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THESIS

DEVELOPMENT OF APOPTOSIS AND CHANGES IN APOPTOSIS-RELATED GENES EXPRESSION IN DEVELOPING MOUSE BRAIN FROM FUSARENON-X-TREATED PREGNANT MICE

SAMAK SUTJARIT

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Samak Sutjarit 2014: Development of Apoptosis and Changes in Apoptosis-related Genes Expression in Developing Mouse Brain from Fusarenon-X-treated Pregnant Mice. Doctor of Philosophy (Genetic Engineering), Major Field: Genetic Engineering, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Amnart Poapolathep, Ph.D. 97 pages.

Fusarenon-X (FX), a type B trichothecene mycotoxin, is mainly produced by *Fusarium crookwellense*, which occurs naturally in agricultural commodities, such as wheat and barley. FX has been shown to exert a variety of toxic effects on multiple targets *in vitro*. However, the embryonic toxicity of FX *in vivo* remains unclear. In the present study, we investigated FX-induced apoptosis and the relationship between the genetic regulatory mechanisms and FX-induced apoptosis in the developing mouse brain of FX-treated pregnant mice. Pregnant mice were orally administered FX (3.5 mg/kg b.w.) and were assessed at 0, 12, 24 and 48 hours after treatment (HAT). Apoptosis in the fetal brain was determined using hematoxylin and eosin staining, the TUNEL method, immunohistochemistry for PCNA and electron microscopy. Gene expressions were evaluated using microarray and real time-reverse transcription polymerase chain reaction (qRT-PCR). Histopathological changes showed that, the number of apoptotic cells in the telencephalon of the mouse fetus peaked at 12 HAT and decreased at 24 and 48 HAT, respectively. FX induced the up-regulation of Bax, Trp53 and Casp9 and down-regulated Bcl2 but the expression levels of Fas and Casp8 mRNA remained unchanged. These data suggested that FX induces apoptosis in the developing mouse brain in FX-treated dams. Moreover, the genetic regulatory mechanisms of FX-induced apoptosis are regulated by Bax, Bcl2, Trp53 and Casp9 or can be defined via an intrinsic apoptotic pathway.

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LIST OF ABBREVIATIONS

FX	=	Fusarenon-X
NIV	=	Nivalenol
DON	=	Deoxynivalenol
DAS	=	Diacetoxyscirpenol
ZON	=	Zearalenone
3a-DON	=	3-acetyldeoxy-nivalenol
15a-DON	=	15-acetyldeoxy-nivalenol
DMSO	=	Dimethyl sulfoxide
EDTA	=	Ethylenediaminetetraacetic acid
Tris-HCl	=	Tris-Hydrochloride
ACN	=	Acetronitrile
MeOH	=	Methanol
TLC	=	Thin layer chromatography
GC	=	Gas chromatography
HPLC	=	High performance liquid chromatography
LC	=	Liquid chromatography
LC-MS/MS	=	Liquid chromatography tandem mass spectrometry
ESI	=	Electrospray ionization
APCI	=	Atmospheric-pressure chemical ionization
ELISA	=	Enzyme -linked immunosorbent assay
ATA	=	Alimentary aleukia
iv	=	Intravenous
ip	=	Intraperitoneal
po	=	Per os / oral
bw	=	Body weight
kg	=	Kilogram
g	=	Gram
mg	=	Milligram

LIST OF ABBREVIATIONS (Continued)

µg	=	Microgram
ng	=	Nanogram
ppb	=	Part per billion
ppm	=	Part per million
wk	=	Week
min	=	Minute
h	=	Hour
°C	=	Degree celcius
M	=	Molar
mM	=	Milli molar
SD	=	Standard deviation
SEM	=	Standard error of mean
CV	=	Coefficient of variation
WHO	=	World Health Organization
WTO	=	World Trade Organization
SPS	=	Sanitary and phytosanitary
IARC	=	International agency for research on cancer
C	=	Carbon
H	=	Hydrogen
JNK	=	Jun N-terminal kinase
ERK	=	Extracellular signal regulated kinase
MAPKs	=	Mitogen-activated protein kinases
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
ICR	=	Imprinting control regions
spp	=	Species
TNF	=	Tumor necrosis factor
TNFR	=	Tumor necrosis factor receptor

LIST OF ABBREVIATIONS (Continued)

TEM	=	Transmission electron microscope
HE	=	Hematoxylin and eosin staining
TUNEL	=	Terminal dUTP Nick-End Labelling
LM	=	Light microscope
RT-PCR	=	Real-Time polymerase chain reaction
HAT	=	Hour after treatment
PCNA	=	Proliferating cell nuclear antigen
ENU	=	Ethylnitrosurea
HU	=	Hydroxyurea
Bcl	=	B- cell lymphoma
CNS	=	Central nervous system
Apaf-1	=	Apoptotic protease activating factor-1
PI	=	Propidium iodide
FITC	=	Fluorescene isothiocyanate
ISEL	=	*In Situ* End-Labelling
CAD	=	Caspase-Activated DNase
pNA	=	p-nitroanilide
RT	=	Room temperature

DEVELOPMENT OF APOPTOSIS AND CHANGES IN APOPTOSIS-RELATED GENES EXPRESSION IN DEVELOPING MOUSE BRAIN FROM FUSARENON-X-TREATED PREGNANT MICE

INTRODUCTION

A toxin can be defined as a substance that is synthesized by a plant species, an animal, or by microorganisms (Turner *et al.*, 2009). Mycotoxins are toxic metabolites produced by fungi, especially saprophytic moulds that grow on a variety of foodstuffs including animal feeds and human foods, they are potentially risky to human and animals (Turner *et al.*, 2009). Mycotoxins can be caused a mycotoxicosis, acute or chronic disease, to humans and animals (Bryden, 2007). Chronic condition is defined as immunosuppression, cancer, reduced growth and development, which result from continual exposure to low level of mycotoxins ingestion (Bryden, 2007). Mycotoxins has a high incidences and impact to human health globally in many developed countries (Bryden, 2007).

Mycotoxins are classified due to their chemical structures, origin of biosynthesis, and the innumerable biological effects and the abundantly production of different fungal species (Bennett and Klich, 2003). Mycotoxins can be classified based on the organ that are affected; hepatotoxins, nephrotoxins, neurotoxins, immunotoxins and others (Bennett and Klich, 2003). Mycotoxin production of fungi is a complex process currently not fully understood (Kokkonen *et al.*, 2010). It is known that both the growth of fungi and their toxigenic potential are affected by environmental factors such as temperature, humidity, water activity, pH and both nutrient composition and availability (Kokkonen *et al.*, 2010). Under field conditions, there are many factors which influence the mycotoxin production such as climate, agricultural practices, host plant and the presence of other microorganisms (Kokkonen *et al.*, 2010). The assessment of mycotoxins contamination for humans and animals is

based on their doubtful identification and accurate quantification in foods and feedstuffs (Peter and Bernhard, 2006). Recently, the mycotoxin-contaminated foods and feed are global attention due to theirs adverse health and economic effects (Loungo *et al.*, 2010). Major food commodities affected are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fruit, particularly apples (Turner *et al.*, 2009). Morover, they may also be found in beer and wine resulting from the use of contaminated barley, other cereals and grapes in their production (Turner *et al.*, 2009).

In 1940s, more than 10% of the populations in Orenburg, near Siberia, were fatally suffered from overwintered millet, wheat and barley (Joffe, 1971), and the syndrome was officially named "Alimentary toxic aleukia"(ATA). During 1970, trichothecenes were used as biological warfare attacks in Afganistan and South East Asia (Laos and Camboda) via aerial application. It has been described "Yellow rain" (Wannenmacher and Wiener, 1997). Up to present, about 148 trichothecenes have been isolated including fusarenon-X (FX), Nivalenol (NIV), Deoxynivalenol (DON), T-2 toxin, diacetoxynivalenol and satratoxins, and their structures have been elucidated (Yagen *et al*., 1993).

Fusarenon-X (FX; 4-acetylnivalenol; 3, -7, -15-trihydroxy-4-acetoxy-12, -13 epoxytrichothec-9-ene) is a non-macrocyclic, type B, trichothecene mycotoxin (IARC 1993). It is mainly produced in large amounts by *Fusarium nivale* and *F. crookwellense*, which occur naturally in wheat, barley and cereal-based products (IARC 1993; Yazar and Omurtag, 2008). These foodstuff-derived products, particularly breakfast cereals, bread and beer, are susceptible to FX contamination, and are very important in the human diet (Yazar and Omurtag, 2008). In France, 1,872 samples were analyzed for FX, with 10 % being positive, at a limit of detection (LOD) of 50 μg/kg (DHCP, 2003). Moreover, Juan et al., (2014) reported that FX can be found in commercial infant formulas and baby foods on the Italian market. FX has demonstrated harmful effects in experimental or farm animals and in humans (Tandon et al., 1990). It resulted in gastrointestinal erosion, nephropathy and reduction of feed intake (Conkova et al., 2003; D'Mello et al., 1999). Poapolathep et al., (2003)

demonstrated that FX was bsorbed from the intestinal tract and exhibited a high toxicity in mice. It has been reported to induce adverse health effects, particularly apoptosis, in organs containing actively dividing cells such as the small intestine, thymus, spleen, bone marrow, testes, reticulocytes and mitogen-stimulated human lymphocytes, as observed with other trichothecenes (Forsell and Pestka, 1985; Miura et al., 1998; Ohta et al., 1978; Poapolathep et al., 2002).It is known to be cytotoxic to many types of mammalian cells (Bondy et al.,1991; Ohotsubo and Saito, 1970; Ueno et al., 1973). Miura et al. (2002) 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.

Apoptosis, or cell suicide, is a genetically controlled cell death (Fink and Cookson, 2005). It normally occurs during development and aging, and functions as a homeostatic mechanism to maintain cell populations in tissues (Nobury and Hickson, 2001). Dysfunction or dysregulation of this program have been implicated in a variety of pathological conditions, such as cancer and autoimmune diseases, AIDS and neurodegenerative disorders, which decrease and increase the occurrence of apoptosis, respectively (Fadeel, 1999). FX can also induce apoptosis in mouse thymocytes *in vivo* and *in vitro* (Miura et al., 1998 and 2002). FX is a particularly potent inhibitor of protein synthesis (Bony et al., 2007), it binds to the peptidyltransferase catalytic center on ribosomes and blocks elongation of the peptide chain (Carter and Cannon, 1978). Toxic effects have been observed in the fetuses of pregnant animals, particularly mice that were exposed to trichothecene mycotoxins such as T-2 toxin, and deoxynivalenol (Debouck et al., 2001, Lafarge-Frayssimet et al., 1990; Rousseaux and Schiefer, 1987). Poapolathep et al., (2004) demonstrated that FX can pass through the placenta of FX-treated pregnant mice.

OBJECTIVES

As mentioned above, there are only a few reports of the adverse effects of FX and molecular pathways of FX-induced apoptosis in developing embryos, particularly in the brain are still unclear, although FX-induced ultrastructural changes of thymocytes characteristic for apoptosis and FX can penetrate to fetal mice via placenta (Miura *et al*., 1998; Poapolathep *et a*l., 2004)

The major purpose of this study is to get insight of the molecular mechanisms of FX-induced cell death in developing mouse brain from FX -treated dams as follows,

1. To indicate FX can induce apoptosis in the developing mouse brain from FX-treated dams.

2. To investigate the relationship between the genetic regulatory mechanisms and FX-induced apoptosis in the developing mouse brain.

LITERATURE REVIEW

1. Background

Toxic compounds especially natural toxins that can contaminate in human food and animal feed are plant secondary metabolites, bacterial toxins, phytotoxins and mycotoxins (Berthiller et al., 2012). Mycotoxins contaminate in human food and animal feed at various stages of food chain (Berthiller et al., 2012). The three most important genera of mycotoxigenic fungi are Aspergillus, Penicillium and Fusarium (Bryden,2007). There are principal classes of mycotoxins that mainly produced by these genera are aflatoxins, ochratoxins, trichothecenes and fumonisins respectively (Bryden,2007). Mycotoxins are toxic secondary metabolites and have low molecular weight natural products (Bennett and Klich, 2003, Turner et al., 2009). They can contaminate with agricultural products in the field or post harvest (Bennett and Klich, 2003, Turner et al., 2009). Agicultural-mycotoxigenic fungi are phytopathogenic and saprophytic organisms that infected plants in the field and/or greenhouse colonized plant products post harvest, respectively (Berthiller et al., 2012). Mycotoxins are unavoidable contaminants in human food and animal feed, so they are a major problem of public health all over the world (Wood, 1982, Woody and Chu, 1992). There are many interactivity factors that involved in pathogenesis of a mycotoxicosis so it is difficult to diagnose mycotoxins exposure in Figure 1 (Bryden,2007). The diseases that are involved with mycotoxigenic fungi in human are shown in Table 1

Mycotoxin production of fungi is a complex process that currently not fully understood (Kokkonen *et al*., 2010). It is known that both the fungal growth and their toxigenic potentials are affected by environmental factors such as temperature, humidity, water activity, pH and both nutrient composition and availability (Kokkonen *et al*., 2010). Under field conditions, there are many factors which influence the mycotoxin production: climate, agricultural practices, host plant and the presence of other microorganisms (Kokkonen *et al*., 2010).

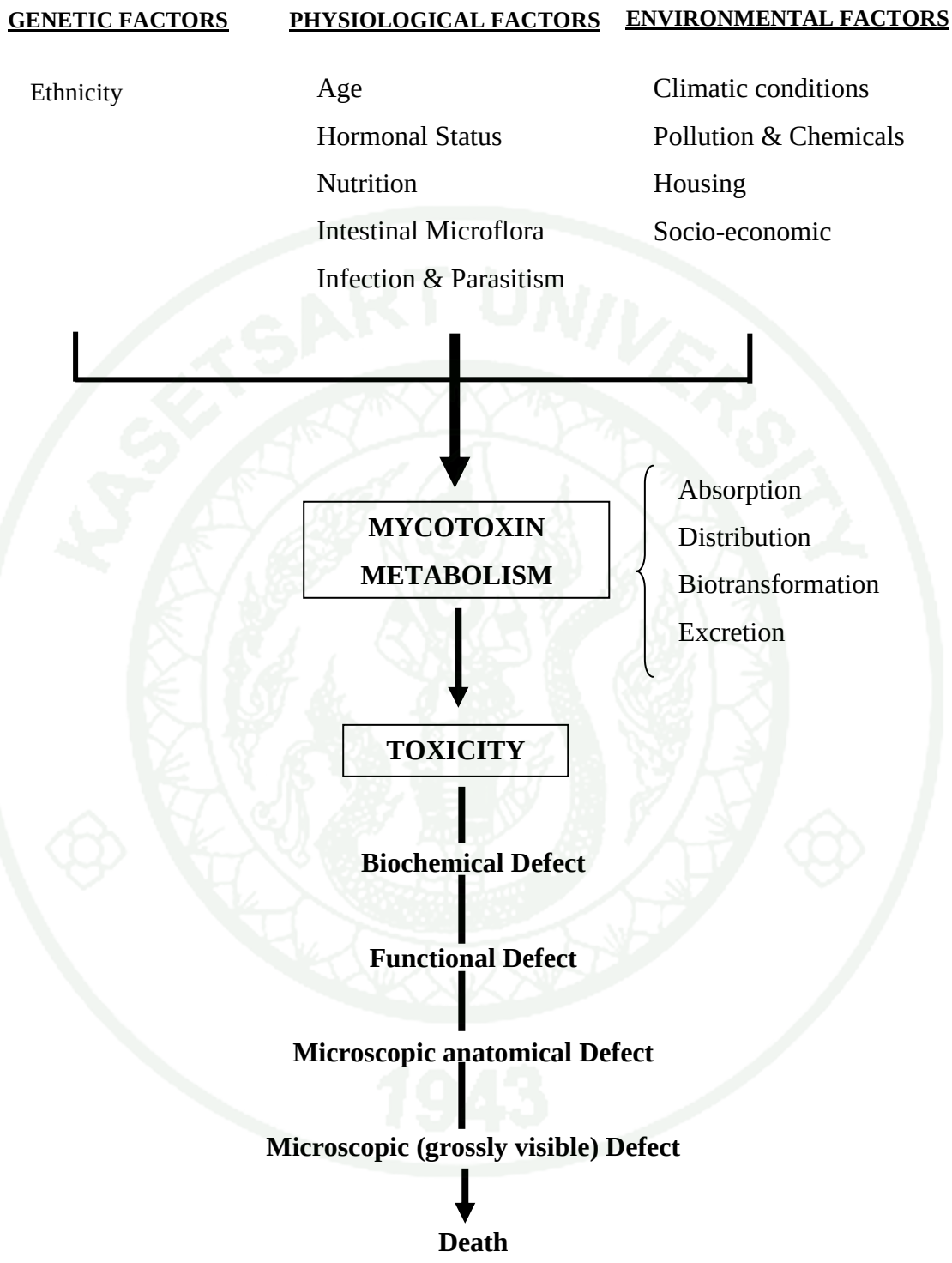


Figure 1 A schematic representation of general relationships in a mycotoxicosis.

Source: Bryden (2007)

Table 1 Mycotoxigenic fungi-related diseases in humans

Disease	Mycotoxin source	Fungus
Akakabio-byo	Wheat, barley, oats, rice	*Fusarium spp.*
Alimentary toxic aleukia	Cereal grains (toxic bread)	*Fusarium spp.*
Balkan nephropathy	Cereal grains	*Penicillium spp.*
Cardiac beriberi	Rice	*Aspergillus spp.*, *Penicillium spp.*
Celery harvester's disease	Celery (Pink rot)	*Sclerontinia*
Ergotism	Rye, cereal grains	*Claviceps purpurea*
Hepatocarcinoma	Cereal grains, peanuts	*Aspergillus flavus*, *A. parasiticus*
Kwashiorkor	Cereal grains	*Aspergillus flavus.* *A. parasiticus*
Neural tube defects	Maize	*Fusarium verticillioides*, *F.proliferatum*
Oesophageal tumors	Corn	*Fusarium verticilloides*, *F.proliferatum*
Onyalai	Millet	*Phoma sorghina*
Reye's syndrome	Cereal grains (grain dust)	*Aspergillus*
Stachybotryotoxicosis	Cereal grains, (grain dust)	*Stachybotrys atra*

Source: Bryden (2007)

Major food commodities affected are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fruits, particularly apples (Turner *et al*., 2009). Mycotoxins might be found in beer and wine resulting from the use of contaminated barley, other cereals and grapes in their production (Turner *et al*., 2009). Various mycotoxins continue to be reported in different commodities in various countries around the world (Ramesh and Vasanthi, 1999).

2. Toxicity

Clinicians often arrange mycotoxins by the affected-organs (Bennett and Klich, 2003). Thus, mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins and others (Bennett and Klich, 2003). Organic chemists have attempted to classify them by their chemical structures (e.g., lactones, coumarins) (Bennett and Klich, 2003). Physicians have attempted to classify by the illnesses they cause (e.g., St. Anthony's fire, stachybotryotoxicosis). Mycologists attempted to classify by the fungi that produce them (e.g., Aspergillus toxins, Penicillium toxins) (Bennett and Klich, 2003). Toxicologists tend to concentrate their efforts on hazardous chemicals such as polyaromatic hydrocarbons, heavy metals, and organic pesticides (Bennett and Klich, 2003). Because they have devoted less effort to natural products, agriculturalists, chemists, microbiologists, and veterinarians who are often unfamiliar with the basic principles of toxicology have conducted most of the mycotoxin research (Bennett and Klich, 2003). The final toxic effects, appearing in consumers exposed to a mixture of mycotoxins, are related to the toxicokinetic behavior, the metabolism, and the toxicodynamic effects of mycotoxins (Monbaliu *et al*., 2010).

Mostly, the main human and veterinary health burden of mycotoxin exposure is related to chronic exposure such as cancer induction, kidney toxicity, immune suppression. However, the best-known mycotoxin episodes are manifestations of acute effects such as turkey X syndrome, human ergotism, stachybotryotoxicosis (Bennett and Klich, 2003). Human exposure to mycotoxins is further determined by environmental or biological monitoring (Bennett and Klich, 2003). In environmental monitoring, mycotoxins were measured in food, air, or other samples. In biological monitoring, the presence of residues adducts and metabolites are assayed directly in tissues, fluids, and their excretion (Hsieh, 1988). The different chemical and physicochemical properties of the mycotoxins require specific extraction, cleanup, separation and detection methods (Krska *et al*., 2008). Therefore, most methods target only individual mycotoxins or at best a group of closely related mycotoxins

(Krska *et al*., 2008). A large variety of analytical methods is used for mycotoxin determination, including chromatographic methods such as thin layer chromatography, Gas chromatograpy or liquid chromatograpy and immunochemical methods such as enzyme-liked immunosorbent assay (Berthiller *et al*., 2012). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the technique of choice for mycotoxin analysis because of highly reliable, versatility, specificity and selectivity (Monbaliu *et al*., 2010; Rubert *et al*., 2012).

3. Structure

Trichothecene mycotoxins are a family of tetracyclic sesquiterpenoid substances that composed of over 200 compounds of widely varying toxicity. All trichothecenes have a basic tetracuclic sesquiterpenoid structure with a six membered -oxygen containing ring, an epoxide group in the 12,13 position and an olefenic bond in the 9,10 position. The essential for toxicity is based on the epoxy group at C-12 and C-13 of trichothecenes structure. The presence or absence of a macrocyclic ring linking between C-4 position and C-15 position is important criteria to classify the trichothecene; non-macrocyclic and macrocyclic trichothecenes. The non-macrocyclic trichothecenes, produced primarily by genus *Fusarium*, are T-2 toxin, HT-2 toxin,deoxynivalenol, nivalenol and fusarenon-X while the macrocyclic ones produced primarily by *Stachybotrys spp*. and *Myrothecium spp*.are roridins, verrucarins and satratoxins. Moreover, trichothecenes are classified into 4 groups based on substitutions at 5 positions of the trichothecenes skeleton Figure 2. Members of group A do not contain carbonyl on C-8. The examples are represented by T-2 toxin, HT-2 toxin, and diacetoxyscirpenol. Hydrolysis of ester groups leads to the formation of a basic trichothecene moiety with one to five hydroxyl groups. Group B differs from group A by the presence of a carbonyl group on C-8. Group C members, such as crotocine, have another epoxy group between the C-7 and C-8 or C-8 and C-9 positions, respectively. Compounds in group D, such as satratoxin G, include a macrocyclic ring between C-4 and C-15 (Wu *et al*, 2010).

The trichothecenes are most commonly found in cereals, and particularly in barley, maize, oats, and wheat (Conkova *et al*., 2003; D'Mello *et al*., 1999; Eriksen and Petterson, 2004). These mycotoxins are chemically stable to heating and survive food processing (Montes *et al*., 2012). Consequently, a frequent contamination can be expected for cereal-based foods, posing a potential risk to human health (Montes *et al*., 2012). Trichothecene levels are depending on climatic, as well as on seasonal and geographic conditions, because the influence of several factors can lead to a change in the Fusarium profile (Ibanes-Vea *et al*., 2012). Trichothecenes occur worldwide in grains and other commodities grown in cooler climates (Haschek and Beasley, 2009). Mild temperatures tend to enhance fungal growth and cool temperatures increase toxin production (0–15°C) (Haschek and Beasley, 2009).

In European agricultural commodities, type-A trichothecenes occur less frequently and at lower concentrations than DON and toxicity of type-A trichothecenes are higher than those of type-B (Ibanes-Vea *et al*., 2012). The primary fungal species producing the trichothecene mycotoxins are listed in Table 2. Trichothecene mycotoxins including Fusarenon-X (FX), non-protein toxins, are highly cytotoxic to eukaryotic cells and act by inhibiting protein and DNA (Miura *et al*., 1998). Thus, they cause specific damage to the tissues containing many rapidly proliferating cells such as lymphoid tissues (Miura *et al*., 1998). Although, trichothecenes identified so far are over 150 members, data of their natural occurrence in foods are mostly limited to T-2 toxin, diacetoxyscirpenol (DAS), deoxynivalenol (DON), nivalenol (NIV), and fusarenon-X (FX) due to their high toxicities and occurrence (Pittet, 1998; Voyksner *et al*., 1987; Yoshizawa *et al*., 1980; Poapolathep *et al*., 2002). DON may be the most commonly occurring trichothecene in nature (Wu *et al*, 2010). T-2 toxin does not occur as much as DON, but its toxicity is higher than that of DON (Wu *et al*, 2010). In addition, the acetyl derivatives of trichothecenes, 3-acetyldeoxy-nivalenol (3-aDON), 15-acetyldeoxynivalenol (15-aDON), and fusarenon-X (FX), co-occur frequenlly all over the world (Wu *et al*, 2010).

4. Toxicokinetics

The toxicokinetics of trichothecenes has been a subjected of various review articles (Swanson and Corley, 1989; Yagen and Bialer, 1993; IARC, 1993). Trichothecenes undergo all four basic reactions in xenobiotic metabolism (Ramesh, 2007). Phase I hydrolysis and oxidation and phase II glucuronide conjugation occur in the body tissues, while reduction of the 12,13-epoxide is thought to occur through microbial action in the gastrointestinal tract; although T-2 toxin is the only trichothecene for which all four basic reactions or pathways occur simultaneously in the same animal (Swanson and Corley, 1989). The ability to remove the epoxide oxygen (deepoxidation) is an important step in the detoxification of trichothecenes (Ramesh, 2007). The toxicokinetics of trichothecenes is important for the understanding potential impact on animals (Ramesh, 2007). The kinetics of all trichothecenes has not been investigated completely, but the existing data shows that the toxins are rapidly absorbed from digestive tract (Erikson and Pettersons, 2004). They do not accumulate to a significant extent in the body and are rapidly excreted within a few days in urine and feces after po administration (Swanson and Corley, 1989). Trichothecenes are rapidly excreted without any accumulation in any tissue, and only traces of the toxins are found 24 h after po or iv exposure (Erikson and Pettersons, 2004). Trichothecenes do not accumulate in animals due to the rapid excretion and only traces (<50 ng/g) can be found in animal-derived food products (Erikson and Pettersons, 2004). The oral bioavailability of trichothecenes is generally low as a result of physiological instability and first-pass metabolism (Yagen and Bialer, 1993). Intestinal microorganisms present an additional pathway for the detoxification of ingested trichothecenes in some animal species (Sudakin, 2003). Reduction of the trichothecene C-12,13 epoxide has been demonstrated to occur within the gastrointestinal tract of rats, cattle, and swine (Swanson *et al*., 1988). In pigs, T-2 toxin, DON and NIV can be detected in blood with in less than half an hour after oral exposure to the toxins (Erikson and Pettersons, 2004). The main route of excretion after iv injection is the urine, but after oral exposure a significant proportion is also excreted in the faeces (Swanson and Corley, 1989; Prelusky *et al*., 1988).

Trichothecenes undergo a variety of different metabolic reactions in animals (Erikson and Pettersons, 2004). The major reactions are hydrolysis to split off side

groups, hydroxylations and de-epoxidation. Acetylated toxins are rapidly metabolised to the de-acetylated form of the toxin (e.g. T-2 toxin to HT-2 toxin, FX to NIV, 3-acetyl DON to DON) (Erikson and Pettersons, 2004). The deacetylation reactions are fast and catalyzed by specific esterases (Swanson and Corley, 1989). A 12,13 de-epoxide metabolite of trichothecenes has been detected after oral exposure to NIV or DON in rats, mice, cattle and sheep (Erikson and Pettersons, 2004). A de-epoxide metabolite was found after iv injection of T-2 toxin in pigs (Erikson and Pettersons, 2004). De-epoxidation of trichothecenes has also been reported to occur by incubating them with the microflora from the intestines of a range of species or with micro-organisms from the rumen of cows (Swanson and Corley, 1989; Swanson *et al*., 1988; Kollarczik *et al*., 1994). These incubation studies have also showed that the microflora is able to deacetylate trichothecenes (Erikson and Pettersons, 2004). De-epoxide metabolites of trichothecenes have also been detected in plasma and urine in sheep and cows, but only at low concentrations (Erikson and Pettersons, 2004). This reduction of the epoxide ring is probably carried out by anaerobic gastrointestinal micro-organisms (Swanson and Corley, 1989). Some trichothecenes, such as DAS, T-2 toxin and HT-2 toxin and metabolites there of, are extensive by conjugatetd with glucuronide, while little or no such conjugation has been found in monogastric animals given DON or NIV (Swanson and Corley, 1989). The absorption of trichothecenes is rapid also in ruminants, but only low proportions of the administered toxin are accounted for in studies with cattle and sheep, even when the de-epoxide metabolite is included (Erikson and Pettersons, 2004). This indicates that other unknown metabolites may be formed in ruminants (Erikson and Pettersons, 2004).

Among a large number of mycotoxins, only a few states that will affect the pigs performances (Viljoen, 2008). The risk of mycotoxins in pig contaminated-feed, depending on the age and health of the pigs and the level of toxins in the extreme is death (Viljoen, 2008). However, low levels of mycotoxins can reduce pig performance and general well-being (Viljoen, 2008). When pigs are fed with mycotoxin harmful, it can affect the central nervous system, pork, liver, kidneys, immune system and reproductive processes. (Viljoen, 2008). Aflatoxin, zearalenone, and tricothecene (vomitoxin and T-2 toxin) are the most often reported mycotoxins in

swine feed. Each toxin is produced by a different mould (Viljoen, 2008). The conditions that promote the growth of fungi are varies. However, high humidity and warm temperatures are required for the growth of most fungi feed (Cranshaw, 2008). The pig is most sensitive to trichothecenes (Erikson, 2003). The kinetic parameters of DON have been related to intravenous and acute and chronic oral DON exposures in pigs (Coppock *et al*., 1985; Prelusky *et al*., 1988, 1990; Goyarts and Dänicke, 2006). In pigs fed graded levels of T-2 toxin in standard pig ration for 8 weeks, no significant differences in body weight gain and feed consumption were observed between the test and control pigs (Agag, 2005). Young pigs refused a ration containing 16 ppm T-2 toxin, but not a diet containing 10-12 ppm (Agag, 2005). The no observed effect level in ration was estimated to be less than 1 ppm based on differences in body weight gain (Weaver *et al*., 1978a). Dietary levels of T-2 toxin as low as 0.5 ppm were found to cause a reduction in feed intake in pigs (Rafai *et al*., 1995a). T-2 toxicosis is due to elevation of tryptophan in the brain. Tryptophan, a mediator of appetite, is a precursor of serotonin (Smith and seddon, 1998). Infertility with some lesions in the uteri and ovaries result from consumption of feed contaminated with 1 to 2 ppm of T-2 toxin (Jacobsen *et al*., 1993). The intravenous administration of T-2 toxin to pigs at doses of 4 or 8 mg/ kg bw, resulted in increased plasma concentrations of epinephrine, norepinephrine, thromboxane B_2 and 6- keto- prostaglandin F (Lorenzana *et al*., 1985; WHO, 1990).

5. Mechanism of action

Health risk associated with human exposure to trichothecenes is widely recognized (Montes *et al*., 2012). The risk depends on the degree they are consumed in a diversified diet (Montes *et al*., 2012). In order to protect consumers health, the European Commission has legislated maximum levels for trichothecenes in cereal grains, flours, and cereal-based products intended for human and animal consumption (Montes *et al*., 2012; European Commission, 2006). Trichothecenes initiate a wide range of toxic effects on farm animals and humans. (Haschek and Beasley, 2009) They causes of apoptosis and/or necrosis in the lymphoid, hematopoietic, and gastrointestinal systems resulting in leukopenia, vomiting, and diarrhea that can be

lethal (Haschek and Beasley, 2009) Alimentary toxic aleukia (ATA), a typical disease for human, was found to be associated primarily with the ingestion of moldy cereal infected with T-2 toxin (Joffe, 1974 and 1978). In addition, trichothecenes are toxic to the skin and testes (Wu *et.al*, 2010). Immune suppression and increased susceptibility to infection may occur, especially in the late phase of the disease (Wu *et.al*, 2010). The toxic effects from trichothecenes largely resemble those following radiation exposure (radiomimetic) due to their effects on rapidly dividing cells in the intestine, bone marrow, and testis (Haschek and Beasley, 2009). Trichothecenes have multiple effects on eukaryotic cells, including inhibition of protein, RNA and DNA synthesis, alteration of membrane structure and mitochondrial function, stimulation of lipid peroxidation, induction of programmed cell death or apoptosis, and activation of cytokines and chemokines. The effects of these toxin on eukaryotic cell function was shown in Table 3. Trichothecenes including deoxynivalenol (DON) are the most common group causing animal disease, effects range from feed refusal and vomiting to immunosuppression and loss of productivity (Viljoen, 2008). Although DON can be acutely lethal when ingested in large quantities, moderate- to low-level ingestion of the toxin can cause poor performance and altered immune function (Pier *et al*., 1980a, 1980b). Monogastric animals, particularly swine, exhibit the greatest sensitivity to DON, while chickens and turkeys, followed by ruminants, appear to have higher tolerance (Prelusky *et al*., 1994). The adverse effects of the trichothecenes mycotoxin in animals are show in Table 4.

Trichothecenes are potent inhibitors of protein synthesis due to binding to the 60S ribosomal unit and this is believed to be the main mechanism of toxicity. (Haschek and Beasley, 2009) Inhibition of protein synthesis occurs through interference with peptidyl transferase activity, with an intact C-9, 10 double bonds and the C-12, 13 epoxide are required for this inhibition. (Haschek and Beasley, 2009) They can also activate MAPKs and induce apoptosis in a process known as the ‘‘ribotoxic stress response’’ (Haschek and Beasley, 2009). In addition, trichothecenes can activate p38, Jun N-terminal kinase (JNK) and extracellular signal regulated kinase (ERK) MAPKs *in vitro* and *in vivo*. (Haschek and Beasley, 2009) They

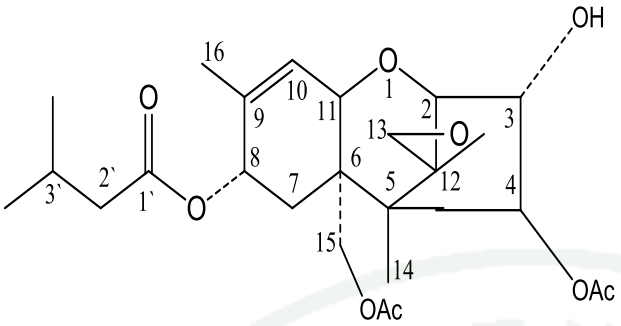
affecton rapidly dividing cells in the lymphoid, hematopoietic, and gastrointestinal systems (Haschek and Beasley, 2009).

6. Fusarenon-X

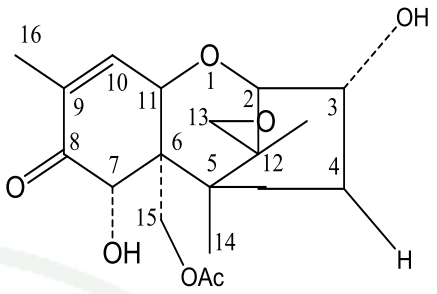
Fusarenon-X (FX; 3, 7, 15-trihydroxy-4-acetoxy-12, 13 epoxytrichothec-9-e-8-one), a type B trichothecene mycotoxin, is one of the 12, 13-epoxytrichothecenes mainly produced by *Fusarium crookwellense*, which naturally occurs in agricultural commodities such as wheat and barley (IARC, 1993; Poapolathep *et al*., 2008). Montes *et al*., (2012) found the contamination of FX in breakfast cereal. This was a first report of FX detection in food, especially in Spain (Montes *et al*., 2012). It has been reported that FX induced adverse health effects, particularly apoptosis, in organs containing actively dividing cells such as the small intestine, thymus, spleen, bone marrow, testes, reticulocytes, and mitogen-stimulated human lymphocytes, as observed in other trichothecenes (Ohta *et al*., 1978; Forsell and Pestka, 1985; Miura *et al*., 1998; Poapolathep *et al*., 2002). FX has been classified as the class 3 by IARC classification. For the definition of class 3 carcinoginicity refers to inadequate or limited evidence available for humans and animals. (IARC, 2011). Recently, the limited pharmacokinetic data are available for trichothecene mycotoxins in animals, especially for FX (Poapolathep *et al*, 2008). Poapolathep *et al*. (2008) reported the FX disposition fit an open 2-compartment pharmacokinetics model in broilers and ducks. FX can be metabolized to NIV via deacetylation in mice, excreted mainly in urine (Poapolathep *et al*, 2003). The liver and kidney are the organs responsible for the FX-to-NIV conversion (Poapolathep *et al*, 2003). The metabolic pathways of FX and NIV are shown on Figure 4.

In general, FX is well known to be cytotoxic to many types of mammalian cells (Miura *et al*., 2002). Although, FX has been observed to occur frequently with deoxynivalenol (DON; 3, 7, 15-trihydroxy-12, 13-epoxytrichothec-9-e-8-on) in agricultural products (Yoshizawa, 1983). The fate and disposition of FX in animals have not been extensively studied as DON (Popolathep *et al*, 2003). It is well known that species differences affect the fate of drugs and chemicals in animals (Walker,

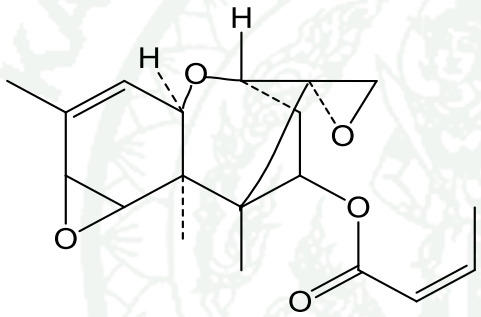
1980). In our previous investigation, we demonstrated that FX is rapidly converted to NIV in mice after po administration (Poapolathep *et al*., 2003, 2004). However, the previous findings in mice cannot be directly extraporated to other animal species. (Poapolathep *et al*., 2002, 2003) Not only FX is absorbed from the gastrointestinal tract rapidly and metabolized to NIV after being absorbed but also shown that the liver and kidney are the organs responsible for the FX- to- NIV conversion in mice (Poapolathep *et al*., 2003). In addition, FX can transfer to fetal mice via placenta (Poapolathep *et al*., 2004). Both *in vivo* and *in vitro* metabolisms of NIV and FX in female ICR mice were investigated by utilizing ^{3}H-NIV and ^{3}H-FX (Poapolathep *et al*., 2003). During *in vivo* study, a large proportion of ^{3}H-FX was found to be excreted as ^{3}H-NIV in urine and feces (Poapolathep *et al*., 2003). ^{3}H-NIV was mostly excreted in the unchanged form, except for an unknown metabolite (Poapolathep *et al*., 2003). FX can be metabolized to NIV via deacetylation in mice poultry and ducks, excreted mainly in urine (Poapolathep *et al*., 2003, 2008).



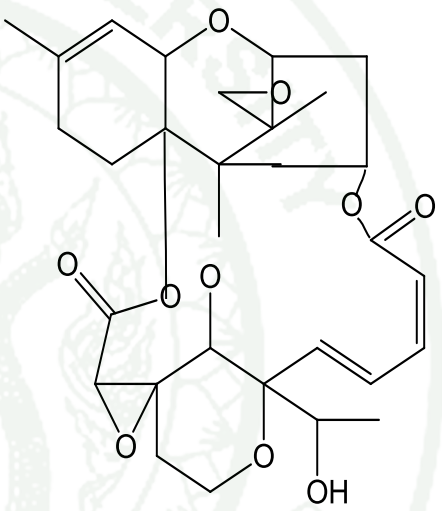
Group A: T-2 toxin



Group B: Deoxynivalenol



Group C: Crotocin



Group D: Satratoxin G

Figure 2 Chemical structures of Trichothecenes classification

Source: Wu *et al* (2010)

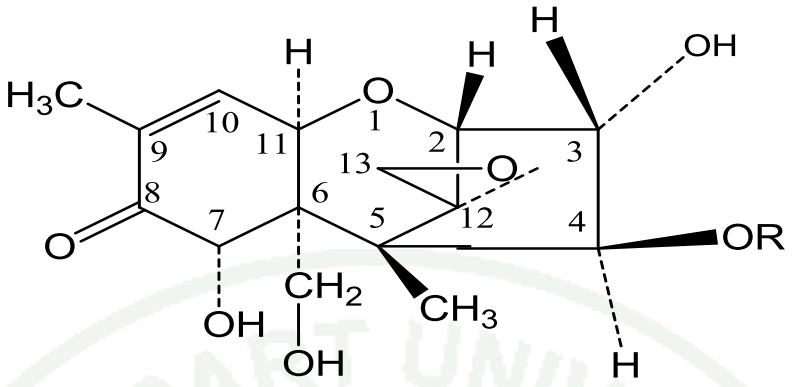


Figure 3 Chemical structures of Fusarenon-X (R= Ac)

Source: Poapolathep *et al* (2003)

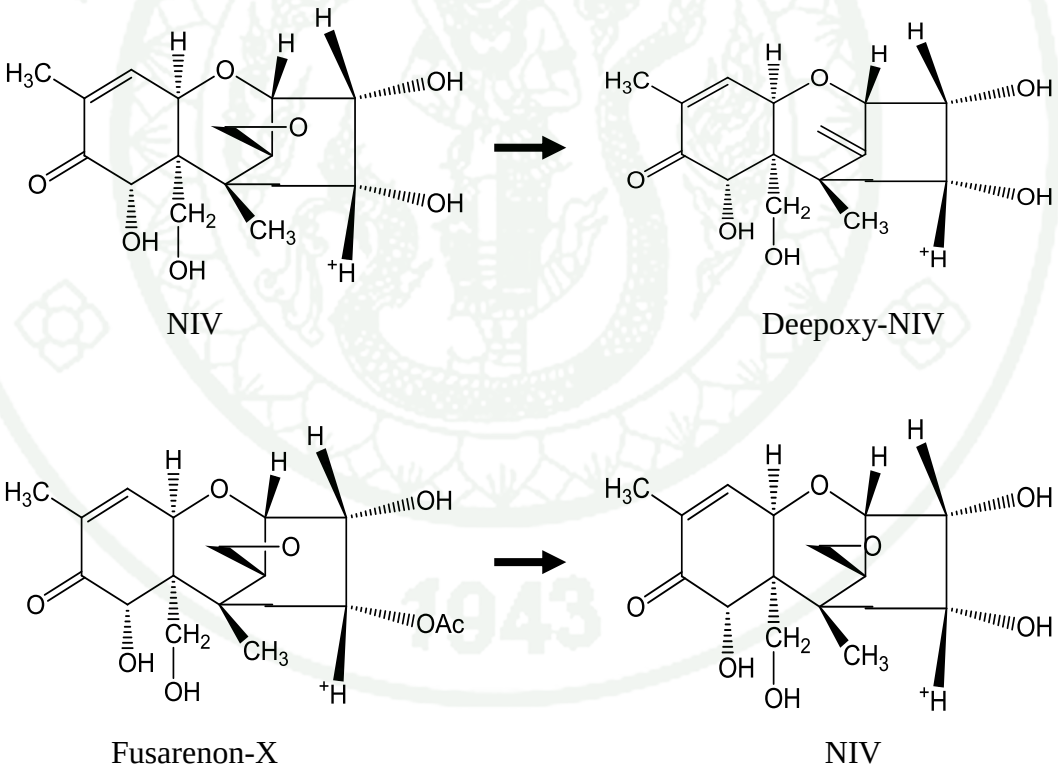


Figure 4 The metabolic pathways of FX and NIV in animals

Source: Wu *et al* (2010)

Table 2 The LD_{50} and lethal dose of trichothecene toxins and their comparative toxicity

Group	Fungi	Trichothecene	Acute LD_{50} Values (mg/kg)				Lethal dose (mg/kg)
			Mouse			Pig	
			iv or ip	po	Inhala tion	iv	inhalation
A	*Fusarium* spp.	T-2 toxin	3.0 – 5.3	3.8 – 10.5	0.16	1.21	1.5 – 3.0
		HT-2 toxin	6.5 – 9.0				
		Diacetoxyscirpenol (DAS)	9.6 – 23.0	15.5 – 46.0		0.37	
		Monoacetoxyscirpenol					
B	*Fusarium* spp.	Deoxynivalenol (DON, vomitoxin)	70.0 – 76.7	46.0			
		Nivalenol	4.0 - 6.3				
		Fusarenon-X	3.4	4.5			
C	*Cephalosporium* spp.	Crotocin	700 – 810	1,000			
D	*Myrothecium* spp.	Verrucarins					
		Verrucarin A and B	0.5(A)-7.0(B)				
		Roridins					
		Roridin A	1.0 (A)				
	Stachybotrys spp.	Satratoxins					

Source: Haschek and Beasley (2009)

Table 3 Trichothecene effects on eukaryotic cell functions

Compound	References
Inhibit protein synthesis	Ehrlich and Daigle (1987), McLaughlin *et al*. (1977)
Inhibit RNA and DNA synthesis	Rosenstein and Lafarge-Frayssinet (1983), Thompson and Wannemacher (1986)
Stimulate lipid peroxidation	Rizzo *et al*. (1994), Vila *et al*. (2002)
Alter cellular membrane function	Bunner and Morris (1988)
Inhibit mitochondrial and electron transport chain function	Pace *et al*. (1988)
Induce apoptosis	Pestka *et al*. (1994), Shinozuka *et al*. (1998), Islam *et al*. (1998)
Activate MAPKs	Zhou *et al*. (2005)
Modulate immune responses	Corrier (1991), Bondy and Pestka (2000)
Alter neurotransmitters	Prelusky *et al*. (1992), Swamy *et al*. (2004)
Induce gene expression of numerous chemokines and cytokines	Azcona-Olivera *et al*. (1995), Zhou *et al*. (1997), Moon and Pestka (2002), Ji *et al*. (1998), Pestka *et al*. (2005)

Source: Ramesh (2007)

Table 4 The adverse effects of the trichothecenes mycotoxins in animals

Compound	Effects
T-2 toxin	Feed refusal; weight loss; decreases red blood cell count; reduces leucocyte count; reproductive disorders; increases mortality of piglets after birth, reduces plasma glucose in piglets, pathological changes in liver and stomach, increases infection rate; alimentary toxic aleukia (ATA); induces apoptosis in the thymus and spleen; inhibits the synthesis of DNA and RNA
DON	Food refusal; vomiting; digestive disorders; weight loss; decreases levels of serum protein; oxidative stress and blood phagocytic activity in broilers; cytotoxic effect on human primary hepatocytes
NIV, FX	Gastrointestinal erosions; nephropathy; reduction of feed intake; cytotoxicity
DAS	Reduces feed intake and weight gain; oral lesions; gastrointestinal lesions; diarrhea

Source: Wu *et al.* (2010)

Table 5 Deoxynivalenol (DON) and T-2 toxicity in various animal species.

Animal species	Dose	Symptoms of toxicity	Reference
Pigs	10 ppm	Neutrophils affected	Takayama *et al*., 2005
	5.7 mg/kg	Elevated liver RNA concentration and increased protein Synthesis	Danicke *et al*., 2006
	0.21 ppm DON + 0.004 ppm ZON or 9.75 ppm DON + 0.358 ppm ZON in diet (sow)	Lesions in spleens and liver of sows No pathological lesions in piglet	Tiemann *et al*., 2008
Poultry	4 or 16 mg/kg diet (7 day oldchicks)	Reduced body weight and feed intake, plaque formation and buccal ulceration	Hoerr *et al*., 1982
Ruminants	15.6 mg/kg DON (sheep)	No effects for 28 days Weight loss (extended feeding)	Harvey *et al*., 1986
	10 - 20 mg/kg T-2 toxin in feed (calves)	Sloughing of papilla and omasum ulceration	Cheeke *et al*., 1998a
	3-5 ppm (dairy cow)	Reduced IgA , serum albumin and globulin	Korosteleva *et al*., 2007

Source: Upadhaya *et al*. (2010)

7. Apoptosis

There are many terms that have the meaning of cell death (Table 6.)

Table 6 Development of cell death concept

Terms	B.C.	References
Necrosis	1665	Robert Hook, light microscope
Necrobiosis, Degeneration	1858	Virchow's Lecture XV
	1858	Gerlach, carmine staining
Cosgulation necrosis (Diptheria)	1877	Weigert and Cohnheim, infiltrate of leukocytes and fibrin
Karyolysis, Karyorhexis	1879	Klebs
Spontaneous cell death, Chromatolysis (ovary)	1885	Flemming: chromatin, mitosis
Chromatin margination	1890	Arnheim
Pyknosis	1894	Schmaus and Albrecht
Autolysis	1900	Jacoby
Oncosis	1910	Von Recklinghausen
Amitosis (yolk sac)	1914	Graper: shrinkage, nuclear division
	1914-1918	World War I
	1938	Ruska, TEM
Physiological cell death (embryo)	1950	Glucksmann: nuclear pyknotic granules, phagocytosis by neighbor 1950s lysosome
Cell suicide	1950s	De Duve
Accidental cell death	1958	Bessis
Programmed cell death	1964	Lockshin and Williams
Apoptosis	1972	Kerr
	1984	Wyllie et al: DNA ladder
Postapoptotic necrosis	1990	Sauders and Fallon

Source : Yoshinori *et al*. (2003)

8. Apoptosis and necrosis

It is generally accepted that there are two principal modes of cell death: apoptosis and necrosis. Apoptosis is defined using several ultrastructural characteristics including condensation of chromatin, cell shrinkage, budding and formation of apoptotic bodies. These morphological changes are mainly observed as nuclear changes, but not cytoplasmic changes. In contrast to apoptosis, necrosis is a general term for cell death. It has been understood as cell death accompanied by cytoplasmic changes including intracellular organelles and cell membrane degeneration, cell swelling, blebbing and inflammatory cells infiltration. Morover, the necrosis has not been defined based on the ultrastructural change. The structures of intracellular organelles such as mitochondria are well preserved during apoptosis, whereas during necrosis can be occured the degeneration of organelles. The mode of cell death might be determined by the concentration of intracellular adenosine triphosphate (ATP). ATP can be generated either via glycolysis or via mitochondria that is required for the induction of apoptosis. The fate of cell death may depend on the intensity of stimuli and/or the concentration of intracellular ATP (Liest *et al.*, 1997; Nicotera and Leist, 1997; Vander-Heiden *et al.*, 1997; Yoshinori *et al*., 2003).

9. Tumor necrosis factor (TNF) and TNF receptor (TNFR) family

Each of the ligands is a type II membrane protein. They are characterized by the confinement of the C-terminus that have 150-amino acid region within the extracellular space. This region is actually the hallmark of the ligand family and bind to their receptors. Although these proteins exist as trimeric or multimeric membrane bound proteins however there are some members such as TNF and FasL that function in a soluble form. There are many ligands that interact with their receptors such as TNFR1, TNFR2, CD40 and Fas. They are type I membrane proteins and are grouped due to the presence of conserved cystein residues in the extracellular ligand domain. Most receptors are found in the soluble form. The proteolytic cleavage is a process that cleaved TNFR1, TNFR2, CD40 and Fas to soluble form. (Locksley *et al.*, 2001; Itoh *et al.*, 1991; Yoshinori *et al.*, 2003).

10. Apoptotic signaling

There are two major apoptotic pathways in mammalian cells: Death signal-induced, death receptor-mediated, extrinsic pathway and stress-induced, mitochondrion-mediated, intrinsic pathway. In the death receptor-mediated pathway, binding of FasL and TNF to their specific receptors lining on the cell membrane Fas and TNFR1 induces receptor clustering through Fas-associated death domain (FADD) or TNFR-associated death domain (TRADD), respectively. FADD and TRADD can be binded with procaspase-8/-10 to form death-inducing signal complex (DISC) resulting in caspase-8/-10 activation. The activated caspase-8 cleaves procaspase-3 to active form (Figure 5). The mitochondrion-mediated pathway is used extensively for responding to both extracellular and internal stimuli especially DNA damage. Proapoptotic proteins including Bid, Bax and Bak that present in the cytoplasm of mitochondria can cause the changing of mitochondrial inner transmembrane potential or permeability transition (PT) resulting in opening of mitochondrion permeability transition pores (MPTPs). MPTPs are divided into two types; one is adenine nucleotide translocator (ANT) found in inner mitochondrial membrane. They function as transmembrane channel to transport ATP-ADP antiport. The other voltage-dependent anion channel (VDAC) or porin that found in outer mitochondrial membrane. They function as non-selective pore. When MPTPs open, the release of cytochrom c, a critical step in this pathway, to the cytosal and combines with cytosolic deoxyadenosine triphosphate (ATP) , apoptotic protease activation factor-1 (Apaf-1) and procaspase-9 to form apoptosome. This complex activate procaspase-9 to form activated caspase-9 that in turn cleaves procaspase-3 to generate active form. Both the extrinsic and intrinsic apoptotic pathways involve the activation of caspase-3. Downstream of caspase-3 can produce products of actin, DNase and lamin degradation. They include apoptotis- characteristic appearances such as DNA fragmentation and chromatin condensation (Figure 5) (Akao *et al.*, 1994; Arnoult *et al*., 2003; Bauer *et al*., 1999; Beutner *et al*., 1998; Cohen., 1997; Cowling *et al*., 2002; Elmore., 2007; Fan *et al*., 2001,2005; Fu *et al*., 2002; Li *et al.*, 1997; Lü et al., 2003; Wang *et al*., 2001,2005; Zhivotovsky and Kroemer., 2004).

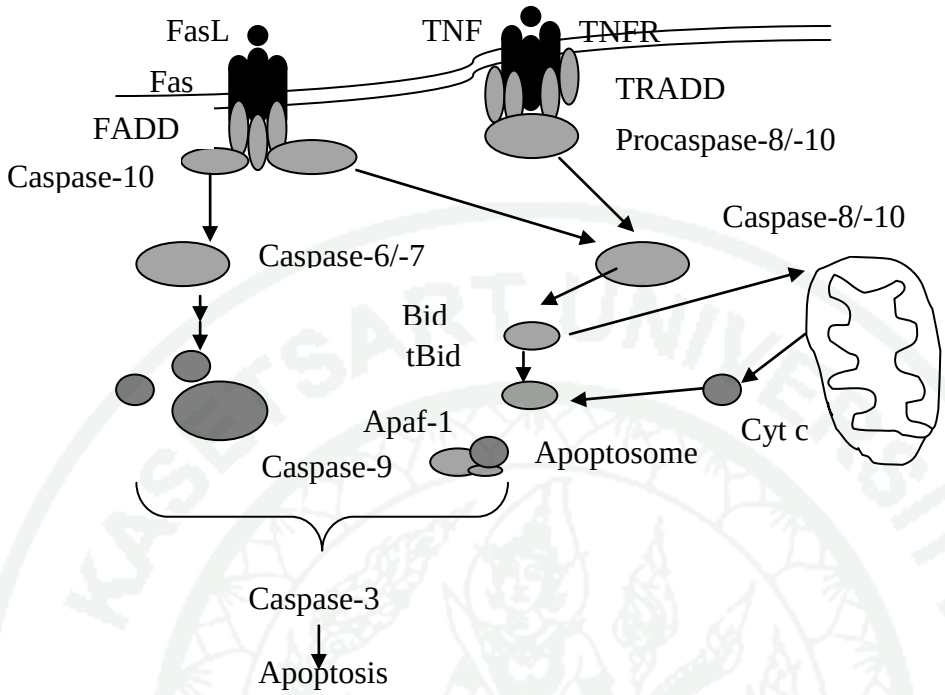


Figure 5 Death receptor- and mitochondrion -mediated pathway

Source: Modified from Fan *et al*. (2005)

11. Methods of morphological detection of apoptosis

11.1 Transmission electron microscope (TEM)

TEM is the most reliable method for detecting apoptosis because apoptosis is defined based on several ultrastructural characteristic changes. Moreover, various materials including fixed and/or living cells can be used for TEM. Several types in the morphology of apoptosis can occur in different cell types and tissues since the fate of apoptotic cells is tissue specific. Although TEM is the best method to detect apoptosis, a few limitations for detecting apoptosis can be pointed out. First, the terminal stage of apoptosis can be detected by TEM. Therefore, TEM cannot classify apoptotic cells to any stage of apoptosis. Second, a condensed chromatin cannot be indicated that nuclear parts contain many fragmented DNA so condensed chromatin and fragmented DNA are independent events of apoptosis detected by TEM. Third, apoptosis can occur in only 2-3% of cells at any one time that have a

tissue regression rate of 25% per day. Fourth, a preparation of ultra-thin sections that used in TEM must require the men who have high skill. (Bursch *et al.*, 1990a,1990b; Kumagai *et al.*, 2001; Yoshinori *et al.*, 2003)

11.2 Histochemical dye staining

HE staining is a useful method for apoptotic detection in a single layer of cells because it provides the information about cell size, contour and chromatin condensation or pyknotic nucleus. However, it is inadequate for use in paraffin-embedded tissue sections and leads to misinterpret the infiltration of lymphocyte as apoptotic bodies since it is very difficult to distinguish between lymphocytes and condensed-chromatin-rich nuclei of apoptotic bodies in thick sections. Epoxy-resin-embedded sections can be clearly showed the contour of cells at a high resolution therefore they can be stained with toluidine blue to difine lymphocytes from apoptotic bodies. (Yoshinori *et al.*, 2003)

11.3 Fluorescence dye staining

Several fluorescence dyes, such as Hoechst 33342 and propidium iodide (PI), specifically bind to DNA and provide a reliable image of chromatin condensation by fluorescence microscopy. Both Hoechst 33342 and PI are often used for the discrimination of dead cells; Hoechst 33342 stains all nuclei and PI stains only nuclei of cells with disrupted membrane integrity. When cells were double-stained with Hoechst 33342 and PI, almost all cells can be classified into the following four groups: viable cells with Hoechst 33342-positive intact nuclei, necrotic cells with H-positive nuclei, early-stage apoptot- ic cells with Hoechst 33342-positive fragmented nuclei, and terminal stage apoptotic cells with PI-positive fragmented nuclei. Phosphatidylserine in healthy cells is located in the interior side of the plasma membrane, but within 1 hr after treatment of Jurkat cells with anti-Fas IgM, phosphatidylserine is redistributed on the cell surface. On the other hand, annexin V, which has a strong anticoagulation activity, is a member of a protein family that has a high Ca^{2+}- dependent affinity for aminophospholipids and therefore, shows a high

affinity for phosphatidylserine. Recently, annexin V labeled with fluorescein isothiocyanate (FITC) is used as a probe for apoptosis induced in various cell lines. Early-stage apoptotic cells are generally stained with annexin V-FITC, but not with PI. Apoptotic cells during the middle stages are stained with the Hoechst 33342 dye, indicating the onset of nuclear fragmentation. During the later stages of apoptosis, PI can be readily transported across cell membranes because of the disrupted membrane integrity. Thus, while early- to middle- stage apoptotic cells are stained green by annexin V on the cell surface, the late-stage, or necrotic cells can be identified by their concurrent intracellular yellow-red staining . Moreover, fluorescence microscopy using both TUNEL-FITC and autofluorescence of methyl green, which binds to double-stranded DNA, clearly reveals the topographic distribution of both free 3'-OH ends of DNA and double-stranded DNA, respectively. Fluorescence dyes are applied not only to fluorescence microscopy but also to flowcytometry. The advantage of flowcytometry is that it enables the determination of various characteristics, such as the size of cells, DNA fragmentation and externalization of phosphatidylserine, in many cells in a short time. However,researchers must pay attention to the following points; the ratio of apoptosis detected by flowcytometry is always higher than that detected by microscopy, because flowcytometry counts each apoptotic body as one apoptotic cell. (Ito *et al.,* 1998; Martin *et al.,* 1995; Shimuzu *et al.,* 1996, Yoshinori *et al.,* 2003).

11.4 TUNEL staining

The TUNEL method using LM, TEM and confocal laser microscopy was developed for the detection of free 3'-OH DNA ends. They are occurred as a nuclear condensed chromatin of apoptotic cells in tissue sections and cell cultures, because many new 3'-OH DNA ends are generated by DNA fragmentation in apoptosis. The TUNEL method offers considerable advantages in the discrimination of apoptotic cells from many cells. First, the TUNELmethod is applicable to any kinds of material: cultured cells, tissues and blood samples. Second, DNA fragmentation is suited for both staining by the TUNEL and DNA laddering. When a large percentage of cells in a tissue are apoptotic, electrophoresis of extracted DNA can be used to validate them.

In contrast, the TUNEL method can more easily detect apoptotic cells than electrophoresis of extracted DNA, when a tissue contains a few apoptotic cells. Third, it can be topographically visualized the location of individual TUNEL-positive cells in materials. Lastly, TUNEL can detect apoptotic cells at the relatively early stage of apoptosis, because the increase in the number of sites of free- 3'-OH DNA ends yielded during the apoptotic process precedes morphological changes of apoptosis However, some researchers have reported that the TUNEL method labeled not only newly formed DNA ends in apoptosis but also the sites of DNA breaks digested in necrosis (Ansali *et al.,* 1993; Arends *et al.,* 1990; Burschuh *et al.,* 1990a, b; Gold *et al.,* 1993; Gorczyca *et al.,* 1993; Inoki *et al.,* 1997; Ito and Otsuki., 1998; Migheli *et al.,* 1995; Thiry., 1991; Wijsman *et al.,* 1993; Yoshinori *et al.,* 2003).

11.4.1 LM

The original TUNEL method was first reported by Gavrieli *et al.,* (1992)The residues of biotinylated dUTP are catalytically added to the DNA by TdT. The TdT catalyzes a template- independent incorporation of deoxynucleotide triphosphate to the 3'-OH DNA ends of double- or single-stranded DNA. The incorporated nucleotides form a polymer of biotin-11-dUTP, at a ratio that has been optimized for extra-avidin peroxidase binding at the reaction site. The localized peroxidase enzyme then catalytically generates an intense signal from chromogenic substances. This molecular, biological histochemical staining allows for sensitive and specific staining of very high concentration 3'-OH DNA ends that are localized in apoptotic cells. Biotin is a vitamin B 2 complex and is contained in various organs, particularly in kidney at high levels. Therefore, the original TUNEL method may show a false-positive reactivity. For this reason, a digoxigenin nucleotide is used, instead of the biotinylated nucleotide, because digoxigenin is derived from the digitalis plant and immunohistochemically similar ligands for binding of the anti-digoxigenin antibody are generally insignificant in animal tissues, ensuring a low background staining. (Yoshinori *et al.,* 2003)

11.4.2 TEM

The procedure of TUNEL that used with the TEM technique is fundamentally technique. It is the same as TUNEL using LM technique, except that nucleotide incorporation is accomplished using immunogold particles, instead of the immunoperoxidase used in LM. The detailed procedures of the TUNEL using the TEM technique have been reported elsewhere. Briefly, all procedures of the TUNEL using TEM are carried out, floating the surface of grids with ultrathin sections on droplets of reagents. The TUNEL using the TEM technique with immunogold staining has some advantages for both the detection of apoptotic cells and semiquantification of free 3'-OH DNA ends. First, the TUNEL using TEM enables to determine of the apoptotic stages. The increase in the number of sites of newly formed free 3'-OH DNA ends precedes morphological changes of apoptosis. The TUNEL using TEM clearly shows apoptotic cells in the early stage, the moderately increased number of incorporated immunogold particles at the initial chromatin condensation and at the late stage, the typical chromatin condensation with a large number of immunogold particles in the nuclei. Second, in situ end-labeling (ISEL)/TEM technique yields objective data through image analysis for quantitative changes of 3'-OH DNA ends in each apoptotic or necrotic cell. (Inoki *et al.*, 1997; Migheli *et al.*, 1995; Thiry, 1991).

11.5 Flow cytometry

In flow cytometry, a suspension of cells is passed through a highly focused beam of laser light. Flow cytometry measures both the deviation of light (light scattering) as afunction of size and optical properties of the cells as well as the absorption/emission of fluorescence from cell components that were stained with a fluorescent dye. One of the characteristics of early apoptosis is cell shrinkage, which can be detected based on the degree of light scattering in the laser beam in flow cyctometry; measures of forward and side light scatters, which are indicators of cell size and cell structure, respectively, decrease in cells undergoing apoptosis. Initially, forward scatter is markedly decreased in early apoptotic cells while a transient increase in side scatter is observed. However, side-scatter signals eventually decrease due to the depletion of apoptotic bodies. The intensity of both the forward- and side-

scatter signals is decreased, suggesting the cells were relatively in late apoptosis. DNA histograms are the most common method of identification and quantification of apoptosis. DNA fragmentation is a feature of apoptotic cells thus the DNA contents in the sub-G1 compartment are smaller and of lower molecular weight than those in G1. Flow cytometry has the advantages of simplicity and low cost, making it an excellent method for screening large numbers of samples for apoptotic cells in terms of percentages. A flow cytometry is able to discriminate not only viable cells from dying cells, but also apoptic cells from necrotic cells using appropriate markers. Various apoptotic markers are applicable to flow cytometry. For example, phosphatidylserine is normally confined to the inner leaflet of the plasma membrane, but this is externalized during apoptosis inmany cell types. Annexin V, which has an affinity to phosphatidylserine, has also shown applicability as a probe to monitor changes in the distribution of phosphatidylserine in the plasma membrane during apoptosis. A microscope-based laser scanning cytometer (LSC) has recently been developed. The LSC is a microscope with a cytofluorometer that combines the multiple advantages of flow cytometry with confirmational analysis of cell morphology. Furthermore, LSC was reported to be a rapid and useful technique for studying many types of stimuli that lead to apoptotic cell death in tissues in vivo as well as in cell culture (DarzynkiewiczA *et al.*, 1998, 1999; Fadok *et al.*, 1992; Laerum, 1991; Pattorossi *et al.*, 2000; Salzman *et al.*, 1990; Vermes *et al.*, 1995; Verdguer *et al.*, 2002; Zhang *et al.*, 1997).

12. Biochemical methods for detecting apoptosis

12.1 Caspase activity

The discovery of the gene CED-3 in the nematode *C. elegans* is the first implicated in apoptosis. It is associated with caspase acitivity. Caspases are a family of cysteine proteases. The term 'caspase' was coined to reflect a cysteine protease mechanism ('c') specific for aspartate ('aspase'). They can be discriminately cleaved only after an aspartic acid moiety rather than randomly destroy proteins during apoptosis. They act together in apoptotic cascade that triggered by various stimuli

and terminated the cleavage of protein in the cell. Mammalian apoptosis can be divided into two major pathways: a death receptor pathway and mitochondrial pathway. Procaspase-8 are activated by Fas-associated death domain (FADD) in a Fas-mediated death receptor pathway and procaspase-9 are stimulated by both cyctochrome c and Apaf-1 in a mitochondrial pathway. Consequently, caspase-3 (previously termed CPP32, Yama or Apopain) is activated and resulting to the activation of caspase-activated DNase (CAD), that participates in DNA fragmentation. Activated caspase-3 also destroys lamin and actin, resulting in chromatin condensation and budding, respectively. Caspase-3 is thus the effector caspase largely responsible for the morphological and biochemical cellular changes that are the hallmarks of apoptosis. (Dales *et al.,* 2001; Enari *et al.,* 1996; Fernandes-Alnemri et al., 1994; Hengartner., 2000 ; Nicholson *et al.,* 1995; Tewari *et al.,* 1995).

12.2 Colorimetric assay

The peptide DEVD (Asp-Glu-Val-Asp) is the cleavage site for capsase-3. It is indicated by the sequence of the cleavage site of poly ADP-ribose polymerase (PARP). Caspase-3 activity is analyzed by conjugating with a colorimetric or fluorometric moiety to DEVD. The colorimetric assay measures light emission of p-nitroanilide (pNA) at 405 nm after DEVD-pNA cleavage. Although anticaspase antibodies are commercially available however the active forms are more significant for apoptotic induction. Western blotting analysis can determine the relative size of caspases that cleaved from procapases whereas immunohistochemistry cannot always discriminate the specific caspase based on the antibodies used. Therefore, the use of combination between colorimetric/fluorometric assays with Western blotting analysis should yield more reliable data. (Enari *et al.,* 1996; Gurtu *et al.* 1997; Los *et al.,* 1995; Nicholson *et al.,* 1995).

13. Molecular biological methods for detecting apoptosis

13.1 DNA agarose gel electrophoresis

Internucleosomal DNA fragmentation resulting from endonuclease digestion are one of the characteristic features of apoptosis. They are about 180-200 bp in length and can be visualized as a DNA ladder on an agarose gel stained with ethidium bromide under UV light. Due to its low sensitivity, a large number of cells and/or high apoptotic rates can be visualized using adequate ethidium bromide staining. However, this method will be able to detect the fragmentation of DNA only when apoptosis occurs at a rate of more than 20 % of the total cell population. In addition, it is the most convenient method because it does save time and manpower for detecting DNA ladder in vitro. (Yoshinori *et al.*, 2003)

13.2 Western blotting

Western blotting is a very useful and commonly employed immunochemical techniques. It is also referred to simply as immunoblotting. Equal amounts of proteins from cells or tissues are separated by SDS-PAGE and electrophoretically transferred from the gel. These membrane are made of nitrocellulose, nylon, or poly vinilidene difluoride (PVDF) and probed with antibodies. Primary polyclonal or monoclonal antibodies react with specifically antigenic epitopes. These epitopes are presented by target proteins that attached to the support membrane. A secondary antibody, conjugated with HRP or alkaline phosphatase, is then applied and binds to the primary antibody-antigen complex. Chromogenic or luminescent substrates are used to visualizethe bound components. Common chromogens are used as 3,3',5,5'-tetramethylbenzidine and DAB. Visualization methods using luminescent substrates are referred to as chemiluminescense. They are usually employed either a luminol substrate for HRP or 1,2-dioxetane phosphates for alkaline phosphatase. Chemiluminescent visualization frequently has better sensitivity than chromogenic or radioisotope labeling methods because they can be allowed for reprobing of the same membrane-bound proteins after rinsing with 0.2 M NaOH. Moreover, a chromogenic substrate can also be applied after using the chemiluminescent reaction. Internal controls such as β-actin should be employed to ensure that samples contain equal amounts of protein. Although, Western blotting are specifically detected and quantified the protein of

interest however it does not detect distribution patterns of the protein *in situ*. Immunohistochemistry are performed to combination enables quantification of levels of protein expression in cells/ tissues and localization of protein(s) of interest, but it can not differentiate sizes of proteins. Therefore, a combination of Western blots and immunohistochemistry is recommended. (Gallagher *et al.,* 1997; Shibata *et al.,* 1999; Yoshinori *et al.,* 2003).

13.3 Real-Time PCR

The detection and amplification of specific mRNAs are one major application of Real-Time PCR. The quantity of obtained RNA was frequently limited and often insufficient for Northern blot analysis. Recently, quantitative Real-Time PCR is being used increasingly as an alternative method to Northern blot analysis or the RNase protection assay for gene expression. It has become a rapid, sensitive, and specific technique that widely used in laboratories to investigate genetic disorders and a variety of human pathogens for providing prognostic and diagnostic information. Preceding the good quality cDNA synthesis that isolated intact (undegraded) total RNA from cultured cells or tissues is one of the most important steps. Isolated RNA are frequently contained genomic DNA. They would be subsequently amplified together with the target cDNA. However, genomic DNA removal can be facilitated by DNase I digestion. The first strand of cDNA is then generated by RT (without RNase H activity) using oligo(dT) primers, random hexamer primers, or gene-specific primers. Incubation with RNase H then digests mRNA templates. The target cDNA is subsequently amplified using two gene-specific primers and Taq DNA polymerase.
Primers are chosen based on the gene of interest. Internal control genes such as 13-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are run separately to validate the PCR conditions. However, it was recently reported that an internal control gene can be coamplified in the same reaction as the target cDNA; this technique is called competitive RT-PCR Roche Molecular Biochemicals of Germany has recently been developed an optimized form of PCR for rapid analysis. They combine between a thermal cycler and a fluorometric component which is called the LightCycler. This instrument can be detected the amplified PCR products through two fluorophores; the

donor fluorophore and an acceptor fluorophore. They can be coupled to two sequence-specificoligonucleotide hybidization probes. After excitation by the donor fluorophore, the acceptor fluorophore are emitted the light at a defined wavelength based on the theory of fluorescence resonance energy transfer (FRET). The LightCycler can be used toquantify gene expression and to detect gene mutation that is applicable to study of apoptosis. (Fille *et al.*, 1997; Powel *et al.* 1987; Yoshinori *et al.*, 2003)

14. Gene transfer for apoptotic induction

The selection of the best transfection method is critical to maximizing gene transfer to a cell lines. Transfection methods include mediation by calcium phosphate, diethylaminoethyl (DEAE)-dextran, and liposomes, and gene transfer through viruses and electroporation. In general, DEAE-dextran- and liposome-mediated transfection and viral gene transfer are recommended for adherent cell lines, while liposome mediation and electroporation are more effective for nonadherent cell lines. The overexpression of proapoptotic proteins have been reported to apply for inducing therapeutic apoptosis in cancers. For example, the transfection of proapoptotic members of the Bcl-2 family (bax or bak gene) in various types of carcinoma cells both *in vitro* and *in vivo* are shown high levels of apoptosis. In addition,introduction of caspase genes have been shown to inhibit the growth of malignant glioma cells through induction of apoptosis in both *in vitro* and *in vivo* systems (Cull *et al.*, 1998; Kiefer *et al.*, 1995; Kingston *et al.*, 1996; Kondo *et al.*, 1995; Nishimura *et al.*, 2001; Oltavi *et al.*, 1993; Orth and Dixit, 1997; Reed, 1994).

MATERIALS AND METHODS

Materials

1. Standard fusarenon-X (Wako® company)
2. 10%Dimethyl sulfloxide (DMSO)
3. 0.9% normal saline
4. 10% neutral buffered formalin
5. Hematoxylin and eosin (HE)
6. Methyl green
7. 2.5% glutaraldehide
8. 0.1 M phosphate buffer (pH 7.4)
9. 1% osmium tetroxide
10. Uranyl acetate
11. Lead citrate
12. Digoxygenin-dUTP
13. Terminal deoxynucleotidyl tranferase (TdT)
14. Peroxidase-conjugated anti-digoxygenin antibody
15. Peroxidase-diaminobenzidine
16. Protinase K
17. Tris acetate-EDTA buffer
18. 2% agarose gel
19. Ethidium bromide
20. Monoclonal anti-PCNA mouse antibody (clone PC10; DAKO, Denmark)
21. Biotinylated anti-mouse IgG antibody
22. Normal horse serum
23. Deionized distrilled water
24. Methanol
25. Ether
26. Xylene

27. Absolute ethyl alcohol
28. 95% ethyl alcohol
29. 80% ethyl alcohol
30. 70% ethyl alcohol
31. Citric acid (pH 6)
32. 30% hydrogen peroxide
33. AxyPrep Multisource Total RNA Miniprep Kit (Axygen Biosciences)
34. Hard paraffin
35. Feeding tube
36. Tuberculin syringe
37. Stainless scissor
38. Stainless forcep
39. Knife
40. Plastic plate
41. Needle No 22
42. Tissue processor
43. Embeder
44. Microtome
45. Coating slide
46. Glass slide
47. Cover slip
48. Water bath
49. Hot plate
50. Light microscope
51. Electron microscope
52. Centrifuge
53. Vortex mixer
54. Incubator
55. Autopipette
56. Pipette tip
57. Eppendroff tube

58. Cooler box
59. Surgical glove
60. pH meter
61. White box
62. Fume hood
63. 4ºC refrigerator
64. -20ºC refrigerator
65. -80ºC refrigerator
66. PCR machine
67. Real-Time PCR machine
68. UV light transilluminator
69. Autoclave
70. Microarray machine

Methods

1. Animals

Forty pregnant female ICR mice (7^{th} day of pregnancy) were purchased from National Laboratory Animal Center, Mahidol University. The mice were housed in individual stainless-steel cages in an air-conditioned animal room with a cycle 12-h-light/12-h-dark cycle at the Laboratory Animal Unit, Faculty of Veterinary Medicine, Kasetsart University, and were acclimatized to the environment for 1 week. The experimental animals were fed commercial feed pellets and had accessed to water *ad libitum* with both being free of FX throughout the experiments. All experimental procedures performed on the animals were approved by the Animal Ethics Research Committee of the Faculty of Veterinary Medicine and the Kasetsart University Research and Development Institute.

2. Toxin preparation

Standard Fusarenon-X (FX, lot no. TCR 7665) was purchased from Wako Pure Chemical Indrustries Ltd. (Kyoto, Japan) and it was dissolved with 10% dimethyl sulfloxide (DMSO) in 0.9% normal saline (NS) to the final concentration of 2 mg/ml for oral administration.

3. Experimental design

FX was orally administered to twenty pregnant mice on the 14^{th} day of pregnancy at a dose of 3.5 mg/kg b.w. The dose level of FX used in this study was selected based on a preliminary study, in which two dosage of FX at 1.75 and 3.5 mg/kg b.w. were examined in pregnant mice, and we found that the high dosage of 3.5 mg/kg b.w. of FX could induce apoptosis in developing mouse brain. Thus, the dosage of 3.5 mg/kg b.w. of FX was selected. Fifteen pregnant mice in each treatment group were sacrificed by heart puncture under carbon dioxide anesthesia at 12, 24 and 48 hours after treatment (HAT). The remaining five mice in the treatment group were

sacrificed by heart puncture under carbon dioxide anesthesia at 12 HAT to investigate gene expression. The remaining twenty mice were administered 10% DMSO in 0.9% NS and sacrificed at each time of treatment to serve as controls. After euthanization, the fetuses of each dam were immediately collected in 10% neutral buffered formalin and liquid nitrogen.

To indicate FX can induce apoptosis in the developing mouse brain from FX-treated dams.

4. Histological examination

Five fetuses from each litter were fixed in 10% neutral buffered formalin. Duplicated paraffin sections of each fetus were stained with hematoxylin and eosin (HE) and observed under a light microscope. The paraffin sections were subjected to *in situ* detection of fragmented DNA and immunohistochemical staining for proliferating cell nuclear antigen (PCNA) as described below.

5. Electron microscopic examination

For electron microscopy, the fetal brain samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer saline (PBS) pH 7.4, post-fixed in 1% osmium tetroxide in the same PBS, and embedded. Ultrathin sections were double stained with uranyl acetate and lead citrate and observed under an transmission electron microscope.

6. *In situ* detection of fragmented DNA

In situ detection of fragmented DNA was performed on the paraffin sections using the modified TUNEL method with a commercial apoptosis detection kit (ApopTag®Peroxidase *In Situ* Apoptosis Detection Kit; Millipore Inc. Canada). The procedure was performed as follows: the sections were deparaffined twice with xylene and were rehydrated in a graded series of alcohol solution: 100% (twice), 95%

(twice), and 70% (once), respectively. Next, the sections were washed with PBS and pretreated by microwaving once at 800 watts for 5 minutes (min) in citric acid, at pH 6.0. The sections were allowed to cool for 20 min at room temperature (RT). The sections were then washed with PBS, and the endogenouse peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at RT. The slides were washed with PBS and incubated in equilibration buffer for at least 20 seconds and then immediately incubated in a prepared deoxygenin labeled-terminal deoxynucleotidyl transferase solution. The sections were covered with film, placed in a humidified box and incubated for 60 min at 37°C. After incubation, the film was removed and the sections were incubated in stop wash buffer for 10 min at RT. Next, the sections were washed with PBS and incubated in anti-deoxygenin-labeled peroxidase. The slides were covered with film, placed in a humidified box and incubated for an additional 30 min at RT. After incubation, the film were removed, washed with PBS and incubated in a diaminobenzidine solution for 5 min at RT. The sections were then counterstained with hematoxylin and apoptotic nuclei were stained dark brown. The apoptotic index (positive cells/100 neuron cells×100) was calculated for each section.

7. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA)

Immunohistochemical staining for PCNA was performed on the paraffin-embedded sections to evaluate the proliferative activity of the cells in the brain using the avidin-biotin-peroxidase complex (ABC) method and VECTASTAIN ABC kit (Vector Laboratories, USA). Monoclonal mouse anti-PCNA antibody (clone PC10; DAKO, Denmark) was used as the primary antibody. The secondary antibody was biotinylated anti-mouse IgG antibody that was obtained from VECTASTAIN ABC kit (Vector Laboratories, USA). PCNA-positive nuclei were visualized by peroxidase-diaminobenzidine (DAB) reaction. Counterstaining was performed with hematoxylin. The PCNA index (positive cells/100 neurons ×100) was calculated for each section.

To investigate the relationship between the genetic regulatory mechanisms and FX-induced apoptosis in mouse brain.

8. DNA extraction

Five randomly selected fetal liver samples from each dam were extracted using the Wizard®Genomic DNA purification kit (Promega,USA). The samples were lyzed with 600µl chilled nuclei lysis solution and immediately incubated at 65°C for 30 min. After lysis, 3µl RNase solution were added to the samples and further incubated at 37°C for 30 min. Proteins were precipitated by adding 200µl of protein precipitation solution, then vortexed, and finally centrifuged for 4 min at 16000xg. The supernatant was transferred into a fresh microcentrifuge tube containing 600µl of isopropanol, mixed, and then centrifuged for 1 min at 16000xg to precipitate the DNA. The DNA was washed once in 600µl 70% ethanol. The ethanol was removed and the DNA pellet was air-dried for 15 min, and then rehydrated in DNA rehydration solution at 65°C for 1 h. The DNA was immediately used for PCR amplification. For quality and quantity control, the sample concentrations were measured using a NanoDrop®Spectrophotometer ND-1000.

9. PCR amplification

An amount of 20µl PCR reaction mixture was used. It included 1 µg of DNA, 10µl SapphireAmp Fast PCR Master Mix (2x premix,Takara, Japan), 6.8µl deionized distilled water (DDW), and 1.2µl mouse specific primer. For male specific Sry primers;FSry-5'TGGGACTGGTGACAATTGTC-3'andRSry-5-GAGTACAGGTGT GCAGCTCT-3') were used (Lambert et al,2000). For the mouse IL3 gene; FIL3-5'-GGGACTCCAAGCTTCAATCA-3'andRIL3-5'-TGGAGGAGGAAGAAAAGCAA-3') were used (Lambert et al,2000). A thermal cycler (iCycler®, Bio-Rad, CA) was used with the following PCR conditions: 94°C for 1 min followed by 30 cycles of 95°C for 5 sec, 50°C for 5 sec and 72°C for 10 sec. PCR products were terminated with a final extension at 72°C for 7 min.

10. Gel electrophoresis

A 5µl aliquot of each PCR product was loaded on 2% agarose gel (Lonza, Rockland, ME, USA) containing 0.44 mg/ml ethidium bromide (Nacalai Tesque, Kyoto, Japan). Electrophoresis was performed in 0.5X Tris-borate-EDTA buffer for 20 min then imaged under ultra-violet transillumination using a CCD camera and molecular imaging software (Figure 6).

11. RNA extraction

Two frozen male mouse brain samples from each dam were extracted using the NucleoSpin®RNA II extraction tissue kit (Macherey-Nagel, Germany). The samples were homogenized with 700µl TRI Reagent® (Sigma-Aldrich, USA) using the Tissue Lyser (Qiagen, UK) for 30 sec. After homogenization, the samples were added with 200µl chloroform (Nacalai Tesque, Kyoto, Japan), shaken, and centrifuged at 4°C 12000xg for 20 min. The supernatant was transferred into a fresh microcentrifuge tube containing 350µl of 70% ethanol. The samples were loaded to a light blue ring filter, and centrifuged at 11000xg for 1 min. Desalt silica membrane, the samples were added with 350µl Membrane Desalting Buffer and centrifuged at 11000xg for 1 min to dry the membrane. To digest DNA, the sample was applied with 95µl DNase reaction mixture directly onto the center of the silica membrane of the column and immediately incubated at room temperature for 15 min. For the first washing, the column was added with 200µl buffer RA2, and centrifuged at 11000xg for 1 min and then the column was placed into a new collection tube. For the second washing, the column was added with 600µl buffer RA3, and centrifuged at 11000xg for 1min. In the third washing, the column was added with 250µl buffer RA3, and centrifuged at 11000xg for 2 min to dry the membrane completely and the column was placed into a nuclease-free collection tube. The RNA content were eluted in 40µl RNase-free water and centrifuged at 11000xg for 1min. RNA quantity and purity were obtained using a NanoDrop®Spectrophotometer ND-1000. All RNA samples showed an A260/280 ratio between 2.0 and 2.1, and an A260/230 ratio between 2.0 and 2.3. The RNA quality was determined using gel electrophoresis (Figure 7).

12. Microarray processing and analysis

Processes for microarray analysis, including the synthesis of double-stranded cDNA, labeling of transcript and fragment using the MessageAmp™ II-Biotin Enhanced Kit (Life Technology, USA), hybridization, washing, staining and scanning of hybridization products, were performed by the GeneChip®Expression Analysis System according to the Affymetrix protocol. Briefly, purified total RNA was synthesized first-strand cDNA, followed by second-strand cDNA synthesis. The cDNA was used in the *in vitro* transcription reaction to generate the biotinylated cRNA. The quality of the cRNA was assessed prior to its fragmentation. After fragmentation, the cRNA was evaluated and hybridized with the Affymetrix GeneChip® Mouse Genome 430A 2.0 Array. After hybridization, the chip were washed, stained, and scanned using the Affymetrix GeneChip® System. Data from digitized images was processed using the Array software. Normalization and comparison analyses of probe values were performed according to the Affymetrix recommended protocol. Fold changes (FC) of gene expression of the treated group was calculated relative to those of the control group. Genes were considered to be significant when their average FC was $\leq$-0.5 or $\geq$ 1.5 and statistically significant when the corresponding p value was $\leq$ 0.05. Information on each gene was obtained from the National Center for Biotechnology Information (NCBI) database.

13. Quantification of apoptosis-related gene expression

RNA was diluted to 200 ng/µl with DDW. The reverse transcriptase (RT) reaction for the first strand cDNA synthesis was performed. A 4µg sample of RNA was mixed with 2µl oligo (dT) 12-18 primer and nuclease-free water (NFW) to a final volume of 28 µl. The mixture was preheated at 70°C for 10 min, and then chilled on ice for 5 min. The RT-reaction mixture contained 16µl of 5X reaction buffer, 32µl of dNTPs mixture, 4µl of RT-trancriptase. The mixture was added with the RNA and primer mixture to each reaction for a final reaction volume of 80 µl/tube. A thermal cycler (iCycler®, Bio-Rad, CA) was used with the following PCR conditions: 42°C for 50 min followed by 99°C for 5 min, and then keeping at 4°C until analysis. The

cDNA concentration was determined using a NanoDrop®Spectrophotometer ND-1000. Real time PCR was performed with pairs of oligonucleotide primers corresponding to the cDNA sequences of mouse mRNA (Table 7). PCR was performed in a 10 µl reaction mixture containing 1 µl of the forward and reverse primer, 4 µl of the cDNA samples, and 5 µl of FastSYBR® Green Master Mix (Applied Biosystems, USA). This was immediately followed by pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 sec, annealing for 30 sec (40 cycles), termination at 95°C for 15 sec and 60°C for 5 sec. The analyzed melting curve was 95°C, with 0.3°C increment steps using the StepOnePlus™Real-Time PCR system thermal cycler (Applied Biosystems, USA). The endogenous control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used for normalization. Each replicate cycle threshold (CT) was normalized to the CT of GAPDH on a per sample basis. The comparative CT method was used to calculate the relative quantification of gene expression (Livak and Schmittgen, 2001). The following formula was used to calculate the relative amount of transcripts in the FX-treated sample (treat) and the vehicle-treated sample (control) and both were normalized to GAPDH

$\Delta\Delta CT = \Delta CT\ (treat) - \Delta CT\ (control)$

where ΔCT is the difference in CT between the target and GAPDH. The fold change for each treated sample relative to the control sample equals $2^{-\Delta\Delta CT}$

Table 7 List of primers for RNA expression

Gene	Forward	Reverse	Accession number	Estimated PCR product (bp)
Trp53	GCTTCTCCGAAGACT GGATG	CTTCACTTGGGCCTTCA AAA	NM_011640.3	195
Bax	TGCAGAGGATGATTG CTGAC	GATCAGCTCGGGCACTT TAG	NM_007527.3	173
Bcl2	CTGCAAATGCTGGAC TGAAA	TCAGGAGGGTTTCCAGA TTG	NM_009741.4	158
Fas	TGTGAACATGGAACC CTTGA	TTCAGGGTCATCCTGTCT CC	NM_007987.2	177
Casp8	GGCCTCCATCTATGAC CTGA	TGTGGTTCTGTTGCTCG AAG	NM_009812.2	152
Casp9	TGCCCTTGCCTCTGAG TAGT	AACAAAGAAACGCCCA CAAC	NM_001277932. 1	163
GAPDH	TCACTGCCACCCAGA AGA	GACGGACACATTGGGGG TAG		186

14. Statistical analysis

Results of the TUNEL, PCNA index, and relative mRNA expression levels are shown as the mean± standard deviation. The means of different groups were compared using a t-test. A P-value less than 0.05 was considered statistically significant.

Place of work

The experiment were examined at the Faculty of Veterinary Medicine, the Faculty of Veterinary Technology, Kasetsart Veterinary Teaching Hospital, Kasetsart University, and Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Japan.

RESULTS AND DISCUSSION

Results

1. Histological findings

In the telencephalon of the FX-treated group, a large number of pyknotic or karyorrhexic nuclei were found in neurons at 12, 24 and 48 HAT. In addition, the number of these neurons peaked at 12 HAT (Figure 8).

2. Electron microscopy findings

The electron microscopy findings showed shrinkage of the cell body, nuclear chromatin fragmentation and marginalization of condensed chromatin along the nuclear membrane. Some apoptotic bodies were also ingested by macrophage (Figure 9).

3. Finding of *in situ* detection of fragmented DNA

The pyknotic or karyorrhectic nuclei of telencephalic neurons were strongly positive for TUNEL at 12, 24 and 48 HAT (Figure 10), and the TUNEL index peaked at 12 HAT (Figure 11).

4. Finding of immunohistochemical staining for PCNA

The PCNA-positive nuclei of telencephalic neurons were presented in the control group, decreased at 12 HAT and subsequently increased at 24 and 48 HAT in the FX-treated group, (Figure 12). In addition, the PCNA index was clearly present at each point of treatment (Figure 13).

5. Gene expression using Microarray technique

In this study, we obtained differentially expressed genes in the mouse fetal brain. The results presented 1,746 and 1,920 up-(≥1.5-fold) and down-(≤0.5-fold) regulated genes, respectively (Figure 14). Categorization of these expressed genes based on gene ontology included three main groups, namely, biological process, cellular component, and molecular activity with 11, 7, and 12 functional categories, respectively (Figure 15). In the present study, we selected six interesting apoptosis-related genes that show up-, normal, and down-regulation as highlights to provide an example of the apoptosis pathway (Table 8).

6. Quantification of apoptosis-related gene expression

The detection of Bax, Bcl2, Trp53, Fas, Casp8 and Casp9 mRNA of FX-induced apoptotic neurons was examined. The results of the relative expression levels of Bax mRNA showed an increase, whereas the relative expression levels of Bcl2 mRNA were decreased when compared to the control group (Figure 16). Moreover, an increase in the relative expression levels of Trp53 and Casp9 mRNA were observed, whereas the relative expression levels of Fas and Casp8 mRNA remained unchanged (Figure 16). Our results showed that the DNA microarray data can be re-confirmed using appropriate primer design followed by RT-PCR (Table 9)

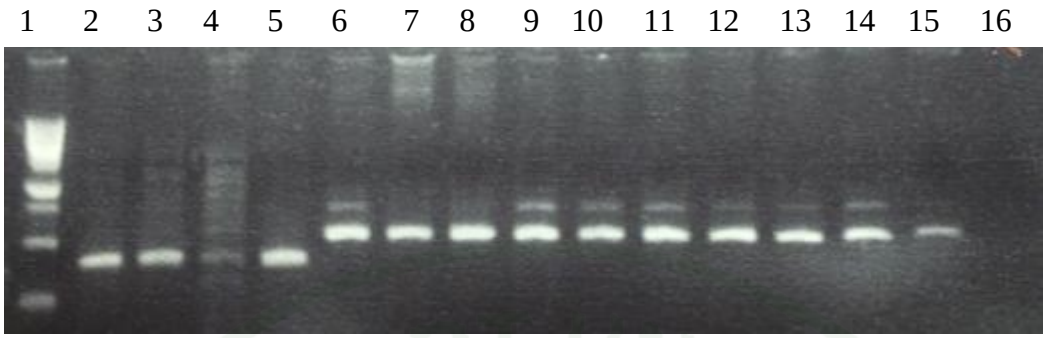


Figure 6 PCR amplification of fetal liver DNA. Lane 1: 1 kb marker. Lane 2-3: male DNA amplified with Sry male-specific primers. Lane 4-5: female and male DNA amplified Sry male-specific primers, respectively. Lane 6-15: male and female DNA amplified with IL3 primers. Lane 16: reagent control (no DNA).

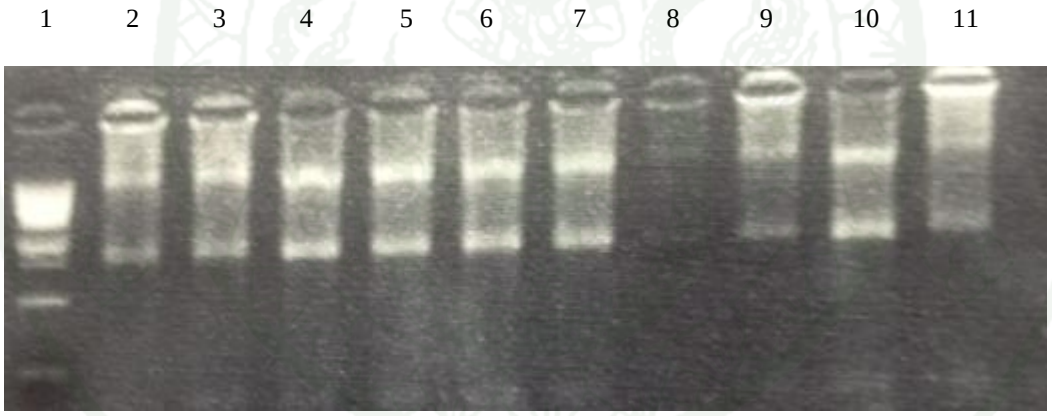


Figure 7 Gel electrophoresis of fetal brain RNA using 2% agarose. Lane 1: 1 kb marker. Lane 2-11: mouse fetal brain RNA.

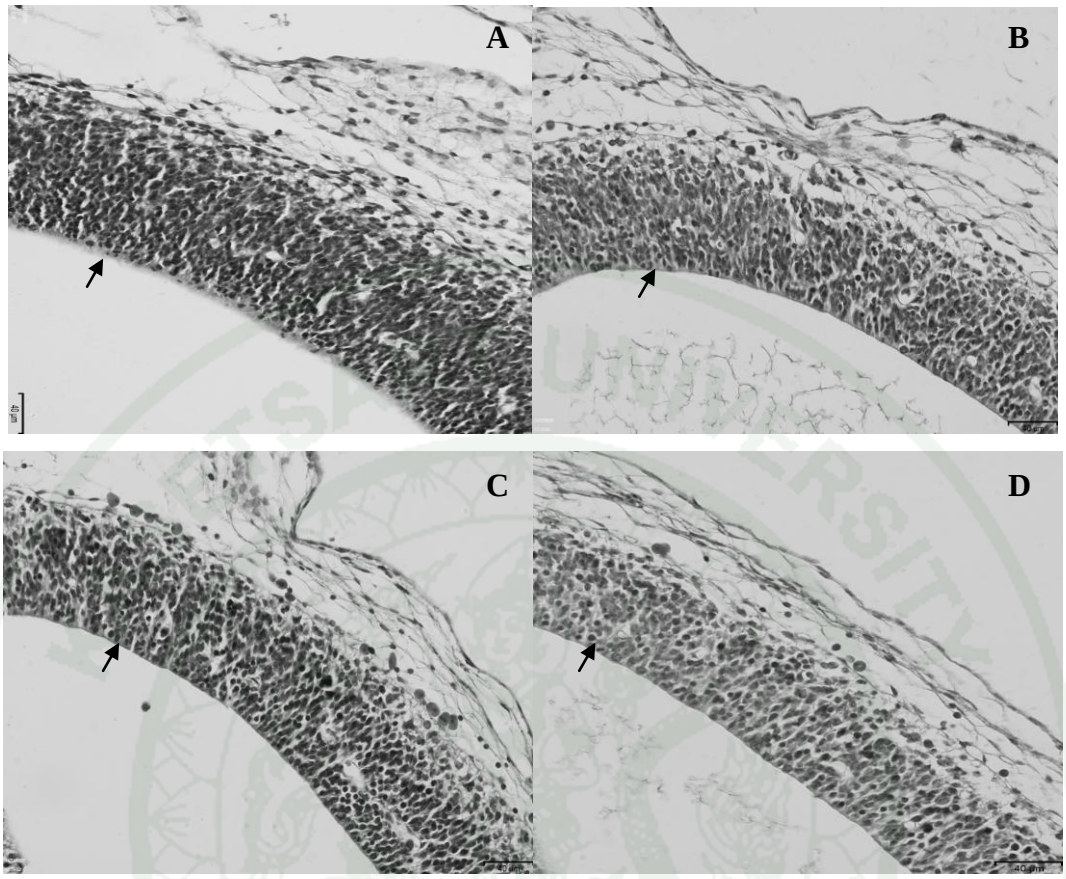


Figure 8 Hematoxylin & eosin staining of telencephalon from mouse embryos after FX-treated pregnant mice**.** (A) Control group, (B) 12 HAT , (C) 24 HAT, (D) 48 HAT. Many pyknotic or karyorrhectic nuclei of neurons are present in (B),(C) and (D). HE x20. Arrow indicated pyknotic nucleus (n=25).

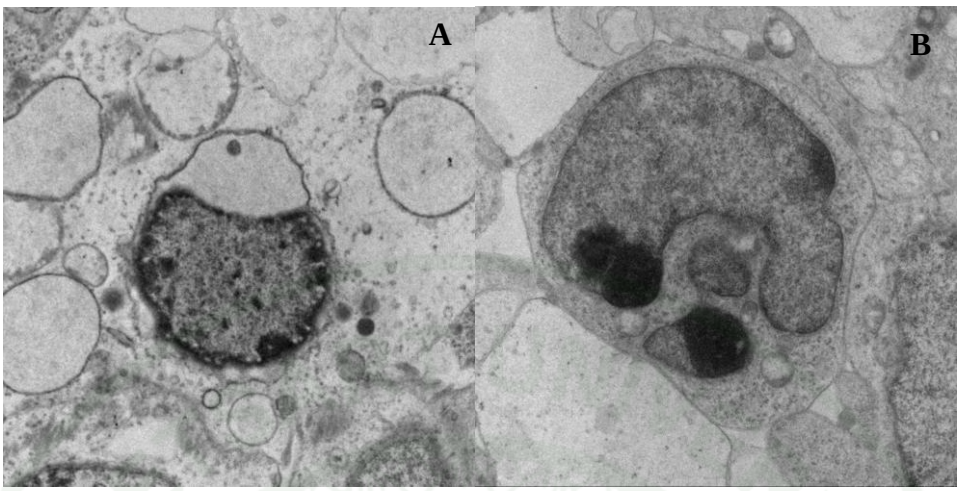


Figure 9 Transmission electeon microscope of neuron of mouse embryos after FX-treated pregnant mice from 12HAT. (A) nuclear chromatin fragmentation and margination of condensed chromatin along the nuclear membrane are seen. X3000. (B) apoptotic body is ingested by macrophage are seen. X3000.

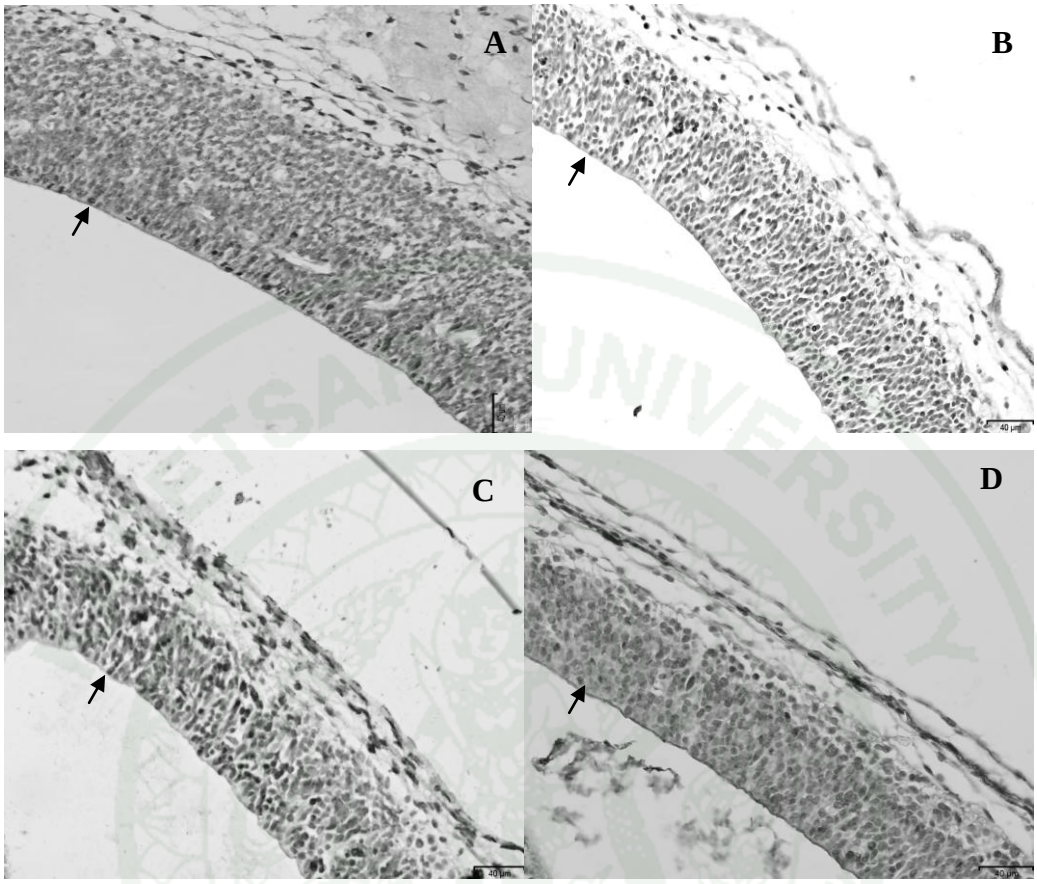


Figure 10 TUNEL staining of telencephalon from mouse embryos after FX-treated pregnant mice**.** (A) Control group, (B) 12 HAT , (C) 24 HAT, (D) 48 HAT. Many TUNEL-positive nuclei of neurons are present in (B),(C) and (D). TUNEL-staining x20. Arrow indicated TUNEL-positive nucleus (n=25).

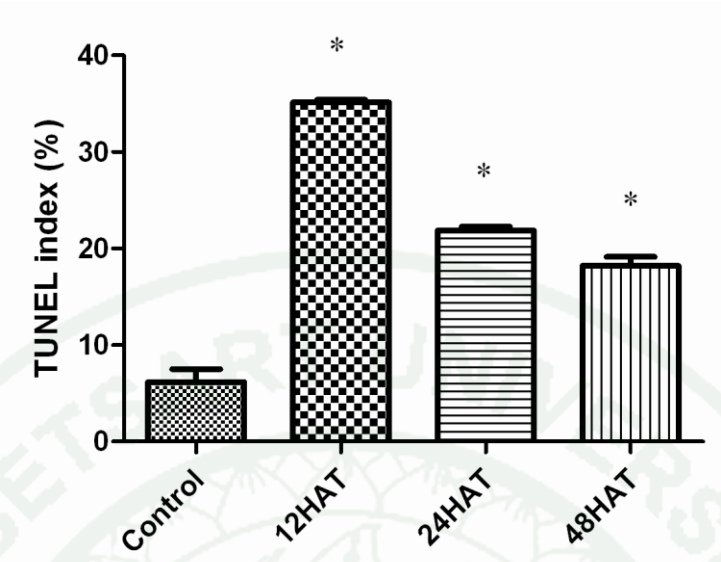


Figure 11 Percentage of TUNEL index in telencephalon of mouse embryos after FX-treated pregnant mice. Each value represents mean±SD from five pregnant mice each group. *p<0.05: Significantly different from the control (n=25).

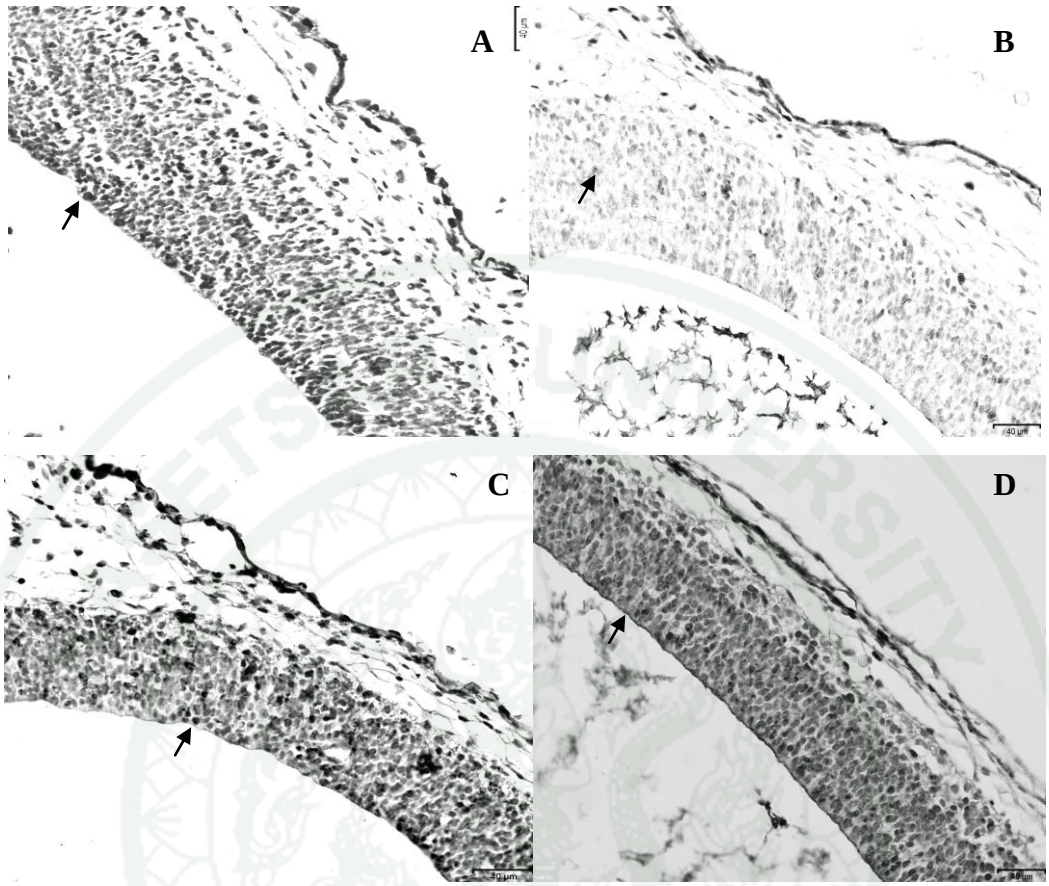


Figure 12 Immunohistochemical staining for PCNA of telencephalon from mouse embryos after FX-treated pregnant mice**.** (A) Control group, (B) 12 HAT , (C) 24 HAT, (D) 48 HAT. PCNA-positive nuclei of neurons are seen. PCNA-staining x20. Arrow indicated PCNA-positive nucleus (n=25).

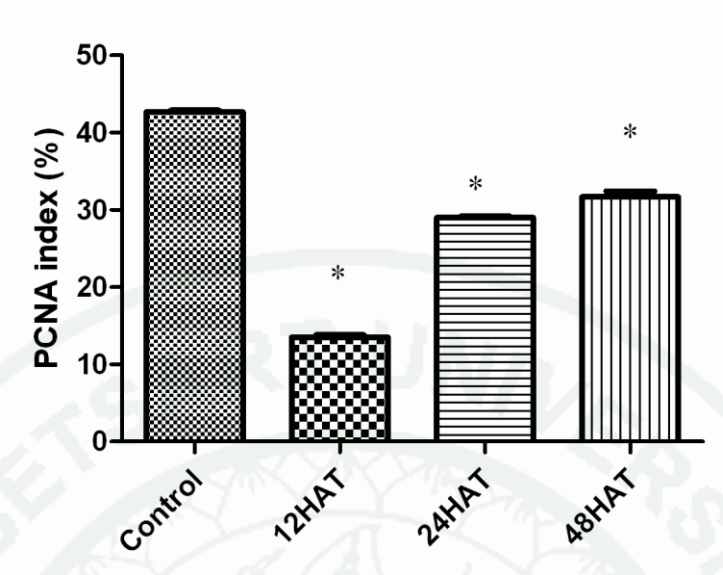


Figure 13 Percentage of PCNA index in telencephalon of mouse embryos after FX-treated pregnant mice. Each value represents mean±SD from five pregnant mice each group. *p<0.05: Significantly different from the control (n=25).

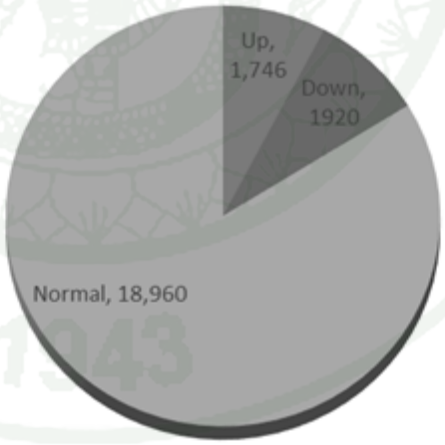


Figure 14 Overview of the differentially expressed FX-responsive transcriptome in the mouse fetal brain.

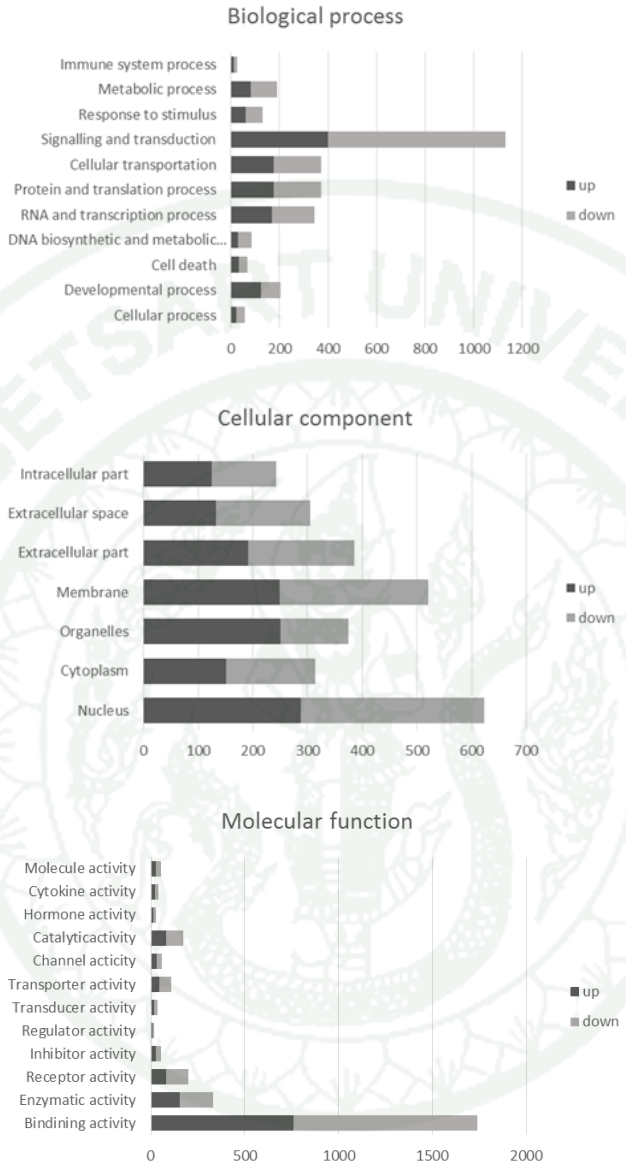


Figure 15 Functional categorization of all differentially expressed genes in the mouse fetal brain. The genes are categorized according to gene ontology in three main groups; biological process, cellular component, and molecular function. up, up-regulated genes; down, down-regulated genes (n=10).

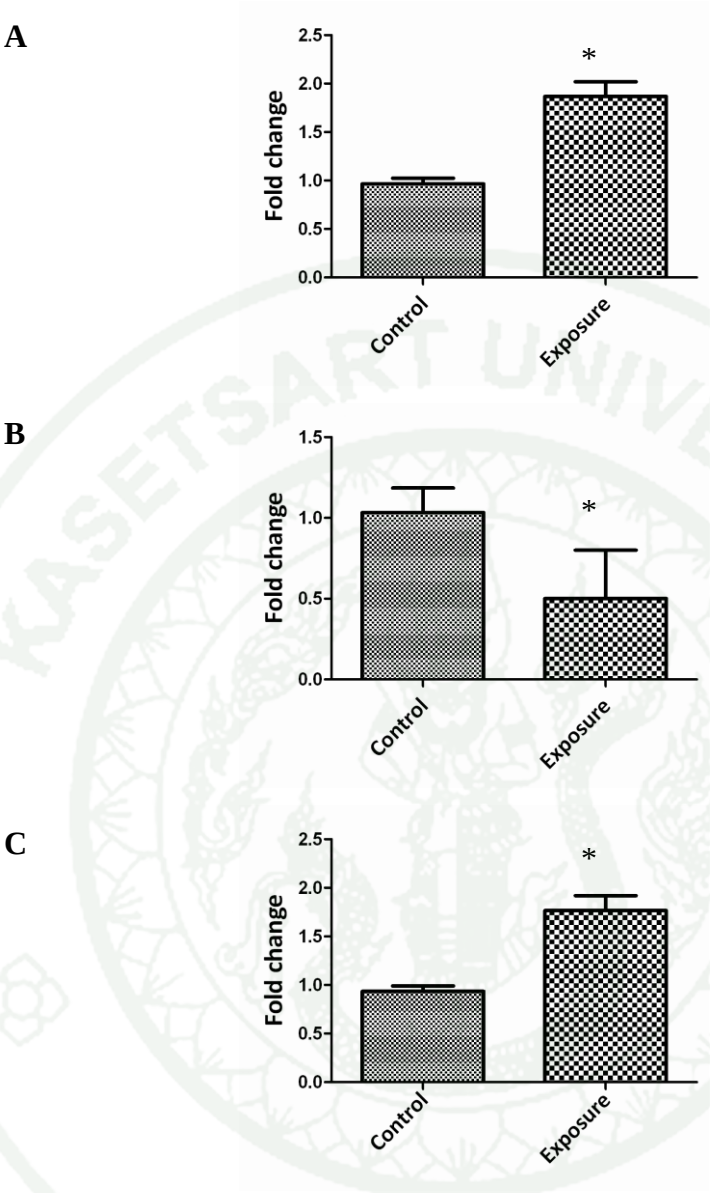
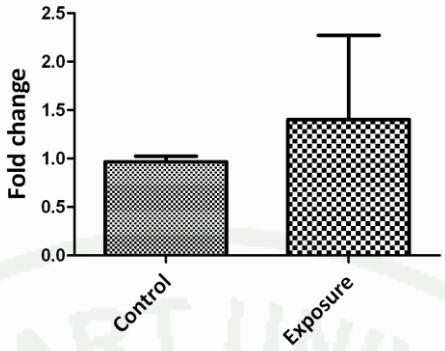


Figure 16 Relative expression levels of (A) Bax, (B) Bcl2, (C) Trp53, (D) Fas, (E) Casp8 and (F) Casp9 in the brain of mouse fetus from FX-treated pregnant mice. Each value represents the mean±SD from five pregnant mice in each group. *p<0.05: Significantly different from the control (n=10).

D



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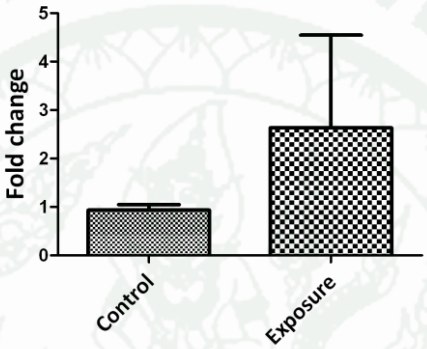

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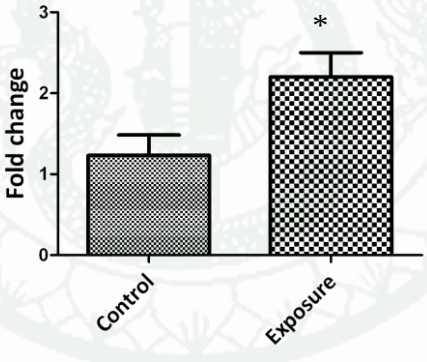

Figure 16 (Continued)

Table 8 List of apoptosis-related genes affected by the FX exposure

Representative public ID	Gene name	Gene symbol	Fold
NM_009425	Tumor necrosis factor (ligand) superfamily, member 10	Tnfsf10	12.8
U10100	Bcl2-like 1	Bcl2l1	6
BG064103	Tnf receptor-associated factor 1	Traf1	5.9
NM_013693	Tumor necrosis factor	Tnf	5.3
BB450072	Transformation related protein 53	Trp53	5.3
NM_007609	Caspase 4, apoptosis-related cysteine peptidase /// hypothetical protein LOC100044206	Casp4 /// LOC100044206	4.3
Y19235	Transformation related protein 73	Trp73	4.0
NM_013609	Nerve growth factor, beta	Ngfb	3.7
M60469	Tumor necrosis factor receptor superfamily, member 1b	Tnfrsf1b	3.4
NM_013542	Granzyme B	Gzmb	3.0
AI326167	B-cell leukemia/lymphoma 2 related protein A1a/A1b/A1d	Bcl2a12,Bcl2a1b,Bcl2a1d	2.6
BB783769	Baculoviral IAP repeat-containing 4	Birc4	2.5
BB828014	Tnf receptor-associated factor 3	Traf3	2.1
U51279	Bcl2-like 1	Bcl2l1	2.1
NM_008391	Interferon regulatory factor 2	Irf2	2.1
U21050	Tnf receptor-associated factor 3	Traf3	2.1
AK013244	Bcl2-like2	Bcl2l2	2

Table 8 (Continued)

Representative public ID	Gene name	Gene symbol	Fold
BG916928	Tnf receptor-associated factor 6	Traf6	2
NM_009743	Bcl2-like1	Bcl2l1	2
NM_016896	Mitogen activated protein kinase kinase kinase 14	Map3k14	1.9
AK011965	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor,epsilon	Nfkbie	1.9
NM_009743	Bcl2-like 1	Bcl2l1	1.7
NM_007522	Bcl-associated death promoter	Bad	1.7
AJ297973	Transformation related protein 53	Trp53	1.5
BI151406	Nerve growth factor receptor (TNFR superfamily, member 16)	Ngfr	1.5
BB815299	Caspase 9	Casp9	1.5
AK013244	Bcl2-like 2	Bcl2l2	1.5
BC006737	Caspase 8	Casp8	1.2
BC018228	Bcl2-associated X protein	Bax	1.2
NM_009741	B-cell leukemia/lymphoma 2	Bcl2	1.1
NM_010177	TNF receptor superfamily member 6	Fas	0.6
U21050	Tnf receptor-associated factor 3	Traf3	0.5
BI151406	Nerve growth factor receptor (TNFR superfamily, member 16)	Ngfr	0.4

Table 8 (Continued)

Representative public ID	Gene name	Gene symbol	Fold
NM_010177	Fas ligand (TNF superfamily, member 6	Fasl	0.4
NM_020275	Tumor necrosis factor receptor superfamily, member 10b	Tnfrsf10b	0.4
AF075434	Transformation related protein 63	Trp63	0.4
NM_008173	Nuclear receptor subfamily 3, group c, member 1	Nr3c1	0.3
BF134200	Baculoviral IAP repeat-containing 4	Birc4	0.3
NM_007544	BH3 interacting domain death agonist	Bid	0.2
NM_011616	CD40ligand	Cd40lg	0.1
NM_020294	Tumor necrosis factor receptor superfamily, member 10b	Tnfrsf10b	0.1
NM_007535	B-cell leukemia/lymphoma 2 related protein A1a /// B-cell leukemia/lymphoma 2 related protein A1b /// B-cell leukemia/lymphoma 2 related protein A1c /// B-cell leukemia/lymphoma 2 related protein A1d	Bcl2a1a /// Bcl2a1b /// Bcl2a1c /// Bcl2a1d	0.04

Table 9 Comparison of apoptosis-related gene expression

Method	**RT-PCR**	**Microarray**
Gene	Mean±SD	
Trp53	1.8±0.2	5.3
Bax	1.9±0.1	1.2
Bcl2	0.5±0.2	1.1
Fas	1.2±0.7	0.6
Casp8	2.7±1.7	1.2
Casp9	2.2±0.3	1.5

Discussion

FX is a type B trichothecene mycotoxins that are widely contaminated in crops. The primary targets of this mycotoxins in the animal body are organ that contain actively dividing cells such as thymus (IRAC, 1993). Toxic effects have been observed in embryos of pregnant animals that give trichothecene mycotoxins such as T-2 toxin and deoxynivalenol (Rousseaux and Schiefer, 1987; Lafarge-Frayssimet *et al.*, 1990; Debouck *et al.*, 2001). Poapolathep *et al*.(2004) demonstrated that FX can be passed through palcenta of FX-treated pregnant mice.

Embryos and fetuses are very sensitive to DNA damage such as that caused by radiation, and DNA-damaging chemicals, which induce congenital anomalies (Adlard et al., 1975; Langman et al.1971; Ozu et al., 1965; Pfafferenorth et al., 1974; Sieber et al., 1978.)

In this study, we examined the toxic effect of FX on the developing mouse brain after a single FX-inoculation to pregnant mice at 12, 24 and 48 HAT. The results showed that apoptotic cells in telencephalon of mouse fetuses 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 a small population of necrotic cells (Detorres et al., 1997; Levin et al., 1990). However these cells exhibited the ultrastructural characteristics of apoptotic cells (Ihara et al., 1998; Shinozuka et al., 1998; Ishigami et al., 2001; Katayama et al., 2001; Poapolathep et al., 2002). Thus, it was reasonable to assume that these TUNEL positive cells in the telencephalon of mouse fetuses from FX-treated pregnant mice were apoptotic cells. The numbers of apoptotic cells peaked at 12 HAT and decreased at 24 and 48 HAT. The same peak time was observed in the fetal central nervous system (CNS) after treatment with ethylnitrosurea (ENU) (Katayama et al., 2001), 5-azacytidine (Ueno et al., 2002), T-2 toxin (Ishigami et al., 2001) and Hydroxyurea (HU) (Woo et al., 2003). However, unlike in pharmacokinetics, the metabolic activation and signaling pathways of chemicals that are involved are in apoptosis include chemicals and differences in the time of initiation and/or termination of apoptosis. The CNS of

mouse fetuses appears to be highly sensitive to genotoxic stimuli and can easily undergo apoptosis, which is consistent with many studies reporting apoptotic cell death in the CNS of fetuses in rats and/or mice treated with the DNA hypomethylating agent, 5-azacytidine (Lu et al., 1998; Ueno et al., 2002), the DNA alkylating agent, ENU (Katayama et al., 2001 and 2002), γ-radiation, and T-2 toxin which are types of trichothecene mycotoxins that exhibit a DNA synthesis inhibitory effect (Borovitskaya et al., 1996).

Accordingly, we wanted to determine the apoptosis pathway that is induced by FX in developing mouse brain of pregnant mice after 12h exposure. We utilized the high-throughput DNA microarray approach to screen differential gene expressions in the fetal brain of male mouse. The results showed that thirty-nine apoptosis-related genes were differentially expressed consisting of twenty up-regulations and eleven down-regulations (Table 2). All of these genes were divided into three major groups; B-cell leukemia/lymphoma 2 family genes (Bcl2), transformation-related protein genes (Trp), and tumor necrosis factor/tumor necrosis factor receptor genes (Tnf/Tnrf).

Apoptosis is a distinct mechanism for the induction of eukaryotic cell killing (Cruickshanks et al., 2013). It comprises the activation of caspase leading to fragmentation of nuclear DNA and the encapsulation of DNA fragments (Cruickshanks et al., 2013). Death receptors in the plasma membrane and at other cellular locations activate the ‘extrinsic’ pathway of apoptosis, and in particular the Fas (CD95/APO-1) and tumor necrosis factor (TNF)-α receptors, in promoting cell death (Cruickshanks et al., 2013). The apoptotic actions of Fas-R are blocked by Flice-like inhibitory protein (FLIP); FLIP proteins inhibit Casp8 (Cruickshanks et al., 2013). The intrinsic pathway is downstream of mitochondria. Toxic multidomain proteins in the same Bcl2 family such as Bax, Bak, and Bok form pores in the outer mitochondrial membrane permitting proteins such as cytochrome c to be released into the cytosol (Cruickshanks et al., 2013)

The Bcl2 family consists of many genes, which can promote either cell survival, such as Bcl2, Bcl-XL, Mcl-1, A1, Bcl-W (Adam and Cory, 1998) or apoptosis, like Bax, Bak, Bcl-XS, Bok (Chao and Korsmeyer, 1998). It is well recognized that the Bcl-2 family, Bax and Bcl2, are central regulators of apoptosis because they function as checkpoints for cell survival and death signals prior to cell fate determination (Cory and Adam, 2002). In the present study, FX was shown to be capable of both up-regulating the pro-apoptotic factor Bax and down-regulating the anti-apoptotic factor Bcl-2 the same as in the study of ethanol exposure (Sari et al., 2012; Moore et al., 1999; Cory and Adams, 2002; Heaton et al., 2003; Young et al., 2003; Ge et al., 2004).

Trp53 is classified as a tumor suppressor gene (McBride et al., 1986). It is a transcription factor that activates vital damage containment procedures to restrict aberrant cell growth in response to DNA damage, oncogene activation, hypoxia and the loss of normal cell contacts (Giaccia and Kastan, 1998; Lohrum and Vousden, 1999). It restricts cellular growth by inducing senescence, cell cycle arrest (at the G1 and/or G2 phase) or apoptosis (Jin and Levine, 2001). In the present study, FX induced apoptosis resulting in an increased level of Trp53 (Table 2), which directly activated Bax expression (Chipuk et al., 2004) These results suggested that activation of the p53-mediated apoptosis signaling pathway played an important role in apoptosis by modulating the Bax/Bcl-2 ratio. Up-regulation of this gene are the same in etoposide-induced apoptosis in the mouse fetal brain (Nam et al., 2006), HU (Woo et al., 2003), and Mercaptopurine (MP) (Kanemitsu et al., 2009). The mode of activated p53-mediated apoptosis may induce two sets of genes upon stress signals. One set, which includes p21/waf-1 and GADD45, mainly functions in the control of cell growth and the other, which includes Bax and Bcl-2, is involved in apoptosis (Agarwal et al., 1998).

Death receptors have been recently identified as a subgroup of the TNF-receptor superfamily with a predominant function in the induction of apoptosis (Schulze-Osthoff et al, 1998). The receptors are characterized by an intracellular region, called the death domain, which is required for the transmission of the

cytotoxic signal (Schulze-Osthoff et al, 1998). Currently, five different such death receptors are known consisting of i tumor necrosis factor (TNF) receptor-1, CD95 (Fas/APO-1), TNF-receptor-related apoptosis-mediated protein (TRAMP) and TNF-related apoptosis-inducing ligand (TRAIL) receptor-1 and -2 (Schulze-Osthoff et al, 1998). Studies on Fas expression presented that the relative expression level of Fas mRNA remains unchanged. These results suggested that the Fas/FasL apoptotic pathway or extrinsic apoptotic pathway might not contribute to FX-induced apoptosis. Caspases, a family of cysteine proteases, are key proteins that modulate the apoptotic response (Lamkanfi et al., 2007). Casp8 is an initiator caspase in the extrinsic apoptotic pathway and Casp9 functions as an initiator caspase in the intrinsic apoptotic pathway (Lamkanfi et al., 2007). We found that FX up-regulated the Casp9. These results suggested that FX induced apoptosis in developing mouse brain via an intrinsic apoptotic pathway.

CONCLUSION AND RECOMMENDATION

In conclusion, the present study indicated that FX not only induced apoptosis, but also reduced cell proliferation to the PCNA staining in the developing mouse brain and the toxic effect are regulated by Bax, Bcl2, Trp53 and Casp9 which are involved in the intrinsic apoptotic pathway (Figure 17).

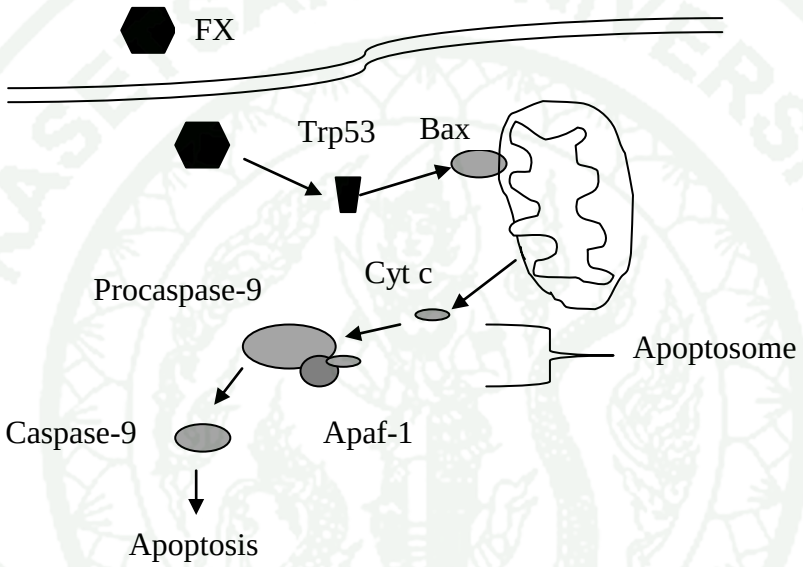

Figure 17 FX- induced apoptosis in developing mouse brain via intrinsic pathway

The present study is the first one that has clarified the toxicological characteristic especially apoptosis and the genetically controlled mechanism of apoptosis in the developing mouse fetus from FX-treated mice. The results of this study are consider to be very useful for further study about embryonic malformation and death in both human and domestic animals that have been attributed to intake of food contaminated with mycotoxins, especially FX.

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APPENDICES

Appendix A

Protocol for TUNEL

1.Deparaffinization

Xylene I (5 min) , Xylene II (5 min), Abs.acl I (10 dip), Abs.acl II (10 dip), 95% alc.I (10 dip), 95% alc.II (10 dip), 70% alc.I (10 dip), 70% alc.II (10 dip), PBS 1X (5 min)

2. Move slides in citric acid jar (pH 6) and pretreat slides by microwave 800 W for 5 min then cool down for 20 min at RT

3.Wash in PBS 3 times

4.Block endogenous peroxide by 3% H_2O_2 10 min (in jar)at RT ; adjust volume

5.Wash in PBS 3 times

6.Drop equilibrium buffer (adjust volume/sample) at least 20 sec then wipe out

7. Immediatedly drop prepared -TDT 10 ul/sample and cover with film (prepared TDT :Reaction buffer 7ul + TDT 3ul)

8.Incubate at 37ºC in humidified chamber for 1 hour

9.Remove film and drop 20 ul/sample stop wash buffer on tissue, then incubate at RT for 10 min

10.Wash in PBS 3 times and leave in jar

11.Drop 10 ul/sample anti-deoxygenin on tissue and cover with film, then incubate at RT for 30 min

12.Wash in PBS 3 times

13.Drop DAB substrate (adjust volume) 5 min at RT (30 in 1000)

14.Wash in tap water

15.Couterstain with hematoxylin 10 sec

Appendix B

Protocol for PCNA

1.Deparaffinization

Xylene I (5 min) , Xylene II (5 min), Abs.acl I (10 dip), Abs.acl II (10 dip), 95% alc.I (10 dip), 95% alc.II (10 dip), 70% alc.I (10 dip), 70% alc.II (10 dip), PBS 1X (5 min)

2. Move slides in citric acid jar (pH 6) and pretreat slides by microwave 800 W for 5 min then cool down for 20 min at RT

3.Wash in PBS 3 times

4.Block endogenous peroxide by 3% H_2O_2 10 min (in jar)at RT ; adjust volume

5.Wash in PBS 3 times

6.Drop normal blocking serum (adjust volume/sample) at RT for 30 min

7.Incubate primary antibody: PCNA at 4ºC in humidified chamber over night (cover with film)

8.Wash in PBS 3 times and leave in jar

9.Incubate secondary antibody at RT for 30 min

10.Wash in PBS 3 times

11.Incubate with ABC at RT for 30 min

12.Wash in PBS 3 times

13.Drop DAB substrate (adjust volume) 10 sec at RT (30 in 1000)

14.Wash in tap water

15.Couterstain with hematoxylin 10 sec

Remark: Normal serum (15+PBS 1000 ul), primary and secondary AB (1:200) and ABC substrate (10+10+ PBS 1000 ul)

Appendix C

RT-reaction mixture

1.RNA 1 µg	5 µl
2.OligodT	0.5µl
3.Deionized Distrilled Water	2.5µl
4.5X reaction buffer	4 ul
5.dNTPs mixuer (final concentration 2.5 mM each dNTP)	8 ul
6.RT-transcriptase	1 ul
Total	**20µl**

Appendix D

RT thermal cyclin protocol

Predenaturation	95ºC, 3 min
Denaturation	95ºC, 15 sec
Annealing	60 ºC, 15 sec
Extention and plate reading	72ºC, 30 sec
Termination	95ºC, 30 sec
Melt curve analysis	65ºC 0.3ºC increment 5 sec/ step
Cycles	40

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