Research in Veterinary Science 102 (2015) 217–222

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/yrvsc

Oral exposure of fusarenon-X induced apoptosis in Peyer's patches, thymus, and spleen of mice

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ARTICLE INFO

Article history:
Received 19 March 2015
Received in revised form 18 August 2015
Accepted 24 August 2015

Keywords:
Apoptosis
Fusarenon-X
Lymphoid tissue
Mice

ABSTRACT

ICR male mice were orally administered once daily with Fusarenon-X (FX) at 0, 0.1, 0.3, and 0.5 mg/kg body weight for 14 days, and examined at 3, 6, 12, 24, and 48 h after last treatment (HAT) on Day 14. FX did not affect body and organ weight, however, at the higher doses FX caused nuclear condensation and fragmentation of lymphocytes in the cortical thymus and germinal center of Peyer's patches. Such effects were not observed in the liver, kidney and brain. Apoptotic lymphocytes evaluated by modified TUNEL method showed dose-dependency and peaked at 12 HAT in the Peyer's patches and thymus of 0.3 and 0.5 mg/kg FX-treated mice, whereas apoptotic bodies engulfed by macrophage were clearly seen by electron microscopy in 0.5 mg/kg FX-treated mice. Thus, repeated exposure to low doses of FX induces apoptosis in the lymphoid tissues of mice but did not affect liver, kidney and brain.

1. Introduction

Fusarenon-X (FX) is a member of the 8-ketotrichothecenes or type B trichothecenes which are produced by the most widely distributed *Fusarium* fungi especially *Fusarium graminearum* and *Fusarium crookwellense* (IARC, 1993; Ueno, 1983). It has been found naturally occurring and contaminated with other type B members such as deoxynivalenol (DON) and nivalenol (NIV) in wheat, barley, and cereal-based products (Eriksen and Alexander, 1998). In Spain, 148 samples of breakfast cereal mostly consumed by Spaniards were analyzed for type B trichothecenes contamination, with two multigrain-based cereal samples being FX positive and having co-occurrence of DON and FX at concentrations of 51 and 42 mg/kg, respectively in one multigrain sample (Montes et al., 2012). Moreover, Rubert et al. (2012) determined 35 samples of commercial baby foods contained FX, at a limit of detection (LOD) of 10–18 μg/kg. In mice and piglets, FX is rapidly metabolized to NIV by the liver and kidney after being absorbed from the gastrointestinal tract and it is detected in the liver, kidney, and spleen at 3 h up to 24 h following oral administration (Poapolathep et al., 2003b; Saengtienchai et al., 2014). In addition, FX also transfers to fetal or sucking mice via the placenta or milk after being metabolized to NIV in the maternal body (Poapolathep et al., 2004a). The LD50 values of FX are 3–5 mg/kg in mice, irrespective of sex and route of administration, whereas for oral administration of NIV, the LD50 is 38.9 mg/kg (Ueno, 1983). Rats are also more susceptible to FX than NIV, the oral LD50 values for FX and NIV in rats are 4.4 and 19.5 mg/kg, respectively (Conkova et al., 2003). Young animals are more sensitive to FX than adults (Ueno, 1983). The recommended tolerance daily intake for FX in infants and children is 0.01 μg/kg body weight/day (Rodríguez-Carrasco et al., 2013). IARC classified FX as group 3, that is, not carcinogenic to humans (IARC, 1993). The primary targets of FX are organs containing actively proliferating cells such as thymus, spleen, intestinal mucosa, testes, and bone marrow (IARC, 1993; Poapolathep et al., 2004a). It has been associated with adverse effects in experimental animals, including anorexia, growth retardation, gastrointestinal erosions, nephropathy, and immunosuppression (D'Mello et al., 1999; Conkova et al., 2003; Wu et al., 2014). Among type B trichothecenes, FX has been found to be more toxic than its metabolite, NIV, and other type B members in cytotoxicity studies (Eriksen et al., 2004). It can provoke a more persistent anorexia in mice particularly at higher dose exposures compared to NIV, DON, 3-acetyl-DON, and 15-acetyl-DON (Wu et al., 2012). Moreover, FX induced more persistent responses on proinflammatory cytokine than other type B trichothecenes, with greater potency in inducing IL-1ß and equipotent in the induction of CCL-2 and TNF-α expression (Wu et al., 2014).

FX has been reported to induce apoptosis *in vitro* and *in vivo* especially in the acute phase. In He-60 cell culture, FX exerts DNA fragmentation in a dose-dependent manner and the percentage of apoptotic cells increases dose- and time-dependently after exposure (Miura et al., 2002). Recently, Sutjarit et al. (2014) reported that single oral administration of FX in pregnant mice induces apoptosis in the developing mouse brain. Moreover, there have been few studies on

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http://dx.doi.org/10.1016/j.rvsc.2015.08.012
0034-5288/

the development of apoptosis following the administration of metabolites of FX. NIV is able to induce apoptosis in the lymphoid tissues of mice and it selectively damages different types of lymphocyte population (Poapolathep et al., 2002, 2003a, 2004b). However, toxicological studies of repeated exposure to FX are still limited and effects are unclear. This study was designed to evaluate the apoptosis potential of FX in mice over repeated exposure.

2. Materials and methods

2.1. Animals

One hundred 2-week-old male ICR mice were purchased and acclimatized to the environment for a week. Five mice each per stainless-steel cage were housed under controlled conditions (temperature: 23 ± 2 °C; 12 h-light/12 h-dark) at the Laboratory Animal Unit, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand. They were fed commercial feed pellets and water *ad libitum* with both being free of FX throughout the experiments. All experimental procedures on animals were approved by the Animal Ethics Research Committee of the Faculty of Veterinary Medicine, Kasetsart University. The experiments were started when the mice were 3 weeks old.

2.2. Chemicals

Standard FX was purchased from Wako Pure Chemical Industries Ltd. (Kyoto, Japan), and dissolved with 10% dimethyl sulfoxide (DMSO) in 0.9% normal saline solution (NSS) to a final concentration of 1 mg/ml for oral administration.

2.3. Experimental design

One hundred mice were divided into four groups. Thirty mice in each FX group were orally administered once daily with FX at dose levels of 0.1, 0.3, and 0.5 mg/kg body weight for 14 days. For histopathological and immunohistochemical studies, five mice of each FX dose group were killed by cervical decapitation under ether anesthesia at each time point of 3, 6, 12, 24, and 48 h after last treatment (HAT) on Day 14 (25 mice/group). Ten mice in the vehicle control (0 mg/kg Fx) were orally administered DMSO in 0.9% NSS once daily for 14 days and five of them were sacrificed at 24 HAT. Five remaining mice in each FX and vehicle control group were separated for electron microscopic evaluation and sacrificed by cervical decapitation under ether anesthesia at 12 HAT on Day 14.

2.4. Histopathology

The thymus and spleen of each mouse were weighed at necropsy. The thymus, spleen, Peyer's patches, liver, kidney, and brain were collected for histopathological evaluation. All tissue samples were fixed in 10% neutral buffered formalin. Paraffin sections (4 μM) were stained using hematoxylin and eosin staining and evaluated under a light microscope. Additional, paraffin sections were subjected to *in situ* detection of DNA fragmentation and immunohistochemical staining for proliferating cell nuclear antigen (PCNA) as mentioned below.

2.5. Evaluation of transmission electron microscopy

Small samples of thymus and Peyer's patch of each mouse were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer saline (pH 7.4), postfixed in 1% osmium tetroxide in the same buffer, and embedded. Ultrathin sections (1 μM) were double stained with uranyl acetate and lead citrate, and evaluated under an electron microscope.

2.6. In situ detection of DNA fragmentation

The *in situ* detection of DNA fragmentation was carried out on the paraffin sections of the thymus, spleen, Peyer's patches, liver, kidney, and brain using the modified TUNEL method with a commercial apoptosis detection kit (ApopTag® Peroxidase *in situ* Apoptosis Detection Kit; Millipore Inc., Canada). Briefly, paraffin sections were deparaffinized with xylene and rehydrated in a graded series of alcohol solution. Next, sections were washed with PBS and pretreated by microwaving at 800 W for 5 min in citric acid (pH 6.0). The sections were cooled down at room temperature (RT) for 20 min and then washed with PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase for 10 min at RT and then washed with PBS. The sections were incubated with equilibration buffer for at least 20 s and then incubated in a prepared deoxygenin labeled-terminal deoxynucleotidyl transferase solution with film overlay at 37 °C for 1 h. After incubation, the film was removed and the sections were incubated in stop wash buffer at RT for 10 min and then washed with PBS. Antideoxygenin-labeled peroxidase was added to sections with covered film and incubated at RT for 30 min. After incubation, the film was removed, washed with PBS, and incubated with diaminobenzidine solution at RT for 5 min. Next, the sections were counterstained with hematoxylin and evaluated under a light microscope. The TUNEL positive nuclei stained dark brown. The ratio of TUNEL positive cells to 1000 lymphocytes counted in 2 randomized fields for each of 5 sections per animal was calculated and presented as the mean percentage ± SD (TUNEL index = positive cells/1000 lymphocytes × 100) for each treatment group at 3, 6, 12, 24 and 48 HAT and 24 HAT for the vehicle control group.

2.7. Immunohistochemical staining for PCNA

Immunohistochemical staining for PCNA was carried out on paraffin sections of the thymus, spleen, and Peyer's patches to determine the proliferative activity of cells using the avidin–biotin–peroxidase complex (ABC) method and a VECTASTAIN ABC kit (Vector Laboratories, USA). Monoclonal anti-PCNA mouse antibody (clone PC10; DAKO, Denmark) was used as the primary antibody. Biotinylated horse anti-mouse IgG antibody from the VECTASTAIN ABC kit served as the secondary antibody. Diaminobenzidine solution was added for visualizing PCNA-positive cells. The sections were counterstained with hematoxylin and evaluated under a light microscope. PCNA-positive cells stained dark brown. The ratio of PCNA-positive cells to 1000 lymphocytes counted in 2 randomized fields for each of 5 sections per animal was calculated and presented as the mean percentage ± SD (PCNA index = positive cells/1000 lymphocytes × 100) for each treatment group at 3, 6, 12, 24 and 48 HAT and 24 HAT for the vehicle control group.

2.8. Statistical analysis

TUNEL and PCNA indices are shown as the mean ± standard deviation (SD) of five mice per group per time point. Student t-test was performed, followed by one-way ANOVA analysis and the Tukey test for pairwise multiple comparisons using GraphPad Prism version 5.0. (GraphPad Software, Inc. CA, USA). A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical findings

No mortality and no clinical signs were observed throughout the experiment. There were no significant differences in body weights and organ weight relative to body weight between the FX-treated groups and the control group.