation of precursor osteoblast from mesenchymal stem cell and OSX is critical for differentiation of RUNX-2-expressing pre-osteoblast into mature and functional osteoblast (Sinha and Zhou 2013). Unsurprisingly, T-2 toxin inhibits osteoblastic differentiation and expressions of differentiation markers with a dose-effect relation. We also found that T-2 toxin repressed Wnt signaling pathway and the activation of Wnt signaling pathway mitigated T-2 toxin-induced differentiation impairment. When the Wnt signaling pathway is activated, β-catenin, binding to GSK-3β, is released and promoting osteoblastic differentiation. Activation of the Wnt signaling pathway has been used as a target for the treatment of many bone loss-related diseases (Onuora 2021). Though the detailed molecular mechanisms by which autophagy regulates osteoblastic differentiation remain unclear, several studies have shown that autophagy promotes osteoblastic differentiation (Li et al. 2018b; Liu et al. 2013). In present research, T-2 toxin activated autophagy and the inhibition of autophagy exacerbated osteoblast differentiation disorders. Evidently, autophagy is the protective role in T-2 toxin-induced osteoblastic differentiation disorders.
Alizarin Red S staining was used as an indicator of mineralization to detect calcium deposition in cells. Col-I is a major component constituting the bone matrix and a marker of early mineralization. OCN is a non-collagenous protein synthesized by osteoblast maintaining normal mineralization (Dong et al. 2018). Our results demonstrated that T-2 toxin decreased the formation of mineralized nodules and reduced the expressions of mineralized markers. Moreover, T-2 toxin repressed Wnt signaling pathway and decreased autophagy. Same as differentiation period, the activation of Wnt signaling pathway mitigated T-2 toxin-induced mineralization impairment, whereas the inhibition of autophagy exacerbated it. There has been little agreement about the effect of Wnt signaling pathway on mineralization. The Wnt signaling pathway promotes mineralization in MC3T3-E1 cell line, mouse primary osteoblast, and human osteoblast (Dong et al. 2020; Nash et al. 2015; Yun et al. 2015). Conversely, the opposite conclusions were reached in IDG-SW3 cell line and dental pulp cell, which may be related to different cell types and treatments (Li et al. 2018a; Zhou et al. 2019b). Studies have shown that autophagy deficiency reduces mineralization capacity, which is probably due to autophagy vacuoles could secrete apatite crystals in osteoblast as carriers (Nollet et al. 2014). In addition, low levels of autophagy also caused the accumulation of damaged molecules and organelles and subsequently leading to abnormal cellular function. Therefore, inhibition of autophagy exacerbated the disorders of T-2 toxin-induced osteoblastic mineralization.

There is somewhat controversial of the relationship between Wnt signaling pathway and autophagy. In a variety of cancer cells, Wnt signaling pathway and autophagy are mutually negatively regulated. Autophagy represses Wnt signaling pathway by accelerating β-catenin degradation in colorectal carcinoma cells (Petherick et al. 2013). In turn, the inhibition of Wnt signaling pathway increased autophagic flux (Nager et al. 2018). Other studies, however, have reached different conclusions. A study of hepatic progenitor cells (HPCs) showed that inhibition of autophagy by downregulating of ATG5 expression impaired differentiation of HPCs and inhibited activation of the Wnt signaling pathway, which was relieved by over-expression of β-catenin (Ma et al. 2019). It is suggested that autophagy promotes HPCs differentiation by activating Wnt signaling. Another study found that rapamycin (an autophagy activator) treatment up-regulated the Wnt signaling pathway in mouse aortic endothelial cell (Liu et al. 2021). While treatment with Wnt3a could not only active Wnt signaling pathway, but also enhance autophagy. Consistent with these studies, our results indicate that there is a positive feedback loop between the Wnt signaling pathway and autophagy.

Our current study also has several limitations. First, it is not clear why T-2 toxin promotes autophagy during osteoblastic differentiation stage but inhibits autophagy during mineralization stage. We speculate that autophagy may simply a protective mechanism during osteoblastic differentiation while participates in secretory function during osteoblastic mineralization. Second, although we found mutual regulation between Wnt signaling pathway and autophagy, we didn't explore specific mechanisms, which will need further study.

In conclusion, T-2 toxin induced differentiation and mineralization impairment of osteoblast. Wnt signaling pathway and autophagy play a synergistic protective role in this process. This research provides new insights into the bone toxicity mechanism of T-2 toxin.
Declarations

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Conflicts of interest

The authors declare no competing financial interest.

Ethical Approval

Ethical approval was obtained from the medical ethical committee of the Northeast Agricultural University (Harbin, China).

Human Ethics

Not applicable

Consent for publication

All authors have agreed to the published version of the manuscript.

Availability of supporting data

Informed consent was obtained where applicable.

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Authors’ contributions

Jian Zhang and Xia Wu conducted the experiment and wrote the main manuscript text. Miao Song and Xuliang Zhang prepared all figures. Siming Huo, Jiayu Du, and Bo Li processed the data. Zheng Cao provided funding for this research. Yanfei Li reviewed, edited, and supervised the whole experiment process. All authors reviewed the manuscript.

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**Figures**

**Figure 1**

Effect of T-2 toxin on MC3T3-E1 cell vitality. (A) Representative cell morphology with different concentrations (0, 0.5, 1, 2, 4, 6, 8, 10, 20, 40, 60, 80 nM) of T-2 toxin treatment. (B) The analysis of cell viability by the CCK-8 assay (n = 6). *P < 0.05, **P < 0.01 versus untreated groups.
Figure 2

Effect of T-2 toxin on osteoblastic differentiation. (A) Representative images of ALP staining with the gross appearance and the microscopic view (40X) (n=6). (B) Representative images of MDC staining (400X) (n=6). (C) Protein expressions of RUNX-2 and OSX (n = 3). (D) Protein expressions of Wnt, β-catenin, and GSK-3β (n = 3). (E) Protein expressions of Beclin 1, ATG 5, and LC3 (n = 3). DCG differentiation control group, DLG differentiation low-dose T-2 toxin treatment group, DMG differentiation
mid-dose T-2 toxin treatment group, and DHG differentiation high-dose T-2 toxin treatment group. *$P < 0.05$, **$P < 0.01$ versus untreated groups.

Figure 3
Role of Wnt signaling pathway and autophagy in T-2 toxin-induced osteoblastic differentiation disorders. (A) Representative images of ALP staining with the gross appearance and the microscopic view (40X)
(n=6). (B) Representative images of MDC staining (400X) (n=6). (C) Protein expressions of RUNX-2 and OSX (n=3). (D) Protein expressions of Wnt, β-catenin, and GSK-3β (n=3). (E) Protein expressions of Beclin 1, ATG 5, and LC3 (n=3). DCG differentiation control group, DTG differentiation T-2 toxin treatment group, DTW differentiation T-2 toxin and Wnt activator treatment group, and DTA differentiation T-2 toxin and autophagy inhibitor treatment group. #P < 0.05, ##P < 0.01 versus untreated groups.

Figure 4
Effect of T-2 toxin on osteoblastic mineralization. (A) Representative images of Alizarin red S staining with the gross appearance and the microscopic view (40X) (n=6). (B) Representative images of MDC staining (400X) (n=6). (C) Protein expressions Col-I and OCN (n=3). (D) Protein expressions of Wnt, β-catenin, and GSK-3β (n=3). (E) Protein expressions of Beclin 1, ATG 5, and LC3 (n=3). MCG mineralization control group, MLG mineralization low-dose T-2 toxin treatment group, MMG mineralization mid-dose T-2 toxin treatment group, and MHG mineralization high-dose T-2 toxin treatment group. *P < 0.05, **P < 0.01 versus untreated groups.
Figure 5

Role of Wnt signaling pathway and autophagy in T-2 toxin-induced osteoblastic mineralization disorders. (A) Representative images of Alizarin red S staining with the gross appearance and the microscopic view (40X) (n=6). (B) Representative images of MDC staining (400X) (n=6). (C) Protein expressions of Col-I and OCN (n=3). (D) Protein expressions of Wnt, β-catenin, and GSK-3β (n=3). (E) Protein expressions of Beclin 1, ATG5, and LC3 (n=3). MCG mineralization control group, MTG mineralization T-2 toxin treatment group, MTW mineralization T-2 toxin and Wnt activator treatment group, and MTA mineralization T-2 toxin and autophagy inhibitor treatment group. #P < 0.05, ##P < 0.01 versus untreated groups.

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