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Title: An Overview of the Toxicology and Toxicokinetics of Fusarenon-X, a Type B Trichothecene Mycotoxin

Running head: Fusarenon-X, a Type B Trichothecene Mycotoxin

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ABSTRACT

Fusarenon-X (FX) is a type B trichothecene mycotoxin that is frequently observed along with deoxynivalenol (DON) and nivalenol (NIV) in agricultural commodities. This review aims to give an overview of the literature concerning the toxicology and toxicokinetics of FX. FX is primarily found in cereals grown in temperate regions, but it can also be found worldwide because of the global transport of products. The major toxicity of FX occurs through inhibition of protein synthesis, followed by the disruption of DNA synthesis. Moreover, FX has also been shown to induce apoptosis in *in vitro* and *in vivo* studies. The targets of FX are organs containing actively proliferating cells, such as the thymus, spleen, skin, small intestine, testes, and bone marrow. FX causes immunosuppression, intestinal malabsorption, developmental toxicity, and genotoxicity. In addition, sufficient evidence of carcinogenicity in experimental animals is currently lacking and the International Agency for Research on Cancer (IARC) classifies it as a group 3 carcinogen.

KEYWORDS: fusarenon-X; toxicity; toxicokinetics; trichothecene mycotoxin

Mycotoxins are secondary metabolites produced by molds that exert adverse effects on human and animal health. Mycotoxin contamination can occur during various steps in food production, including pre-harvest, harvest, and storage. The Food and Agriculture Organization of the United Nations (FAO) estimated that approximately 25% of cereals produced worldwide are contaminated by mycotoxins [33]. The primary genera of fungi that produce mycotoxins are those of the *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Claviceps* species. Low-level contamination by *Fusarium* toxins is very common and co-contamination is frequently observed in animal feed [57]. The most relevant groups of mycotoxins that contaminate agricultural crops are aflatoxins, ochratoxins, trichothecenes, zearalenones, fumonisins, and ergot alkaloids.

Trichothecenes are a group of sesquiterpenoid mycotoxins that are commonly produced by *Fusarium* fungi. More than 180 derivatives of trichothecenes have been identified and divided into four types—A, B, C, and D—depending on their functional groups. Type A is characterized by a functional group other than a ketone at the C-8 position, whereas trichothecenes that have a carbonyl function at this position are identified as type B. The third group, type C, is characterized by a second epoxide ring at C-7,8 or C-9,10, whereas type D contains a macrocyclic ring system between C-4 and C-15 with ester linkages. Among trichothecene mycotoxins, types A and B are frequently found as contaminants in food for human and animal consumption [67]. A variety of adverse effects of trichothecenes, including emesis, growth retardation, immunotoxicity, neuroendocrine changes, and interference with reproductive and growth hormone signaling, have been reported in experimental animals [48].

Fusarenon-X (FX) is a member of the 8-ketotrichothecenes, or type B trichothecenes, and is produced by several *Fusarium* species. FX has been frequently observed, along with deoxynivalenol (DON) and nivalenol (NIV) [13, 22], as a contaminant in agricultural

commodities. Compared with that of other type B mycotoxins, oral administration of FX provoked a more profound anorexia in mice. In contrast, feed refusal induced in mice after intraperitoneal (i.p.) administration of FX was not as significant as that induced by NIV [70]. This was consistent with the results in the mink model, where FX produced emetic responses similar to DON, but of stronger potency than other DON congeners, following oral (p.o.) administration [68]. These findings indicated that, of the type B trichothecenes, FX is potentially toxic in experimental animals and humans after ingestion.

OCCURRENCE AND MODE OF ACTION

Chemical structure: The molecular structure of FX (3,7,15-trihydroxy-4-acetoxy-12,13 epoxytrichothec-9-en-8-one) includes a tetracyclic 12,13-epoxy-trichothec-9-ene skeleton with an epoxide ring at C-12,13 and a double bond at C-9,10. Its chemical structure is characterized by a hydroxyl (OH) group at the C-3,7,15 position and an acetyloxy (-$OCOCH_3$) group at the C-4 position (Fig. 1) [22, 63].

Occurrence of FX: The production of trichothecene metabolites depends on many factors, including the substrate, temperature, and humidity [8, 13, 22, 26, 47, 63, 66, 72]. FX was first isolated from the *Fusarium nivale* strain, Fn-2B, which primarily produced FX at a temperature between 25°C and 27°C, but was also found to produce FX at 15°C [63]. The closely related species, *F. culmorum* and *F. crookwellence*, also generated FX in both cool and warm areas [22]. Moreover, *F. sulphureum*, *F. sambucinum*, and *F. solani* have been reported as capable of either producing or accumulating FX [32]. FX was found to be generated during an early stage of fungal growth, then deacetylated during further growth [63]. Furthermore, room temperature storage (20°C) was more likely to encourage accumulation of FX and other trichothecenes (T-2, diacetoxyscirpenol (DAS), 3-acetyldeoxynivalenol (3-ADON)) than storage at cooler temperatures [32]. FX was found

101 together with other *Fusarium* toxins produced by the same fungal species in cereals,
102 including wheat, barley, corn, rye, oats, maize, and multigrain. It was also observed in maize
103 silage and extracted oil seed [8, 13, 26, 47, 66, 72]. FX has been found most commonly in the
104 temperate regions of Europe and Asia (Table 1) because these regions provide conditions
105 suitable for *Fusarium* growth and FX production. However, FX can be found in agricultural
106 commodities worldwide due to global product transport. Regarding health concerns, the
107 European commission (EC) has established maximum levels of *Fusarium* toxins allowed in
108 cereals and cereal products for human and animal consumption [15, 16]. The maximum level
109 of DON in cereals intended for direct human consumption is 750 μg/kg [16]. The European
110 Food Safety Authority (EFSA) has set the tolerable daily intake (TDI) of NIV at 1.2 μg/kg
111 body weight (BW) [17]. In 2010, the Food Safety Commission in Japan (FSCJ) set a TDI for
112 DON and NIV of 1 and 0.4 μg/kg BW, respectively [18]. However, guidance limits and TDI
113 recommendations are currently not available for FX [12, 15, 16].

114 *Mechanism of action:* Several mechanisms of action have been reported for FX.
115 Generally, FX is known to evoke a ribotoxic stress response, which inhibits protein and DNA
116 synthesis in eukaryotic cells. In detail, it caused the disaggregation of eukaryotic
117 polyribosomes *in vitro* at high concentrations [45, 65]. FX bound to ribosomes and inhibited
118 the second peptide bond formation, but not the polypeptide chain initiation [10, 11, 41].
119 Furthermore, FX dose-dependently encouraged DNA strand breakage of both dividing and
120 differentiated Caco-2 cells. This action was stronger than that produced by its metabolite,
121 NIV [7]. These proposed mechanisms of action suggest that FX is genotoxic to intestinal
122 cells, although, in a previous study, FX exhibited a weak clastogenic effect on Chinese
123 hamster V79-E cells [62]. The mechanisms of action of FX are yet to be fully understood. To
124 elucidate this issue, further studies are needed.
125

TOXICOKINETICS

Absorption and distribution: FX is a highly lipid-soluble compound that is rapidly absorbed from the gastrointestinal tract of mice [50], broilers, ducks [52], and piglets [54]. The maximum plasma concentration of FX occurred after approximately 5 min in piglets [54], 12 min in ducks [52], and 30 min in mice [50]. Its oral bioavailability was higher in piglets (74%) [54], than in ducks (19.5%) and broilers (9.8%) [52]. Furthermore, the oral bioavailability of FX was reported to be higher than that of NIV in mice [9, 50].

Metabolism: The maximum concentration of FX was detected in the liver, kidney, and spleen of piglets 3 hr after oral exposure [54]. Its metabolite, NIV, was found in plasma as soon as 10 min after p.o. administration of FX in broilers and ducks [52]. These results concur with an *in vitro* study of microsomal nonspecific carboxyesterase in rats and rabbits that demonstrated that the C-4 acetyl residues of FX were hydrolyzed by microsomal carboxyesterase to yield NIV [44]. Altogether, these findings suggested that FX was rapidly metabolized to NIV (Fig. 2) after being absorbed from the gastrointestinal tract. In addition, *in vitro* studies concerning FX metabolism indicated that FX to NIV conversion occurred in the liver and kidney [44, 50, 52, 54]. Indeed, in *in vivo* studies in mice, piglets, broilers, and ducks [50, 52, 54], the liver and kidney were observed to be the primary organs for FX to NIV conversion. The highest conversion percentage was observed in the liver rather than the kidney in mice (93.99% vs 27.91%). The conversion percentage was similar in the liver and kidney of ducks (98.95% vs 94.32%) and piglets (90.91% vs 89.72%), whereas the pattern was reversed in broilers (94.39% in the kidney vs 70.12% in the liver) [50, 52, 54]. It is noteworthy that NIV was found in fetal and suckling mice via the placenta and the mother's milk, respectively, after being metabolized to NIV in the maternal body [51]. In addition, NIV was reported to be metabolized to a de-epoxidated form by microorganisms in the gastrointestinal tract [69]. The intestinal microflora is important for the biotransformation of

trichothecenes. The presence or absence of particular intestinal microflora species can influence the extent to which an animal is sensitive to NIV because the de-epoxidated products were shown to be less toxic than the parental molecules [28].

Elimination: After intravenous (i.v.) and p.o. administration of FX in piglets, both FX and NIV were observed in the urine and feces for up to 24 and 48 hr, respectively. Large amounts of NIV were detected in the urine after FX exposure [54]. FX and NIV were also detected in excreta of broilers and ducks after i.v. and p.o. administration of FX [52]. An early study [50] that administered ^{3}H-FX to mice reported high and low radioactivity of NIV and FX in urine, respectively. Similarly, the feces of mice administered ^{3}H-FX revealed a similar radioactivity pattern (high for NIV and low for FX). These findings suggested that FX was rapidly excreted (before 24 hr) or almost totally transformed into NIV [28, 69] and excreted in urine. FX tissue concentrations were found to be similar among the tissues tested in mice (heart, lung, liver, stomach, kidney, spleen, thymus, mesenteric lymph nodes, bone marrow, small and large intestine, cecum, muscle, brain, and skin) [50]. This led researchers to speculate that the toxicity of FX on thymus, spleen, bone marrow, and mesenteric lymph nodes was not strictly related to FX accumulation, but also to that of its metabolites.

TOXICITY

Table 2 summarizes the 50% lethal doses of FX to animals. In mice, signs of acute toxicity were similar following administration of a single dose of FX via a variety of routes [63]. Oral exposure of FX exerted equipotent toxicity in newborn mice and rats [63]. FX primarily affected organs containing rapidly proliferating cells, including the thymus, spleen, small intestine, testes, skin, and hematopoietic tissues [22]. It led to a variety of adverse effects reported below.

Cytotoxicity: FX alone showed greater toxicity than NIV and other type B trichothecenes in various cell lines, including U-937 macrophages [27], HL-60 cells [39], RAW 264.7 murine macrophages [43], 3T3 fibroblast cells [14], SF-9 insect cells [19], and Caco-2 cells [2, 7]. Binary combinations of DON-FX and NIV-FX administered at low concentrations exhibited synergistic toxicity on Caco-2 cells, but a tertiary combination (DON-NIV-FX) exhibited antagonistic effects [2].

Immunotoxicity: FX is toxic to organs containing actively proliferating cells. Lymphocyte apoptosis was induced in lymphoid tissues, including Peyer's patches, thymus, and spleen, after 14 repeated ingestions of low doses of FX (0.1, 0.3, and 0.5 mg/kg BW) in mice [4]. Accordingly, after i.p. injection of FX (3 mg/kg BW), the thymus showed severe atrophy with loss of thymocytes and the thymic cortex [40]. Recently, Sutjarit and Poapolathep [59] demonstrated that orally administered FX (4 mg/kg BW) induced apoptosis in hematopoietic cells in the red pulp area of the spleen, hepatocytes around the central lobular zone of the liver, and proximal tubular cells of the kidney in mice. FX also caused apoptosis in Jurkat cell lines [49]. In human promyelocytic leukemia (HL60) cells, FX stimulated cytochrome c release, followed by activation of multiple caspases [39], which induced apoptosis. Significantly, FX evoked immunosuppressive effects similar to those of NIV, T-2 toxin, and 3-ADON in human peripheral blood mononuclear cells by depressing T or B lymphocyte activity in a dose-dependent manner [5]. FX exposure suppressed T-cell mitogen and macrophage responses in the spleens of mice [34, 35]. Forsell and Pestka [20] demonstrated that FX was more potent than other type B trichothecenes to human lymphocyte blastogenesis; this effect was associated with their C-4 substituent order (acetyl > hydroxyl > hydrogen). At a molecular level, FX increased the relative mRNA expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 in clonal human macrophages [27]. Wu *et al*. [71] found that FX was a potent and persistent inducer of IL-1ß and TNF-α

mRNA expression in splenic mice. Furthermore, FX was found to be an effective inducer of cyclooxygenase (COX)-2 mRNA expression through a selective increase in transcription and stabilization of COX-2 genes in murine macrophages [43].

Gastrointestinal toxicity: The gastrointestinal tract is a common target for p.o. administration of FX. In mice, FX ingestion caused a remarkably persistent feed refusal to a greater extent than that caused by DON, NIV, 3-ADON, and 15-acetyldeoxynivalenol (15-ADON) [70]. FX exerted an emetic potency greater than other tested mycotoxins (DON, NIV, 3-ADON, and 15-ADON) in mink [68]. FX (1.5 mg/kg BW) induced apoptosis in basal chief and parietal cells of rat gastric mucosa 1 hr after i.p. administration [31] and 1.5 hr after ingestion [30]. FX disrupted glycolysis and induced intestinal malabsorption by causing hypoglycemia and inhibiting mitosis of intestinal crypt cells [56]. Furthermore, FX damaged the active transport system of monosaccharides and impaired diffusional movements between the intestinal epithelial layer and mesenteric vein [29]. FX caused diarrhea by increasing the permeability of either blood vessel walls or intestinal epithelium [36] or by altering sugar translocation mechanisms [37], but did not modify the cyclic nucleotide system [38].

Genotoxicity: FX caused cell cycle delay, chromosomal aberrations, and sister chromatid exchanges in Chinese hamster V79-E cell lines through the inhibition of protein synthesis [62]. In addition, FX produced DNA strand breaks in dividing and differentiating human intestinal (Caco-2) cells in a dose-dependent manner [7].

Carcinogenicity: Many studies have evaluated the carcinogenicity of FX, as its carcinogenic properties have long been suspected. Among the tested toxins (aflatoxin B_1 and G_1, sterigmatocystin, and *O*-acetylsterigmatocystin), FX failed to induce mutagenesis by the Ames test assay [64]. In male Donryu rats, daily ingestion of FX (7 or 3.5 mg/kg FX in the diet) for 1 or 2 years showed slight incidences of tumorigenicity [55]. These facts might indicate that FX lacks mutagenic and tumorigenic abilities. Furthermore, treatment with FX

in medakas (*Oryzias latipes*) demonstrated no evidence of carcinogenetic effects, whereas other toxins (aflatoxin B_1 and G_1, sterigmatocystin, ortho-aminoazotoluene, methylazoxymethanol acetate, and *N*-nitrosodiethylamine) induced hepatic carcinomas [21]. Despite these findings, there is insufficient evidence to show that FX is carcinogenic in experimental animals. In addition, FX has been classified as a group 3 carcinogen by the International Agency for Research on Cancer (IARC), which indicates it is not carcinogenic to humans [22].

Developmental toxicity: FX can transfer its toxicity to fetuses via the placenta after being metabolized to NIV in the maternal body [51]. A single subcutaneous injection of FX (0.63-2.6 mg/kg BW) caused abortion in pregnant mice in a dose dependence manner, whereas FX ingestion (5, 10, and 20 mg/kg BW) inhibited embryonic implantation during the early phase and throughout the pregnancy period [25]. Oral administration of FX (3.5 mg/kg BW) to pregnant mice induced apoptosis in fetal brains, especially in the telencephalon [58].

Other toxicities: Other toxicities of FX have been also reported. Application of FX (5 μg/site) alone on the shaved skin of guinea pigs induced erythema and hardening due to degenerating fibrocytes and infiltrating cells in the corium [6]. Furthermore, when mycotoxin mixtures were tested, a synergism appeared after DAS-FX treatment, whereas T-2 toxin-FX mixtures provoked an antagonistic effect [6].

The antiviral activity of FX was demonstrated against herpes simplex virus type 1 (HSV-1) (50 *n*g/m*l*) and HSV type-2 (HSV-2) (26 *n*g/m*l*). This occurred at the viral replication stage after virus adsorption in the host cells [61].

CONCLUSION

FX is a type B trichothecene and is produced by several *Fusarium* species. This toxin is predominantly found in temperate regions, but is likely present worldwide because of the

global movement of products. FX is usually found as a co-contaminant with DON and NIV in agricultural commodities. Although FX is detected at low levels as a contaminant, its toxicity in experimental animals has been found to be stronger than that reported for other members of the same trichothecenes type B family. However, no regulations or guidelines currently exist for FX. This toxin primarily impacts organs containing actively dividing cells. Common adverse effects include immunosuppression and intestinal malabsorption. The major mechanism of action of FX is inhibition of protein synthesis, but FX can induce apoptosis and alter genetic material causing cell cycle delays, chromosomal aberrations, and sister chromatid exchanges. Moreover, FX exhibits a developmental toxicity by inducing abortion and inhibiting embryonic implantation. Carcinogenicity in experimental animals and humans is yet to be completely clarified. For this reason, additional research in this field is warranted.

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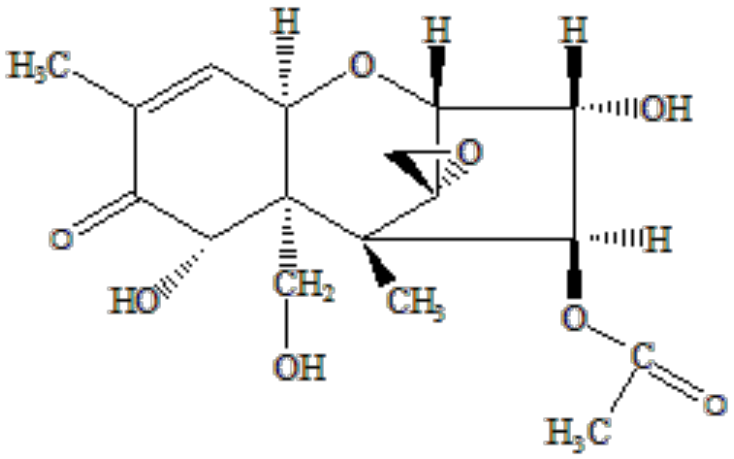

Fig. 1. Chemical structure of FX

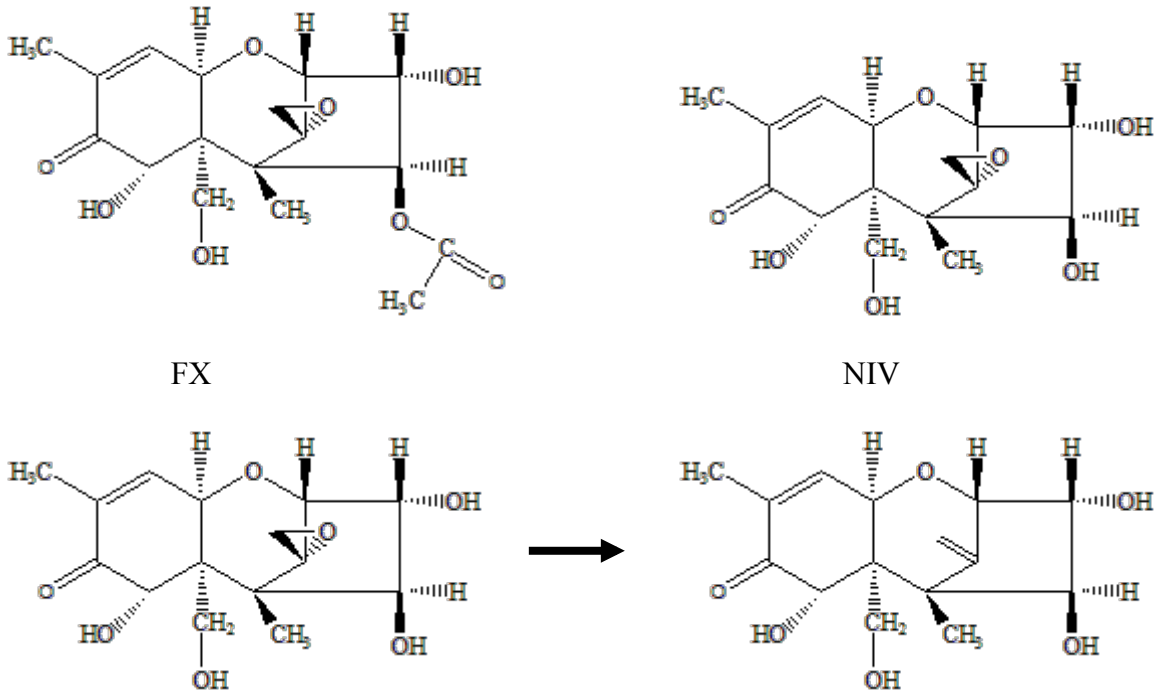

Fig. 2. Metabolic pathways of FX and NIV in animals [69]

500 **Table 1.** Natural occurrence of FX

501

Country	Sample	Year	Positive samples/ Total numbers	Mean content (μg/kg)	References
Belgium	Barley	2014	1/65	NQ	[66]
Belgium	Wheat	2014	1/93	508	[66]
Belgium	Bread	2014	2/25	505	[66]
Belgium	Breakfast cereals	2014	3/20	796	[66]
Czech&UK	Extracted oil seed	2008-2012		114	[72]
Czech&UK	Maize silage	2008-2012		77	[72]
Czech&UK	Complex feed for daily cows	2008-2012		80	[72]
Italy	Maize	2005	5/31	137	[8]
Italy	Oat	2013	3/7	23 ± 30 (26-75)	[26]
Italy	Splet	2013	2/3	91.8 ±54 (53.7-130)	[26]
Italy	Wheat	2013	14/57	18.44 ± 27 (12.5-102)	[26]
Italy	Barley	2013	4/9	18.43 ± 20 (27.5-47.3)	[26]
Italy	Rye	2013	5/11	28.52 ± 31 (42.4-70.2)	[26]
Italy	Whole cereals	2013	5/6	40 ± 38.4 (23.4-102)	[26]
Korea	Conventional cereals	2009	9/99	10.7 (6.8-18.7)	[46]
Korea	Organic cereals	2009	16/88	7.3 (0.9-18.7)	[46]
Korea	Rice	2009	10/65	9.1	[47]
Korea	Glutinous rice	2009	2/11	5.4	[47]
Korea	Brown rice	2009	1/48	18.7	[47]
Korea	Barley	2009	6/39	6.8	[47]
Korea	Mixed grains	2009	13/40	11.0	[47]
Korea	Corn	2009	6/25	8.7	[47]
Korea	Wheat	2009	4/54	7.9	[47]
Korea	Wheat flour	2009	2/38	9.0	[47]
Korea	Breakfast cereals	2009	7/18	7.1	[47]
Japan	Rice	2005		1900	[60]

Poland	Corn	2014		7.9-36.47	[3]
Saudi Arabia	Commercial animal feed	1997-2000		3.13-600	[1]
Spain	Barley	2007	2/100	17.45	[23]
Spain	Barley	2008	1.5/100	3.6	[24]
Spain	Multigrain	2009	2/46	27.2 (15.2-42.4)	[42]
Spain	Wheat-based cereals	2012	1/119	10.8	[53]

502

503 NQ: not quantifiable

504

505

506 **Table 2.** Comparative LD_{50} values (mg/kg) of FX by various routes of administration in
507 different animal species [63]

Animal species	FX LD_{50} (mg/kg)				
	IV	IP	SC	PO	IM
Mouse	3.4	3.4	4.2		
Newborn mouse			0.2	4.5	
Rat			0.5	4.4	
Guinea pig		0.5	0.1		
Cat			< 5.0		
Duckling			2.0		
Chick				33.79	

508