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Mechanism and effects of Zearalenone on mouse T lymphocytes activation *in vitro*☆

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ABSTRACT

Zearalenone (ZEA) is particularly toxic to the female reproductive system. Nevertheless, the effect of ZEA on the immune system is still not fully understood. The following study investigates the effects and mechanism of ZEA on mouse T cell activation in vitro. Briefly, T lymphocytes were extracted from primary splenic lymphocyte in mice, activated by concanavalin A, and then were exposed to different concentrations of ZEA for a certain period of time. Flow cytometry was used to detect the expression of activating and co-stimulatory molecules, and the secretion of cytokines in T cells at various stages. The expression of initiation regulatory protein in T cell activation, nuclear factor protein and co-stimulatory molecule related PI3K-Akt-mTOR signaling pathway proteins were detected by western blot. Our data showed that ZEA exposure inhibits the activity of T cell, and inhibits the expression of different activation signals in T cell. Additionally, ZEA exposure reduces the expression of initiative regulatory protein, i.e. LAT, Lck, Zap-70 during the activation of T cells. Thus, the results showed that ZEA exposure inhibits the formation and transmission of activated signal in T cells, interferes with signal pathway of T cell activation nuclear factor NFAT and NFκB, and decreases the secretion of cytokines after activation. Moreover, ZEA exposure interferes with co-stimulatory molecule CD28 during T cell activation, and with the activity of the PI3K-Akt-mTOR signaling pathway downstream of CD28. To conclude, our results indicated that ZEA toxin interferes with the activation of mouse T lymphocytes by affecting TCR signal and co-stimulatory signal, thus playing an essential role in immune toxicity.

1. Introduction

Zearalenone, also known as F-2 toxin, is one of the mycotoxins produced by fusarium spp which is commonly found in feed and food. Crops such as maize, wheat, oats and barley are vulnerable to ZEA pollution (Ji et al., 2016). ZEA concentration of 11.88 mg/kg has been detected in maize, while similar concentrations have been detected in oats, barley, wheat and sorghum (Mortensen et al., 2006). Also, ZEA has shown to possess estrogenic effects (Zinedine et al., 2007). Preclinical studies using rat model have revealed that ZEA is distributed in estrogen target tissues, such as uterus, Leydig cells and follicles. In addition, the intraperitoneal injection of 2 mg/kg ZEA in mice have confirmed the presence of DNA covalent internal transfer in the kidneys and liver (Grosse et al., 1997). Besides affecting DNA, ZEA also affects the normal structure of chromosomes. 94 μM ZEA and its derivatives increase chromosomal abnormalities in bovine oocytes (Minervini et al., 2001). *In vitro* experiments have shown that 30 μg/mL ZEA induces significant necrosis in peripheral blood mononuclear cells without or with mitogen treatment (Vlata et al., 2006). Moreover, ZEA over 1 μM concentration can obviously inhibit the GJIC function of HaCaT cells, which means they could potentially function as tumor promoting agents. 50 nM ZEA could significantly increases the activity of cytochrome enzyme and the expression of cytochrome enzyme mRNA in MCF-7 cells; cytochrome enzyme has shown to be the main mechanism behind the etiology of breast cancer formation (Z et al., 2004). Also, ZEA toxicity tests performed on bovine lymphocytes

Abbreviations: ZEA, Zearalenone; Con A, Concanavalin A; CCK-8, Cell Counting Kit-8; TCR, T cell receptor; FITC, peridinin chlorophyll protein; PE, phycoerythrin; Per-CP, peridinin chlorophyll protein

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indicated that 0.5 μM ZEA leads to rupture and fragmentation of bovine lymphocyte chromatid and decreased viability of bovine lymphocyte. These data suggested that ZEA can interfere with cytoactivity by interfering with mitosis (Lioi et al., 2004). ZEA can reduce the nutritional value of feed, decline the growth performance and reproductive performance in pigs. It can also have immunosuppressive effects by reducing animal infectious disease resistance (Yin et al., 2015), and can lead to chronic infection or reduce the effects of vaccination and drug treatment (Cheng et al., 2006). *In vivo* experiments showed that ZEA at high dose (40 mg/kg) significantly reduces splenic lymphocyte count in mice, which in turn results in swelling and necrosis of the spleen, atrophy of the white pulp and swelling of the red pulp, thus inducing damage to the immune system by decreasing IgA, IgG and CD3, CD4, CD8 and CD56 in peripheral blood of mice (Abbès et al., 2006). ZEA and its metabolites can also inhibit bovine neutrophils and the proliferation of B and T lymphocyte induced by mitogen (Muratori et al., 2003).

T cells are the major lymphocytes in cellular immune function, and their activation is an important prerequisite for their immune function. Non activated T cells are quiescent in vivo and are activated by lymphoblastic antigen or lymphocyte mitogen, which allows them to perform corresponding immune effect (Lee et al., 2002). The production of activation-induced molecules during each phase of T cell activation can directly reflect the activation of T cells at different stages. During the activation of T cells, the expression and activation of NFAT and NF-κB are essential for the development, maturation, and functioning of the immune system (Fisher et al., 2006). Therefore, the detection of these nuclear factors and their corresponding signaling pathways can directly help us to evaluate the activation effect of T cells.

Furthermore, co-stimulatory molecule is the second signal of T cell activation and cell response. In combination with TCR signaling, it can upregulate the transcription and translation of multiple cytokines and the proliferation of T cells. CD28 regulates the activation of T cells by participating in the expression and function of the negative regulatory factor CTLA4 (CD152). PI3K and Akt kinase are recently discovered signal transduction molecules (Chang et al., 2003). Existing data show that the activity of PI3K and Akt kinase are closely related to the regulation of cell life activities, i.e. participation in the target cell response induced by some growth factors or lymphokine, which also affects intracellular carbohydrate transport, protein and glycogen synthesis (Alessi et al., 1997); participate in the activation of immune cells (Tilton et al., 1997) and inhibits cell apoptosis (Ahmed et al., 1997; Kennedy et al., 1997).

Currently, there are only few *in vitro* and *in vivo* studies that have described the toxic effects of ZEA on the immune system (Ren et al., 2016; Yang et al., 2016). In addition, these mechanisms remain unclear. In this study, mice splenocytes was used to investigate the effect of ZEA on the activation of T lymphocytes, and to provide the theoretical basis for the study of the effect of ZEA mechanism on immune system toxicity which could be helpful for clinical prevention and control.

2. Results

2.1. ZEA toxin inhibits the cytoactive of T cell

Obvious cell aggregations were observed in Con A group compared to Control cells. These data suggested an obvious activation of T cells by Con A. For cells treated with ZEA, cell aggregation decreased in a dose-depend way (Fig. 1A). Furthermore, T cell activity was further quantified (OD^{450}): significant increase in cell activity was found in Con A group compared to Control group ($P < 0.01$). For cells treated with ZEA, the cell activity began to decrease in a dose-dependent manner. The IC-50 of ZEA was 20 μM (Fig. 1B). These data indicated that ZEA exposure could decrease the activity of T cells stimulated by Con A, and inhibit the transformation of T cells from resting stage into lymphoblastoid cells.

2.2. ZEA toxin exposure inhibits the expression of activation-induced molecules CD69, CD25 and CD71 in T cell

To determine the interference of ZEA in the process of T cell activation, we investigated the effects of ZEA on the expression of activation-induced molecules in T lymphocytes at different stages of activation. Mouse splenocytes were treated with Con A and different concentrations(0, 10, 20, 40 μM) of ZEA for 6, 30, 72 h and the expression of activation-induced molecules CD69, CD25 and CD71 in T lymphocytes were detected by double antibody staining combined with flow cytometry, respectively (Fig. 2A-C). Briefly, compared with the Control, in the Con A-treated group, the expression of CD69, CD25 and CD71 in T lymphocytes increased significantly (CD69, 57.93 ± 6.85% vs. 4.31 ± 3.88%, $P < 0.01$; CD25, 54.43 ± 12.83% vs. 6.53 ± 2.63%, $P < 0.01$; CD71, 59.38 ± 3.71% vs. 7.66 ± 5.23%; $P < 0.01$). Furthermore, after ZEA exposure, the expression of CD69, CD25 and CD71 in T cells decreased in dose-dependent manner (Fig. 2D).

2.3. ZEA toxin inhibits the secretion of cytokine in T cell

Mouse T lymphocytes were cultured for 48 h and 72 h, and the supernatant of the cells was collected. The secretion of five cytokine was then detected by Cytometric Beads Array. Briefly, compared with the Control, five cytokines IL-2, IL-3, IL-5, IL-6 and GM-CSF (Fig. 3A-E) began to produce large amounts of secretion after Con A stimulation ($P < 0.01$). After ZEA exposure, the secretion of five cytokines decreased in a dose-response manner ($P < 0.01$). These results indicate that ZEA exposure inhibits cytokine secretion after T cell activation and interferes with the autocrine action of T cells by inhibiting the secretion of IL-2.

2.4. ZEA exposure reduces the expression of initiation regulatory protein in T cell activation

High purity T cell was sorted by immunomagnetic beads (Fig. 4A). The purity of T lymphocytes detected by flow cytometry was 92.4 ± 3.4%(Fig. 4B). The cell protein was extracted after 24 h of ZEA (20, 40 μM) exposure. The initiation regulatory proteins of T cell activation such as LAT, Lck, Zap-70 and p-Zap-70 were detected by Western blot (Fig. 5A,C). Briefly, ZEA exposure inhibited the expression of Lck and Zap-70 and its phosphorylation levels; the expression of LAT, Lck and Zap-70, as well as p-Zap-70 decreased in a dose-dependent manner ($P < 0.01$) (Fig. 5B,D). These results indicated that ZEA exposure can interfere with the expression and phosphorylation modification of the initial regulatory protein of T cell activation, and in turn can affect the activation of T cell signaling.

2.5. ZEA toxin exposure interferes with nuclear factor signaling pathway of T lymphocyte

We further investigated the mechanisms of the inhibition of T cell activation following ZEA exposure. Briefly, the effects of ZEA exposure on T cell nuclear factor NFAT and NF-κB signaling pathways was examined. The cell total protein was extracted after 24 h of ZEA (20, 40 μM) exposure. The detection of key regulatory proteins in NFAT and NF-κB signaling pathways are shown in Fig. 6A-B. Compared with the Control, NFAT and NF-κB signaling pathways were activated. After ZEA exposure, the translation level of NFAT, NF-κB and its upstream regulatory protein decreased significantly in a dose-dependent manner (Fig. 6D-E). The cell nuclear protein was extracted after 24 h of ZEA (20, 40 μM) exposure. The expression of NFAT and NF-κB in the nucleus is shown in Fig. 6C. After ZEA exposure, the translation level of NFAT, NF-κB in the nucleus decreased significantly in a dose-dependent manner (Fig. 6F).