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Short Communication

Fusarenon-X-induced apoptosis in the liver, kidney, and spleen of mice

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Abstract: Fusarenon-X is a non-macrocyclic type B trichothecene mycotoxin. It occurs naturally in agricultural commodities, such as wheat and barley. We investigated fusarenon-X-induced apoptosis in the liver, kidney, and spleen of male and female mice after a single exposure. Thus, mice were orally administered fusarenon-X (4 mg/kg body weight) and were assessed at 0, 3, 9, 18, 24, and 48 hours after treatment. Apoptosis in the liver, kidney, and spleen was determined using hematoxylin and eosin staining, the terminal deoxynucleotidyl transferase dUTP nick end labeling method, immunohistochemistry for proliferating cell nuclear antigen, and electron microscopy. Fusarenon-X-induced apoptosis at 9 hours after treatment, particularly hepatocytes around the central lobular zone of the liver, in proximal tubular cells of the kidney, and in hematopoietic cells in the red pulp area of the spleen in both male and female mice. The results of this study should be very useful with regard to the toxicity of fusarenon-X in both humans and domestic animals, which has been attributed to the intake of food contaminated with mycotoxins, especially fusarenon-X. (DOI: 10.1293/tox.2015-0063; J Toxicol Pathol 2016; 29: 207–211)

Key words: apoptosis, fusarenon-X, liver, kidney, spleen, mice

The *Fusarium* genus of fungi is a common contaminant in cereals in the temperate climatic zones of the world[1]. Among the mycotoxins produced by this genus, the wide family of trichothecenes is extremely prevalent[1]. Fusarenon-X (FX) or 4-acetyl-nivalenol (3, 7, 15-trihydroxy-4-acetoxy-8-oxo-12, 13-epoxy-Δ^9-trichothecene), is a non-macrocyclic type B trichothecene mycotoxin[2]. It is mainly produced in large amounts by *Fusarium nivale* and *F. crookwellense*, which naturally occur in wheat, barley, and cereal-based products[2, 3]. These foodstuff-derived products, particularly breakfast cereals, bread, and beer, are susceptible to FX contamination and are very important in the human diet[3]. In France, 1,872 samples were analyzed for FX, with 10% being positive, at a limit of detection (LOD) of 50 mg/kg [4]. Moreover, Juan *et al.*[5] reported that FX can be found in commercial infant formulas and baby foods on the Italian market. FX is known to be cytotoxic to many types of mammalian cells[6–8]. Miura *et al.*[9] reported that FX-induced apoptosis occurred in HL-60 cells by stimulating cytochrome c release, followed by its downstream events including the activation of multiple caspases involved in apoptosis. Its harmful effects have been demonstrated in experimental animals[10]. Poapolathep *et al.*[11] demonstrated that FX was absorbed from the intestinal tract and exhibited high toxicity in female mice. It resulted in anorexia in female mice[12]. It has been reported to induce adverse health effects, particularly apoptosis, in organs containing actively dividing cells such as the thymus and Peyer's patches in male and female mice[13, 14]. However, apoptosis in the liver, kidney, and spleen has not been elucidated.

To evaluate the apoptosis potential of FX in the liver, kidney, and spleen of male and female mice after a single exposure, 70 four-week-old male and female ICR mice were randomly allocated into 14 groups of five animals each, with 10 treated groups and four control groups. FX was orally administered to the 50 mice at a dose of 4 mg/kg body weight (b.w.). The dose level of FX used in this study was selected based on acute LD_{50} values (mg/kg), and 10 mice were administered 10% dimethyl sulfoxide in 0.9% normal saline (NS). The remaining ten mice were administered 0.9% NS and served as the control animals. All animals were sacrificed by heart puncture under ether anesthesia at 0, 3, 9, 18, 24, and 48 hours after treatment (HAT). After euthanization, the liver, kidney, and spleen were immediately collected in 10% neutral buffered formalin.

For histological microscopy, 4 μm paraffin sections of each organ were stained with hematoxylin and eosin (HE)

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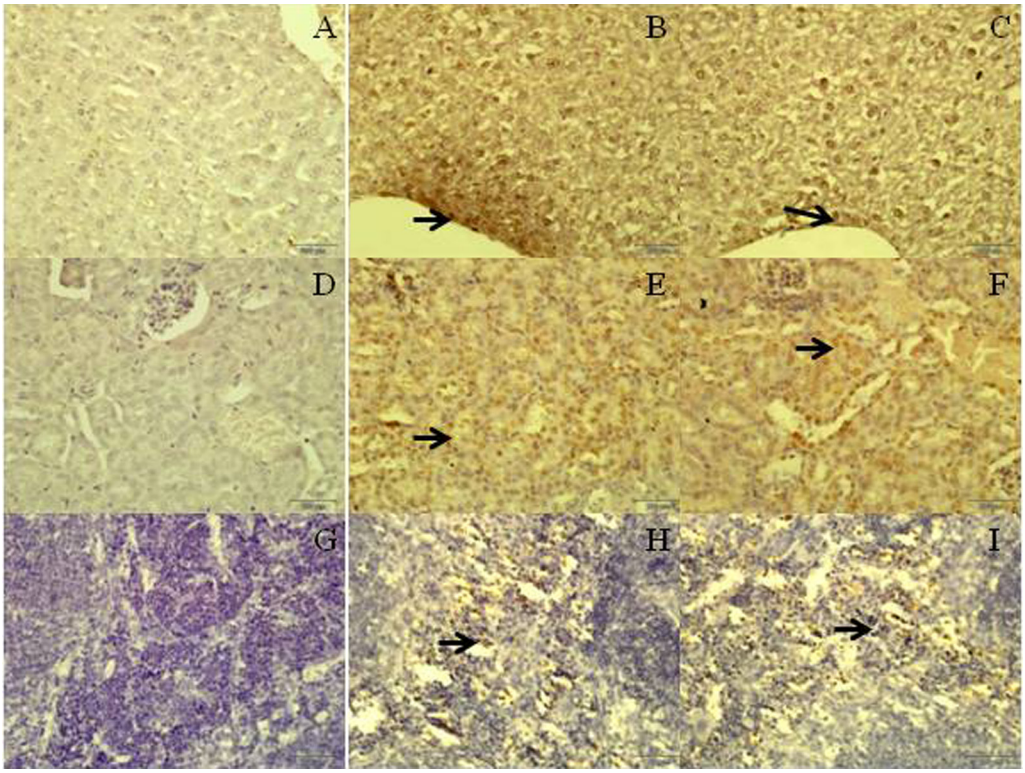

Fig. 1. Representative TUNEL images of the organs from FX-treated mice (9 HAT) and vehicle-treated mice. A. Liver from a vehicle-treated mouse. B. Liver from an FX-treated male mouse. C. Liver from an FX-treated female mouse. D. Kidney from a vehicle-treated mouse. E. Kidney from an FX-treated male mouse. F. Kidney from an FX-treated female mouse. G. Spleen from a vehicle-treated mouse. H. Spleen from an FX-treated male mouse. I. Spleen from an FX-treated female mouse. TUNEL staining ×40. Many TUNEL-positive nuclei are visible (B and C, E and F, and H and I). Arrow indicates TUNEL-positive nuclei (n = 5).

and observed under a light microscope. The results showed pyknotic nuclei of hepatocytes around the central lobular zone of the liver, in proximal tubular cells of the kidney, and in hematopoietic cells in the red pulp area of the spleen in treated animals in both male and female mice (data not shown). Some of the paraffin sections were subjected to *in situ* detection of fragmented DNA using the modified terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method with a commercial apoptosis detection kit (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit, Millipore Corporation, Billerica, MA, USA), and immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was performed on the paraffin sections to evaluate the proliferative activity of the cells in the organs using the avidin-biotin-peroxidase complex (ABC) method and a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA). The pyknotic nuclei of hepatocytes, proximal renal tubular cells, and hematopoietic cells of the spleen in treated animals were markedly positive for TUNEL in the central lobular zone of the liver, in the proximal tubular cells of the kidney, and in the red pulp area of the spleen (Fig. 1), and the TUNEL index peaked at 9 HAT (Fig. 2). However, FX-induced apoptotic cells were found in both the proximal and distal renal tubular cells, but the amounts of TUNEL-positive cells were notable in the proximal tubular cells. Accordingly, pyknotic nuclei and PCNA were negligible in the distal tubular cells. There were PCNA-positive nuclei of the liver, kidney, and spleen cells in both the control and treated animals, and the PCNA index decreased at 9 HAT and subsequently increased at 18, 24, and 48 HAT. The PCNA index at each point of treatment is shown in Fig. 2. For electron microscopy, partial samples of the liver, kidney, and spleen were fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4), postfixed in 1% osmium tetroxide PBS, and embedded. Ultrathin sections were double stained with uranyl acetate and lead citrate and observed under an electron microscope. Our electron microscopy findings showed nuclear chromatin fragmentation and marginalization of condensed chromatin along the nuclear membrane, and apoptotic bodies were ingested by macrophages (Fig. 3)

Apoptosis, or cell suicide, is a genetically controlled cell death[15]. It normally occurs during development and aging, and it functions as a homeostatic mechanism to maintain cell populations in tissues[16]. Dysfunction or dysregulation of this program has been implicated in a variety of pathological conditions, such as cancer and autoimmune diseases,

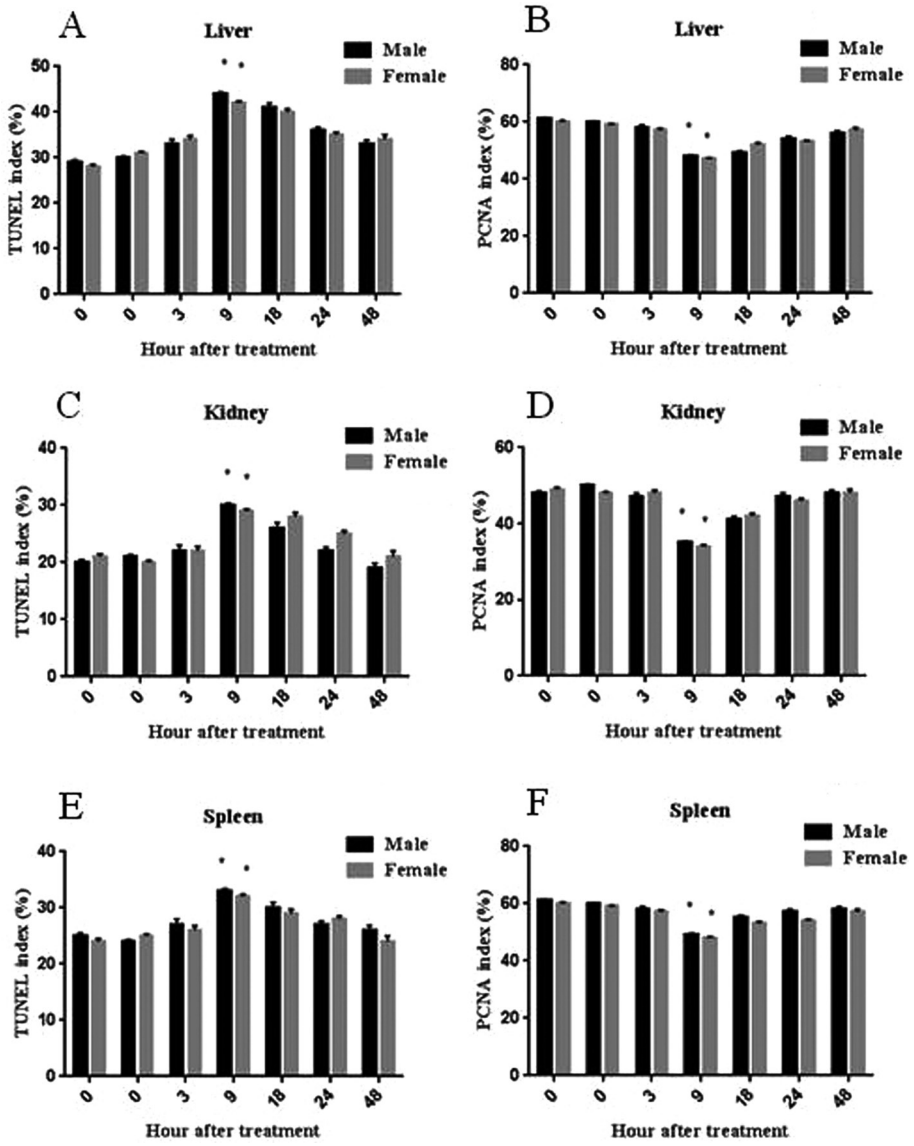

Fig. 2. Percentages of the TUNEL index and PCNA index in liver, kidney, and spleen cells from FX- treated mice. Each value represents the mean ± SD from five mice in each group. A. TUNEL index in liver cells. B. PCNA index in liver cells. C. TUNEL index in spleen cells. D. PCNA index in spleen cells. E. TUNEL index in kidney cells. F. PCNA index in kidney cells. Five fields were used as the counting areas, with 100 cells counted in each area at 10× magnification under a light microscope. *Significantly different from the control animals ($p<0.05$).

and AIDS and neurodegenerative disorders, which decrease and increase the occurrence of apoptosis, respectively[17]. In this study, we examined the acute toxic effects, particularly apoptosis, induced by FX in the liver, kidneys, and spleen of male and female mice. Light and electron microscopy have identified the various morphological changes that occur during apoptosis[18]. In the early phase of apoptosis, cell shrinkage and pyknosis are visible by light microscopy[19]. Pyknosis is the result of chromatin condensation, and this is the most characteristic feature of apoptosis[19]. In this study, the results of histological examination with HE staining showed that the apoptotic cells appeared as dense purple nuclear chromatin fragments in both the control and treated-animal groups. However, there were few apoptotic cells in control animals. Transmission electron microscopy (TEM)-the gold standard for examination of apoptosis-can better define the ultrastructural subcellular characteristic changes including the aggregation of the electron-dense, nuclear material peripherally under the nuclear membrane[20]. In this study, the results from TEM showed the nuclear chromatin fragmentation and marginalization of condensed chromatin along the nuclear membrane. The TUNEL assay was developed for the detection of free 3′-OH DNA ends[20]. They occur as a condensed nuclear chromatin of apoptotic cells in tissue

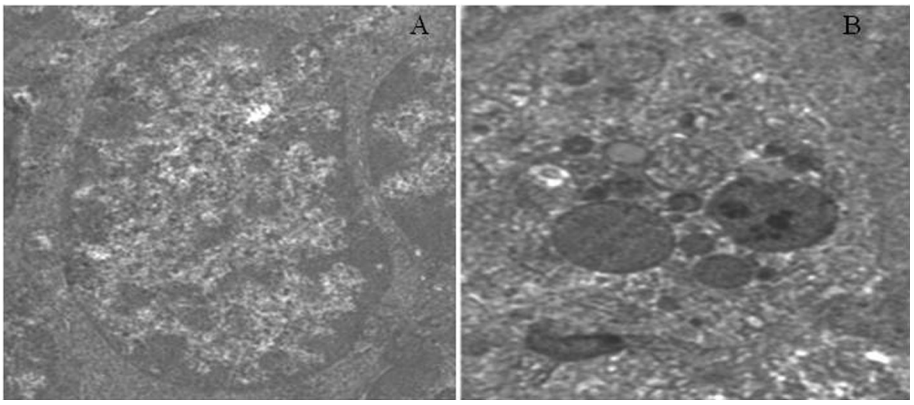

Fig. 3. Representative transmission electron microscopic images of the spleen from FX-treated male and female mice (9HAT). A. Nuclear chromatin fragmentation and marginalization of condensed chromatin along the nuclear membrane. B. Apoptotic bodies were ingested by macrophages ×3000.

sections, because many new 3′-OH DNA ends are generated by DNA fragmentation in apoptosis[20]. Our results showed that the apoptotic cells of hepatocytes, proximal renal tubular cells, and hematopoietic cells of the spleen were positively stained using the TUNEL method. Several studies have demonstrated that the TUNEL technique is not specific for the detection of apoptosis, which can be present in only a small population of necrotic cells[21, 22]. However, these necrotic cells have been shown to exhibit the ultrastructural characteristics of apoptotic cells[23–26]. Thus, it was reasonable to assume that these were TUNEL-positive cells. In the present study, TUNEL-positive cells were particularly presented around the central lobular zone of the liver, in the renal tubular cells of the kidney, and in the red pulp area of the spleen. Based on the physiology of the liver, spleen and kidney, the important function of the liver is as a detoxifier, being the central organ for the detoxification of exo- and endogenous substances. From the physiology of the kidney, the function of nephrons is to filter toxic nitrogenous compounds from the plasma in the same manner as in the spleen. The red pulp of the spleen has a specialized structure in the venous system leading to its unique capacity to filter the blood. In addition, Poapolathep *et al*.[11] demonstrated that FX can be metabolized to nivalenol (NIV) via deacetylation in mice and excreted mainly in urine. Moreover, the liver and kidney are the organs responsible for the FX-to-NIV conversion. Sutjarit *et al*.[26] found that FX induced apoptosis in the developing mouse brain in an FX-treated dam. The genetic regulatory mechanism of FX-induced apoptosis is regulated by Bax, Bcl2, Trp53, and caspase 9, and it can be defined via an intrinsic apoptosis pathway. Based on this, we can assume that the molecular mechanism of apoptosis in mice may involve the intrinsic apoptosis pathway. Taken together, these results and those of the present study reveal that FX can induce apoptosis, particularly around the central lobular zone of the liver, in the renal tubular cells of the kidney, and in the red pulp area of the spleen in male and female mice. The results of this study are considered to be very useful with regard to the toxicity of FX in both humans and domestic animals that have consumed food contaminated with mycotoxins, especially FX.

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Disclosure of Potential Conflicts of Interest: The authors declare that there are no conflicts of interest.

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