MECHANISM OF RESISTANCE TO FUMONISIN $B_1$-INDUCED CYTOTOXICITY

by

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(Under the Direction of Raghubir P. Sharma)

ABSTRACT

Fumonisin $B_1$ ($FB_1$) is a mycotoxin produced by *Fusarium verticillioides* present on corn worldwide. Fumonisin $B_1$ inhibits ceramide synthase and induces expression of cytokines in liver. Fumonisin $B_1$ induces apoptosis in some cells whereas other cells are resistant to its apoptotic effects. We hypothesized that sphingolipid metabolism and interaction of different cytokines may be important in rendering some cells resistant to fumonisin $B_1$ induced apoptosis.

We found that despite the accumulation of sphinganine, human embryonic kidney (HEK-293) cells are resistant to fumonisin $B_1$ toxicity and DL-*threo*-dihydrosphingosine, the sphingosine kinase inhibitor (SKI), considerably increased the sensitivity of HEK-293 cells to fumonisin $B_1$. Results indicated that HEK-293 cells are resistant to fumonisin $B_1$ due to rapid conversion of sphingosine to sphingosine-1-phosphate, which imparts survival properties.

Cellular interactions in fumonisin $B_1$ induced toxicity were investigated using co-cultures of murine macrophages (J774A.1) and nonparenchymatous liver epithelial cells (NMuLi). Treatment of the co-cultures with fumonisin $B_1$ produced cytotoxicity whereas either J774A.1 or NMuLi cultures alone showed no response to the mycotoxin. Expression of cytokines was increased in co-cultures but not in individual cultures. Results indicated that macrophages and

liver epithelial cells interact in response to fumonisin $B_1$ and potentiate the cytokines expression, which may have implications in making hepatocytes responsive to cytotoxicity of fumonisin $B_1$.

Fumonisin $B_1$ induced apoptosis and activated c-Jun NH2-terminal kinase (JNK) in primary culture of liver cells. JNK inhibitor (SP600125) and anti-TNFα reduced the apoptosis induced by fumonisin $B_1$. The role of JNK signaling in fumonisin $B_1$ induced apoptosis is downstream of TNFα production, as fumonisin $B_1$ mediated activation of JNK was reduced by the presence of anti TNFα in the medium, whereas presence of JNK inhibitor did not change the fumonisin $B_1$ induced TNFα expression.

To determine the role of T cells in fumonisin $B_1$-induced apoptosis in mice liver, we depleted T cells in wild-type mice by injecting monoclonal antibodies directed against Thy-1.2 surface antigens of mature T cells. Results obtained in this study indicated that T cell activation followed by production of proinflammatory cytokines is an important mechanism for fumonisin $B_1$-induced hepatotoxicity in mice.

INDEX WORDS: Fumonisin $B_1$, Resistance, Hepatotoxicity, Tumor necrosis factor α, Sphingosine kinase, c-Jun NH2-terminal kinase, T cell depletion,

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DEDICATION

To

My Parents

Dr. S. B. Sharma and Mrs. Kusum Sharma,

My Brothers

Manish and Sheelesh Sharma

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I would like to thank God for giving me the opportunity to explore his creation and appreciate its artistry, truthfulness, and originality. I would like to thank my major professor Dr. Sharma for introducing me to amazing world of fumonisin and sphingolipids; I am honored to have been his student. I have learned the very basic lesson of hard work, discipline and attention to detail from him. I thank him for his professional and financial support and, as importantly, for his great mentorship.

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# CHAPTER 1

# INTRODUCTION

Fumonisins are toxic and carcinogenic mycotoxins produced by *Fusarium verticillioides* and related species, commonly found on corn (Riley *et al.*, 1993). Fumonisin $B_1$ is the most abundant fumonisin and occurs naturally in contaminated foods and feeds. The toxicity of fumonisin $B_1$ has been described in a number of farm and laboratory animals and depends on different factors such as animal species, gender, age, dose and route of administration (Diaz and Boermans, 1994). It has been established as a cause of equine leukoencephalomalacia and porcine pulmonary edema. Fumonisin $B_1$ has been shown to be hepatotoxic and nephrotoxic in mice and rats (Voss *et al.*, 2001). It was hepatocarcinogenic in male BD IX rats (Gelderblom *et al.*, 2001) and a nephrocarcinogen in male F344/N rats, whereas it was hepatocarcinogenic in only female B6C3F1 mice (Howard *et al.*, 2001). In the Transkei region of South Africa where incidence of esophageal cancer is high, an association between fumonisin $B_1$ consumption and esophageal cancer was reported (Sydenham *et al.*, 1990).

Fumonisin $B_1$ is structurally similar to sphingoid bases and causes inhibition of ceramide synthase (sphinganine or sphingosine *N*-acyltransferase) leading to accumulation of free sphingoid bases, sphingoid base metabolites, and depletion of more complex sphingolipids (Riley *et al.*, 1996, Wang *et al.*, 1991). Physiological consequences possible after ceramide synthase inhibition include apoptosis caused by accumulation of free sphingoid bases (sphinganine and sphingosine), increased proliferation caused by increased sphingosine-1-phosphate, or/and decreased ceramide and altered lipid raft function due to disruption of complex sphingolipids synthesis/transport (Merrill *et al.*, 2001). Studies in pig renal epithelial cells (LLC-$PK_1$) human keratinocytes (Tolleson *et al.*, 1999) and human colonic cells (HT29) (Schmelz *et al.*, 1998) have shown that fumonisin $B_1$-induced apoptosis, necrosis, and

inhibition of proliferation are sphinganine dependent. There is a close association between increased apoptosis in liver and kidney and the disruption of sphingolipid metabolism (Tsunoda *et al.*, 1998). A previous study demonstrated that fumonisin $B_1$ induced cell cycle arrest in the G1 phase in African green monkey kidney cells (CV-1) but CV-1 cells transformed by the simian virus 40 (SV40) large T antigen (COS-7) were unaffected by the same levels of fumonisin $B_1$ (Wang *et al.*, 1996; Ciacci-Zanella *et al.*, 1998). Wang *et al.* (1991) and Schmelz *et al.* (1998) showed that concentrations of fumonisin $B_1$ as low as 1 μM caused an almost complete inhibition of ceramide formation by primary hepatocytes, while much higher (>75 μM) concentrations of fumonisin $B_1$ did not induce cytotoxicity. In sensitive cell lines, different studies have reported different doses (HT29: 10 μM, LLC-PK1: 35 μM, CV-1: 5 μM) to be cytotoxic in 24 to 72 hr treatment. It remains to be understood why increased levels of sphinganine do not cause toxicity in some cells and why high dose of fumonisin $B_1$ and long incubation periods are required in sensitive cells in which sphinganine levels rise significantly within hours of as low as 1 μM fumonisin treatment**.**

Levels of free sphingoid bases decrease consistently in fumonisin $B_1$-treated cells between 48 and 72 h indicating the induction of sphingoid base metabolism (Yoo *et al.*, 1996). Catabolism of sphingoid bases requires two independent and sequential steps. The first step involves phosphorylation of sphingoid bases to their 1-phosphate derivative and is mediated by sphingosine kinase (Keenan *et al.*, 1969). Second step involves cleavage of the resulting product by sphingosine-1-phosphate lyase into ethanolamine phosphate and corresponding aldehyde (Van Veldhoven *et al.*, 1991). Due to relative low levels of sphingosine-1-phosphate in cells, it is evident that phosphorylation of sphingosine by sphingosine kinase is the rate-

limiting step in this process. Sphingosine kinase in human tissues is widely expressed with highest levels in adult lung, spleen, kidney, heart, and brain (Melendez *et al.*, 2000). Activation of sphingosine kinase and formation of sphingosine-1-phosphate are linked to cell growth and survival (Spiegel *et al.*, 2002; Spiegel *et al.*, 2002). Diverse external stimuli, particularly growth and survival factors stimulate sphingosine kinase and intracellularly generated sphingosine-1-phosphate has been implicated in their mitogenic and anti-apoptotic effects (Xia *et al.*, 2002; Pitson *et al.*, 2000).

In our preliminary experiments, we found that human embryonic kidney (HEK-293) cells were resistant to cytotoxic effects of up to 50 μM fumonisin $B_1$ whereas porcine kidney (LLC-$PK_1$) cells show significant toxic response with 10 μM fumonisin $B_1$ (Yoo *et al.*, 1992). We hypothesized that the HEK-293 cells are resistant to apoptotic effects of fumonisin $B_1$ due to rapid conversion of accumulate sphinganine or sphingosine to their respective phosphates by sphingosine kinase and the antiapoptotic and proliferative role of the 1-phosphate derivatives.

Fumonisin $B_1$ treatment caused an increased expression of tumor necrosis factor α (TNFα), interferon γ (IFNγ) and interleukin (IL)-12, in liver, where cells involved in TNFα production were identified as Kupffer cells (Bhandari *et al.*, 2002). Increase of proinflammatory cytokines from immune cells has been implicated in fumonisin $B_1$-induced hepatotoxicity. Depletion of Kupffer cells by gadolinium chloride attenuated fumonisin $B_1$ induced hepatotoxicity (He *et al.*, 2005). Deletion of tumor necrosis factor receptors 1 and 2 and interferon γ genes reduced fumonisin $B_1$-induced hepatotoxicity in mice (Sharma *et al.*, 2001, 2003).

The role of inflammatory cytokines in the initiation and development of liver toxicity has been well documented. Liver associated immune cells such as lymphocytes, Kupffer cells and their cytokine products; especially TNFα and IFNγ are involved in liver injury during alcoholic hepatitis (Batey and Wang, 2002). TNFα and IL-1α are responsible for certain pathological manifestation of acetaminophen-induced hepatotoxicity in mice (Blazka *et al.*, 1995). IFNγ and TNFα released from T lymphocytes and macrophages, respectively, are involved in concanavalin A (Con A)-induced hepatitis and in liver toxicity in response to repeated challenges of lipopolysaccharide (Wolf *et al.*, 2001).

Interaction of different types of cells in liver may amplify the cytokine production in response to fumonisin $B_1$ as reported by Bhandari *et al*. (2002) after treatment of mice with fumonisin $B_1$. In various models of acute liver injury a positive amplification loop involving TNFα, IFNγ and IL-12 has been implied and involves Kupffer cells (liver macrophages) hepatic lymphocytes and nonparenchymatous liver epithelial cells (NPECs). A remarkable crosstalk between macrophages and T cells involving TNFα and IFNγ has been suggested using *in vitro* co-culture experiments (Gantner *et al.*, 1996). Interactions of this kind have been reported in several co-culture experiments involving alveolar macrophages and type II pneumocytes (Tao *et al.*, 2002) or microglia and neurons (Hemmer *et al.*, 2001). We hypothesized that interaction of macrophages and non-parenchymatous epithelial cells of liver may amplify the cytokine production in co-culture and make them more susceptible to fumonisin $B_1$ induced cytotoxicity.

Reports on the effects of fumonisin $B_1$ on the immune system are conflicting. Fumonisin- $B_1$ was found to be both immunosuppressive and immunostimulatory in BALB/c

mice, depending on the dose administered and induced an antigenic response (Martinova and Merrill, 1995). Fumonisin $B_1$ was shown to alter the expression of the CD3 receptor, which is integral to T lymphocyte activation. Recently Taranu *et al*. (2005) reported that ingestion of fumonisin $B_1$ alters the Th1/Th2 balance leading to reduced vaccinal antibody response in pigs.

Despite many reports confirming the role of proinflammatory cytokines in fumonisin $B_1$ induced liver damage, little is known about the contribution of T cells in this regard. We hypothesized that fumonisin $B_1$ hepatotoxicity in mice will be compromised by depletion of T cells.

The objective of the research under this dissertation is to test the overall hypothesis that pathways of sphingolipid metabolism and lack of cytokine interactions are responsible for observed differences to apoptotic effects of fumonisin $B_1$ in resistant cultured cells. The following specific aims were attempted to achieve this objective.

1. **Evaluate the role of sphingosine kinase, the enzyme that converts sphingoid bases to their respective phosphates, in rendering some cells resistant to fumonisin $B_1$-induced apoptosis.**
2. **Investigate the effect of interaction of macrophages (J774A.1) and nonparanchymatous liver epithelial cells (NMuLi) in a co-culture on fumonisin $B_1$-induced apoptosis**
3. **Evaluate the role of TNFα and mitogen activated protein kinases in fumonisin $B_1$-induced apoptosis in murine primary hepatocytes**
4. **Examine the fumonisin $B_1$-induced hepatotoxicity in mice depleted of T cells.**

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# CHAPTER 2

# LITERATURE REVIEW

**Fumonisin $B_1$: A fusarium mycotoxin**

Fumonisins are environmental toxins produced by the molds *Fusarium moniliforme (F. verticillioides), F. proliferatum*, and other *Fusarium* species that grow on maize in the field or during storage. These mycotoxins have been found as contaminants worldwide, mainly in corn. There are many other toxins produced by the same fungus but only fumonisin $B_1$ has been implicated in fusarium induced toxicities. More than ten types of fumonisins have been isolated and characterized. Of the identified fumonisins produced by the fungus *F. verticillioides*, B1, B2, and B3 are the most abundant in contaminated food and feed. Fumonisin B1 is the most common, comprising approximately 75 percent of the total fumonisin and is believed to be the most toxic.

**Structure of fumonisin $B_1$**

Fumonisin $B_1$ is water-soluble polar molecule comprised of a long-chain hydroxylated hydrocarbon backbone (Blackwell et al., 1996). Similar to sphingolipids, it is also formed by condensation of long chain fatty acid and amino acid serine. The structure of $FB_1$ consists of a 20-carbon backbone; an amine group at C2; three hydroxyl groups at C3, C5, and C10; two methyl groups at C12 and C16; and two tricarballylic acid monoester groups at C14 and C15 (Fig 2.1). Fumonisin $B_1$ is a water soluble and polar compound due to the presence of amine and carboxylic groups. Fumonisin $B_1$ has a remarkable structural similarity to sphinganine and sphingosine, the long-chain sphingoid base backbone of sphingolipids (Fig. 2.1).

**Species and gender specificity of fumonisin $B_1$**

Fumonisin $B_1$ is known to cause equine leukoencephalomalacia (ELEM) (Kellerman et al. 1990, Marasas et al. 1988, Marasas 2001). Exposure of swine to $FB_1$ containing diet results in

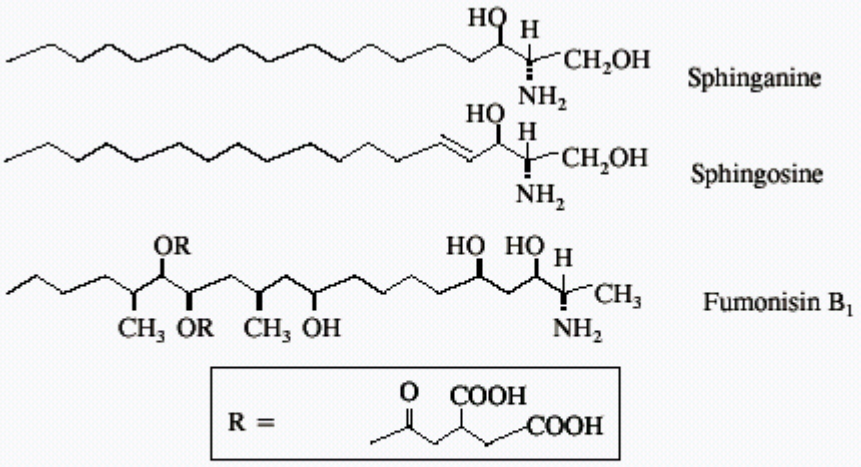


Fig. 2.1. Structures of sphinganine, sphingosine, and fumonisin $B_1$

specific porcine pulmonary edema (PPE) along with commonly observed general hepatotoxicity (Casteel et al., 1993). The animals fed feed with higher levels of $FB_1$ die of pulmonary edema and those exposed to lower levels exhibit subacute hepatotoxicosis. Pure $FB_1$ was shown to cause porcine pulmonary edema (PPE) by intravenous injection (Colvin and Harrison 1992). Due to abnormal climate conditions during the fall of 1989 and spring of 1990, widespread, large-scale outbreaks of ELEM and PPE occurred in the United States. Many horses and pigs died from ingesting fumonisin-contaminated corn (Osweiler *et al*. 1992, Ross *et al*. 1991a, b, 1992). Fumonisin $B_1$ is cardiovascular toxin, which is assumed to responsible for the PPE (Haschek *et al*. 2001, Smith *et al*. 1999). Liver injury has been observed in all animals tested so far, and kidney is also affected by fumonisins in most animals (Riley *et al*. 1994, Sharma *et al*. 1997, Voss *et al*. 1995, 1998, 2001). Fumonisin $B_1$ is immunotoxic in female mice

(Johnson and Sharma 2001), and rats (Theumer *et al*. 2002). It has been suggested that the observed adverse effects of $FB_1$ toxicity are the result of general immunosuppression (Chatterrje et al., 1995; Martinova et al., 1995). In poultry reduced body weight gain, hepatic necrosis, biliary hyperplasia, diarrhea and thymic cortical atrophy was shown, as in the case of one day-old broiler chickens fed $FB_1$ in doses from 0 to 400 mg/kg (Brown et al., 1992). Ducklings exposed to *Fusarium* isolates, showed high mortality, loss in weight, low body fat and swollen and reddened livers (Dutton, 1996) and in quail severe paralysis was observed (Grimes et al., 1993).

Toxicity of fumonisin $B_1$ manifests in various types of diseases in different affected species, although liver is thought to be a target organ in all animals regardless of other co-existing symptoms. High levels of $FB_1$ in human food have been correlated with increased incidence of neural tube defects (NTD) in parts of Texas, USA (Hendricks 1999). In mouse embryo cultures, $FB_1$ has been shown to produce embryotoxicity and NTD (Sadler *et al*. 2002). It has been documented or plausibly suggested that high incidences of NTD occur in some regions of the world (Guatemala, South Africa, and China) where substantial consumption of fumonisins (Marasas *et al*. 2004).

**Carcinogenic potential of fumonisin $B_1$**

Treatment with $FB_1$ in rats causes acute and chronic liver toxicity, bile duct proliferation, fibrosis further developing into cirrhosis, cholangiogenesis and hepatocellular carcinoma and/or cholangiocarcinoma (Gelderblom et al., 1988; Voss et al., 1993). Although, the findings regarding the $FB_1$-related tumor genesis in humans are speculative, $FB_1$ has been associated with the high incidences of esophageal cancer, first diagnosed by Burrell in 1957

and endemically re-occurring in Transkei, South Africa (Dutton, 1996) and later reported in China (Li et al, 1980). A different form of tumor, primary liver cancer, was also linked to $FB_1$ consumption in Haimen province, China. Carcinogenic properties of $FB_1$ observed in rodents are not associated with its direct genotoxicity. As a potent promoter of apoptosis, $FB_1$ causes further regeneration that is characterized by increased cell proliferation in affected tissues. Consequently, the constant elevation of DNA replication in exposed tissues has the potential for increasing the likelihood of cancer induction (Riley et al., 2001). Considering the available evidence the International Agency for Research on Cancer has declared toxins of *Fusarium verticillioides* as potential human carcinogens graded as class 2B (Vainio et al., 1993).

**In vitro studies on the toxicity of Fumonisin $B_1$ in different cell lines**

Different cell lines respond differently to fumonisin $B_1$ treatment. Diverse cellular responses credited to fumonisin $B_1$ exposure include apoptosis, proliferation and cell cycle arrest. $FB_1$ induces apoptosis in various cell lines such as HT29 cells (human colonic cell line) (Schmelz et al., 1998), and porcine renal epithelial (LLC-PK1) cells (Yoo et al., 1992), while Murine or Human leukemia cells (U937) do not undergo apoptosis, when treated with fumonisin $B_1$ (Ciacci-Zanella et al., 1998; Bose et al., 1995). Primary rat hepatocytes and liver slices (Norred et al., 1996) are quite resistant to fumonisin $B_1$-induced apoptosis.

FB1 induced cell cycle arrest in the G1 phase in African green monkey kidney cells (CV-1) but CV-1 cells transformed by the simian virus 40 (SV40) large T antigen (COS-7) are unaffected by the same levels of fumonisin $B_1$ (Wang et al., 1996). Hepatocyte mitotic activity, oval cell proliferation and formation of regenerative nodules were demonstrated in

rats fed with fumonisin $B_1$ containing diet (Lemmer et al., 1999). Taken together these studies indicate that some cells are responsive to fumonisin $B_1$ induced apoptosis while some are resistant and the ability of fumonisin $B_1$ to induce apoptosis may select for cells resistant to apoptosis, providing a way to induce cancer.

**Fumonisin $B_1$ and sphingolipid biosynthesis:**

Structural similarity of fumonisin $B_1$ to one of the intermediates in the *de novo* pathway of sphingolipid biosynthesis implicated the possibility of $FB_1$-related interference with sphingolipid metabolism. Indeed, it has been shown that $FB_1$ is a potent and specific inhibitor of ceramide synthase (sphinganine and sphingosine-N-acyl tranferase), a key enzyme in sphingolipid synthesis (Wang et al., 1991; Merril et al., 1993). Sphingolipids are integral part of all eukaryotic cell membranes, providing the highly resistant protective layer on the outer side of the lipid membrane. Their crucial role in signal transduction pathways, modulating cell growth, differentiation and death is known and expanding (Merrill, 2002). Their *de novo* synthesis proceeds in a number of steps modifying the core-structure (sphinganine) into further intermediates (i.e. ceramide) and more complex final products such as sphingomyelin and gangliosides, which are vital components of the brain white matter (Riboni et al., 1997).

The events resulting in the deregulation of sphingolipid metabolism include inhibition of dihydroceramide synthesis, an increase in free sphinganine, a decrease in reacylation of sphingosine derived from complex sphingolipid turnover (and consequent increase), an increase in sphingoid base degradation products (i.e. sphingosine (sphinganine) 1-phosphate, ethanolamine phosphate and fatty acid aldehydes). The elevation in the levels of sphinganine

and sphingosine in tissue and serum has been established as an accepted biomarker of exposure to $FB_1$ (Solfrizzo et al., 2004)

Initially, the ceramide levels do not decrease after $FB_1$-related inhibition of ceramide synthase, which may be due to the breakdown of sphingomyelin caused by TNFα-dependent activation of sphingomyelinase, resulting in compensatory ceramide synthesis but later there is depletion of complex sphingolipids (Tsunoda et al., 1998). The $FB_1$-induced disruption of sphingolipid metabolism usually precedes and is tightly correlated with the occurrence and severity of the *in vivo* toxicity (Voss et al., 1996).

**Role of cytokines in fumonisin $B_1$ toxicity:**

Fumonisin $B_1$ treatment caused an increased expression of TNFα, interferon (IFN) γ, and interleukine (IL) 12 in liver without any change in kidney or spleen, suggesting the localized site of their production (Bhandari et al., 2002). Cells involved in TNFα production following fumonisin $B_1$ treatment were identified as Kupffer cells in liver (Bhandari et al., 2002). Macrophages derived from animals treated with fumonisin $B_1$ also produce higher amounts of TNF α in response to lipopolysaccharide activation (Dugyala et al., 1998). TNFα was also induced in macrophages directly treated with fumonisin $B_1$ and pretreatment of mice with anti TNFα antibodies prevented acute hematological effects of fumonisin $B_1$ (Dugyala et al., 1998). There is considerable evidence that TNFα and interferon γ signaling pathways play an important role in fumonisin $B_1$ induced toxicity *in vitro* (in sensitive cells) and *in vivo*. Deletion of tumor necrosis factor receptors 1 and 2 and interferon γ genes reduced fumonisin $B_1$-induced hepatotoxicity in mice (Sharma et al., 2001, 2003).

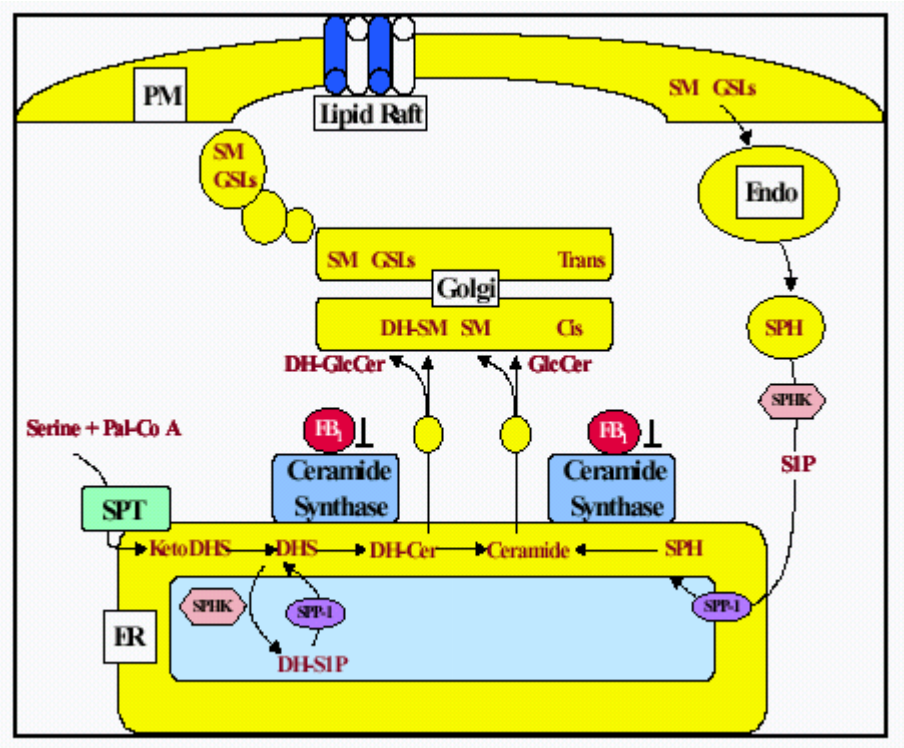


**Figure. 2.2** Scheme depicting the bioactive intermediates of sphingolipid biosynthesis. Dihydroceramide and ceramide biosynthesis takes place at the cytosolic surface of the ER. In the membrane recycling/salvage pathway, sphingosine produced from sphingolipids in the lysosome is phosphorylated by sphingosine kinase in the cytosol to S1P and dephosphorylated in the ER by SPP-1, where it is reutilized for synthesis of ceramide. Inhibition of ceramide synthase by $FB_1$ results in accumulation of sphingoid bases and disruption of complex sphingolipid metabolism. Abbreviations: SPHK- Sphingosine Kinase, SPP1- Sphigosine-1-phosphate phosphatase, Endo-Endosomes, SPT- Serine Palmitoyl Transferrase, PM- Plasma membrane, ER- Endoplasmic Reticulum, SPH– Sphigosine, S1P- Sphingosine-1-Phosphate, DHS- Dihydrosphingosine, SM- Sphigomylin, GSL- Glycosphingolipids. GlcCer– GlycosylCeramide, Pal-CoA– Palmityl Coenzyme A.

However, fumonisin $B_1$ hepatotoxicity was enhanced by deletion of *TNFα* gene or unchanged in mice lacking both TNFα receptors, which suggests that TNFα has paradoxical role in fumonisin $B_1$ toxicity (Sharma et al., 2002). $FB_1$ alters expression of a wide array of cytokines and mediators of apoptotic pathway. Fumonisin $B_1$ increased expression of mediators of apoptosis such as caspase 8 in the liver, which are involved in the TNFα signaling pathway and oncogenic transcription factors such as b-Myc, c-Myc and Max, in the kidney (Bhandari et al., 2002).

**Crosstalk between macrophage and other cell types**

In normal liver, close proximity of different cells such as hepatocytes, Kupffer cells, T cells and other nonparenchymal cells provide an excellent microenvironment for physiological interactions between these cells (Kmiec, 2001). It has been increasingly recognized that both under normal and pathological conditions, substances released from neighboring nonparenchymal cells regulate many hepatocyte and Kupffer cell functions. Kupffer cells are the most abundant macrophage population in the body and when activated by various toxins produce proinflammatory cytokines which have been implicated in liver damage.

In various models of acute liver injury by concavalin A (Con A) or *Pseudomonas* exotoxin A (PEA), where T cells seem to be responsible for liver damage it has been shown that absence of Kupffer cells restricts the area of necrosis (Schumann et al., 1998). A remarkable cross talk between macrophages and T cells involving TNFα and IFNγ has been suggested in other in vitro co culture experiments.

It was shown that TNFα and IL-12 produced by the Kupffer cells in liver can act on NK cells or Th 1 cells to produce IFNγ (Gantner et al., 1996). IFNγ can then further activate the Kupffer cells to produce more TNFα, and IL-12 eventually producing more IFNγ from the Th1 cells, thus generating a positive feedback loop between the two cells. These two cytokines, TNFα and IFNγ have synergistic effects in producing liver damage (Ozmen et al., 1994). Similar crosstalk between macrophages and cell types other than the T cells is also important. Interactions of this kind have been reported in several *in vitro* co culture experiments involving alveolar macrophages and type II pneumocytes (epithelial cells) (Tao et al., 2002) or microglia and neurons (Hemmer et al., 2001), where cytokine production was augmented due to induction of cross-talk between cells in response to toxins.

**Liver nonparenchymal epithelial cells (NPECs)**

Liver lobule is formed by parenchymal cells i.e. hepatocytes and nonparenchymal cells. Among nonparenchymal cells there are small epithelial cells that are phenotypically distinct from either hepatocytes or biliary epithelial cells and seem to have stem cell like potential to differentiate into hepatocytes and biliary epithelial cells (Grisham, 1995). Role of these cells in regenerative response following loss of cells through apoptotis or necrosis has been extensively studied (Thorgeirsson, 1996). Recent studies on liver regeneration have demonstrated that apart from hepatocytes, multipotential NPEC system also generates differentiated lineages needed for liver regeneration under situations where hepatocytes are unable to respond or are functionally compromoised (Thorgeirsson, 1996). Rats treated with 2-acetylaminofluorene (2-AAF) for 2 weeks have mitotically inhibited hepatocytes, under these

conditions powerful activation of the NPECs and their differentiation to hepatocytes has been established (Evarts et al., 1987)

**Cytokines and liver regenerartion**

According to widely accepted multi step model of liver regeneration, cells require priming by cytokines such as TNFα and IL-6 which make them responsive to growth factors (Michalopoulos et al., 1997). Administration of anti TNFα antibodies inhibits liver regeneration (Akerman et al., 1992) and mice lacking TNFR-1 receptors show reduced DNA replication after partial hepatectomy (Yamada et al., 1997). TNF injected in to rats at relatively high doses induces cell replication, which occurs first in NPECs followed by hepatocytes (Fausto, 2000).

**Sphingosine kinase (SK):**

Catabolism of sphingosine requires two independent and sequential steps. In cell free system dihydrosphingosine is cleaved to ethanolamine phosphate and palmitaldehyde (Keenan et al., 1969). First step involves phosphorylation of hydroxyl group on the first carbon of sphingosine and is mediated by Sphingosine Kinase. Sphingosine-1-phosphate lyase cleaves the product into ethanolanime phoshate and corresponding aldehyde. Due to relative inabundance of sphingosine-1-phosphate in cells, phosphorylation by sphingosine kinase is the rate-limiting step in this process.

Sphingosine kinase is highly conserved enzyme found in organisms as diverse as plants, yeast, worms, flies and mammals. Two distinct isoforms of SK, designated SK1 and SK2, have been cloned (Kohama et al., 1998; Liu et al., 2000). Although highly similar in amino

acid sequence and possessing five conserved catalytic domains related to that of the diacylglycerol kinase family (Pitson et al., 2000), SK2 diverges in its amino terminus and central region. These two isozymes have different kinetic properties and also differ in developmental expression (Liu et al., 2002), implying that they may have distinct physiological functions. Human sphingosine kinase is widely expressed with highest levels in adult liver, kidney, heart, and skeletal muscles. About 70% of sphingosine kinase activity was found in cytosol and only about 30% was membrane associated (Nava et al, 2000).

Recent studies suggest that SK1 and formation of sphingosine-1-phosphate (S1P) are linked to cell growth and survival (Spiegel and Milstien 2002). Diverse external stimuli, particularly growth and survival factors stimulate SK1 and intracellularly generated S1P has been implicated in their mitogenic and anti-apoptotic effects. TNFα stimulates SK1 leading to activation of extracellular signal-regulated kinase (ERK) (Pitson et al., 2000) and of NF-κB, critical for prevention of apoptosis (Xia et al., 2002). Similar to platelet-derived growth factor (PDGF) (Olivera et al., 1999), the potent angiogenic factor vascular endothelial growth factor (VEGF) can also stimulate SK1, producing S1P, which mediates VEGF-induced activation of Ras and consequently, ERK signaling and cell growth (Shu et al., 2002). Indeed, Ras transformation requires SK1 activation (Xia et al., 2000).

Moreover, expression of SK1 enhanced proliferation and growth in soft agar, promoted the G1-S transition, protected cells from apoptosis (Olivera et al., 1999: Edsall et al., 2001), and induced tumor formation in mice (Xia et al., 2000). Inhibition of this enzyme activity markedly inhibits cell growth or induces apoptosis (Meyer zu et al., 1998).

**Sphingosine-1-phosphate lyase (S1P lyase):**

S1P lyase is pyridoxal 5 phosphate dependent enzyme with a conserved pyridoxal dependent decarboxylase domain. Once formed S1P is rapidly degraded by S1P lyase to hexadecenal and phoshoethanolamine (Stoffel et al, 1973). Hence intracellular S1P levels are determined by the balance of Sphingosine kinase mediated synthesis and it's degradation by S1P lyase and S1P phosphohydrolase (Kihara et al, 2003). S1P lyase is thought to play central role in keeping intracellular S1P or dihydroS1P levels low.

Inhibition of *Caenorhabditis elegans* S1P Lyase expression by RNA interference causes accumulation of phosphorylated and unphosphorylated long-chain bases and leads to poor feeding, delayed growth, reproductive abnormalities, and intestinal damage (Mendel et al., 2003). Disruption of S1P Lyase gene induced differentiation to primitive endoderm in mouse F9 embryonal carcinoma cells, which suggests role of S1P regulation by S1P lyase in differentiation (Kihara et al, 2003).

Sphingosine-1-phosphate lyase is ubiquitously distributed, being present in yeast, protozoa, and mammals. One notable exception is platelets from man, rabbit and pig (Yatomi et al, 1996). Highest activity was found in microsomal fractions with some activity in mitochondrial and nuclear fractions. Lyase is an integral membrane protein, the catalytic sites or essential domains of which face the cytosol in the cell, which implies that substrate does not have to cross the membrane and reaction products are released into the cytoplasm (Van Veldhoven et al., 1991).

Primary breakdown products of phosphorylated sphingoid bases are actively metabolized *in vivo*. Ethanolamine phosphate is incorporated in phosphotidylethanolamine and subsequently can be transferred to phosphotidylcholine and sphingomyelin. The other fragment, fatty aldehyde is oxidized to fatty acid. After activation, the acyl-CoA is either degraded via β-oxidation or esterified in glycerolipids (Van Veldhoven 2000).

**Sphingosine-1-phosphate posphohydrolase (SPP):**

Besides cleavage, dephosphorylation of sphingosine-1-phosphate might be another attenuation mechanism. The importance of dephosphorylation is perhaps illustrated by the severe toxicity of sphinganine-1-phosphonate, which cannot be dephosphorylated but is still cleaved by the lyase. S1P being an important mediator of signal transduction is tightly regulated through its synthesis catalyzed by sphingosine kinase and degradation by S1P lyase and Phosphohydrolase. Recently two different kinds of enzymes SPP-1 (Le Stunff et al., 2002) and SPP-2 (Ogawa et al, 2003) with sphingosine phosphate phosphohydrolase (SPP) activity have been characterized in human embryonic kidney (HEK) 293 cells. Both SPPs reside predominantly in ER, thus it is likely that SPP is one of the enzymes for the regulation of sphingoid bases and ceramide levels as ER also contains the other enzymes of ceramide biosynthesis and sphingolipid metabolism.

Dihydro-S1P is also a substrate for SPP forming dihydrosphingsine, which is then converted by a fumonisin $B_1$ sensitive ceramide synthase to dihydroceramide. Stunff et al. (2002) have shown that when S1P or dihydro S1P are added to SPP transfected cells, S1P is dephosporylated to sphingosine which forms ceramide and induces apoptosis whereas dihydro

S1P also forms dihydroceramide but it does not accumulate due to it's more efficient use for sphingomyelin or glycosphingolipid biosynthesis.

**Sphingoid bases and apoptosis:**

Intensive investigation in past decade has confirmed the role of sphingolipid metabolites, ceramide, sphingosine and sphingosine-1-phosphate in controlling apoptosis. There is considerable debate about which is primary inducer of apoptosis, ceramide or sphingosine (Cuvillier et al., 2000). Uptake and distribution within the cell of exogenously added sphingolipids is complex, direct correlation between effects of exogenous sphingolipids and increase in endogenous levels cannot be made.

Ceramide and sphingosine are interconvertable sphingolipid metabolites, ceramide synthase can form ceramide from sphingosine and ceramide can be degraded to sphingosine by ceramidase. Ceramide generated from sphingomyelin by activation of sphingomyelinase has been implicated in apoptosis induced by diverse apoptotic stimuli, such as TNFα or Fas ligand (Kolesnick and Kronke 1998). In addition, administration of exogenous ceramide analogs can mimic apoptotic cell death. However many have challenged this direct role of ceramide based on the observations that ceramide increase in many such situations is followed by increase in sphingosine levels which is proapoptotic and is itself capable of inducing apoptosis when added exogenously to many cell types (Ohta et al., 1994; Hung et al., 1999).

Recent data suggest that ceramide and sphingosine could signal mitochondrial apoptosis by inhibiting the protein kinase Akt, responsible for Bad phosphorylation, hence leading to inhibition of anti-apoptotic protein Bcl-2 by Bad (Chang et al, 2001). Although some believe that due to it's lysosomotropic properties sphingosine may work by relocating lysosomal

hydrolases to cytosol and this partial leakage may lead to early events of apoptosis (Kagedal et al, 2001), little is known overall about potential function of sphingosine in mitochondria and how it signals apoptosis.

**Sphingosine-1-phosphate (S1P): Regulation of proliferation and survival:**

S1P is a lysophospholipid derived from phosphorylation of sphingosine by-sphingosine kinases. As a specific ligand for a family of five G protein coupled receptors (S1P1-5) (Chun et al, 2002), which are ubiquitously expressed and couple to various G proteins (Goetzl and An 1998), S1P regulates a wide variety of important cellular processes, including cytoskeletal rearrangements and cell movements (Graeler et al, 2002), angiogenesis and vascular maturation (Garcia et al, 2001), and heart development (Kupperman et al, 2000). An important function for S1P in lymphocytes and immune responses emerged from studies with the immunosuppressive drug FTY720, a sphingosine analogue, which is phosphorylated by SK1 (Brinkmann et al, 2002). Although there is no doubt that S1P acts extracellularly, several studies suggest that this important bioactive lipid, like its precursors sphingosine and ceramide (N-acyl sphingosine), may also have intracellular functions important for calcium homeostasis (Meyer zu et al., 1998), cell growth (Olivera et al., 1999), and suppression of apoptosis (Cuvillier et al., 1996). Because intracellular targets of S1P have not yet been identified, its intracellular function is still a matter of debate (Hla et al, 2001).

There are many reports which suggest relationship between S1P production and early innate inflammatory activation, many external stimuli are capable of activating sphingosine kinase, including TNFα and IL1-β and PDGF. It is interesting to note that both the precursors for S1P (sphingomyelin) and some of the receptors for S1P (S1P1) colocalize in the same

plasma membrane microdomain, the caveolae (Rosen and Liao 2003). Mitogenic response of S1P have shown to be unrelated to it's binding with cell surface receptor as microinjection of SPP in cell causes increased DNA synthesis with out binding to surface receptors (Lee et al, 1998).

**The ceramide/S1P rheostat**

While ceramide acts as mediator of stress response, causing cell cycle arrest and /or apoptosis (Hannun et al, 1996) S1P counteracts these effects (Cuvillier et al, 1996). Thus, it has been suggested that the balance between cellular concentration of ceramide and S1P determines physiological fate of cell. Deletion of long chain sphingoid base phosphate phosphatase in *Saccharomyces* leads to accumulation of phosphorylated long chain sphingoid bases and reduced ceramide levels and concomitant enhancement of survival upon severe heat shock (Mandala et al, 1998). Therefore in addition to it's role as a ligand for Edg-1 (endothelial differentiation gene) receptor, the cellular level of S1P is important for cell proliferation and survival.

**Mitogen activated protein kinases (MAPKs) in cell survival and apoptosis**

MAPKs are one of the Ser/Thr protein kinases, which are responsible for transmitting extracellular signals in to the nucleus (Robinson et al., 1997; Schaeffer et al., 1999). Five different types of MAPKs have been identified, which include extracellular signal-related kinase (ERK1/2), c-Jun $NH_2$ terminal kinase (JNK) and p38MAPK, ERK3 and ERK5 (Schaeffer et al., 1999). Cellular effects downstream to MAPK signaling are diverse and include cell growth, cell differentiation and cell death (Robinson et al., 1997).

ERK is activated by a variety of growth factors, insulin and oxidative stress and leads to a wide array of cellular responses (Bergmann et al., 1998). ERK pathway can transmit both anti-apoptotic and pro-apoptotic signals, probably depending on the nature of the extra cellular stimuli (Bergmann et al., 1998).

Deactivation of p38 MAPK has been shown to lead to both anti-apoptotic and pro-apoptotic responses. Nerve growth factor induced dephosphorylation of p38 prevents Bcl-2 phosphorylation and apoptotic response in lymphoblastoid CESS B cell line (Rosini et al., 2004), whereas, anticancer desipeptide, FR901228, induced apoptosis of ras-transformed 10T1/2 cells through suppression of p38 pathway (Fecteau et al., 2002).

JNK is a member of the mitogen-activated protein (MAP) kinase family and is activated by a variety of extracellular stimuli through JNK kinases and multiple MAP kinase kinase kinases (Chang et al., 2001). Activated JNK, phosphorylates and activates c-Jun, a component of the transcription factor AP-1 and many other targets as well (Chang et al., 2001). The role of JNK activation in mediating the TNF receptor signaling is uncertain, ranging from being protective, irrelevant or pro-apoptotic, which depends on the stimuli and cell types (Liu, 2003). However, there is strong evidence indicating that JNK activation could increase TNFα-induced apoptosis in hepatocytes (Liu et al., 2002; Schwabe et al., 2004). JNK-mediated apoptosis in hepatocytes was shown to involve mitochondrial permeability transition, release of cytochrome c and activation of caspase-3, -8 and -7, suggesting that JNK acts upstream of the mitochondria (Liu et al., 2002). A recent report provided strong evidence that JNK activation in TNFα signaling involves release of Smac/DIABLO from the mitochondria, but not cytochrome c (Deng et al., 2003). The released Smac/DIABLO then disrupts the TRAF2-

cIAP1 complex, relieving the inhibition imposed by TRAF2-cIAP1 on caspase 8 activation and induction of apoptosis (Deng et al., 2003). TRAF2 also mediates the activation of the JNK pathway, as shown by JNK inhibition by TRAF2Δ and in TRAF2 (−/−) mice (Natoli et al., 1997).

Increases in hepatocellular oxidation stress such as those that occur with hepatic overexpression of cytochrome *P*-450 isoforms promoted TNFα mediated apoptosis in hepatocytes through increased activity of c-Jun $NH_2$-terminal kinase (Liu et al., 2002)

There are only few reports indicating the role of MAPKs in fumonisin $B_1$ induced toxicity in mammalian cells (Pinelli et al., 1999; Rentz et al., 2005; Wattenberg et al., 1996). A differential activation of JNK but not of ERK or p38 was reported in LLC-PK1 cells by fumonisin $B_1$ (Johnson et al., 2003). Recently, a decrease in ERK has been reported in LLC-PK1 cells at 24 h (Rentz et al., 2005). In our other studies, we have noticed the induction of both total and phosphorylated JNK in livers of fumonisin $B_1$-treated mice (unpublished data).

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# CHAPTER 3

## SPHINGOSINE KINASE ACTIVITY CONFERS RESISTANCE TO APOPTOSIS BY FUMONISIN $B_1$ IN HUMAN EMBRYONIC KIDNEY (HEK-293) CELLS [1]

[1]Sharma N., He Q., and Sharma R.P. 2004. *Chemico-Biological Interactions*. 30;151(1):33-42. Reprinted here with permission of the publisher.

**Abstract**

Fumonisin $B_1$ induces cytotoxicity in sensitive cells by inhibiting ceramide synthase due to its structural similarity to the long-chain backbones of sphingolipids. The resulting accumulation of sphingoid bases has been established as a mechanism for fumonisin $B_1$ cytotoxicity. We found that despite the accumulation of sphinganine, human embryonic kidney (HEK-293) cells are resistant to fumonisin $B_1$ toxicity; 25 μM fumonisin $B_1$ exposure for 48 h did not increase apoptosis in these cells while it did so in sensitive porcine kidney epithelial (LLC-$PK_1$) cells. In this study DL-*threo*-dihydrosphingosine, the sphingosine kinase inhibitor (SKI), considerably increased the sensitivity of HEK-293 cells to fumonisin $B_1$. Treatment of these cells with 25 μM fumonisin $B_1$ and 2.5 μM SKI increased apoptosis. Sphingoid bases, sphinganine or sphingosine, added to cell cultures induced apoptosis by themselves and their effects were potentiated by SKI or fumonisin $B_1$. Addition of physiological amounts of sphingosine-1-phosphate prevented the toxic effects induced by SKI inhibition and fumonisin $B_1$. Results indicated that HEK-293 cells are resistant to fumonisin $B_1$ due to rapid formation of sphingosine-1-phosphate that imparts survival properties. Taken together these findings suggest that sphingoid base metabolism by sphingosine kinase may be a critical event in rendering the HEK-293 cells relatively resistant to fumonisin $B_1$-induced apoptosis.

Key words: Fumonisin $B_1$; Sphingosine-1-phosphate; Human embryonic kidney cells; Sphingosine kinase

## 1. Introduction

Fumonisins are structurally related toxic and carcinogenic mycotoxins produced by *Fusarium verticillioides*, a common fungal contaminant of maize. Fumonisin $B_1$ is the most abundant fumonisin and occurs naturally in contaminated foods and feeds [1]. Fumonisin $B_1$ toxicity is specific to tissue and also depends on species and gender of the treated animal. Fumonisin $B_1$ causes equine leukoencephalomalacia and porcine pulmonary edema [2, 3]. In vivo studies demonstrated that fumonisin $B_1$ induced apoptosis in kidney and liver of rodents [4-6]. It was hepatocarcinogenic in male BD IX rats [7] and a nephrocarcinogen in male F344/N rats, whereas it was hepatocarcinogenic in only female B6C3F1 mice [8, 9]. An association between human esophageal cancer and fumonisin $B_1$ in areas highly contaminated with these mycotoxins was reported [10].

Fumonisins are structurally related to sphingoid bases and cause inhibition of ceramide synthase (sphinganine- or sphingosine-*N*-acyltransferase) leading to accumulation of corresponding free sphingoid bases, sphingoid base metabolites, and depletion of more complex sphingolipids [11, 12]. Physiological consequences possible after ceramide synthase inhibition include apoptosis caused by accumulation of free sphingoid bases (sphinganine and sphingosine), increased proliferation caused by increased sphingosine-1-phosphate, or/and decreased ceramide and altered lipid raft function due to disruption of complex sphingolipids synthesis/transport [12].

It has been shown that fumonisin $B_1$-induced apoptosis, necrosis, and inhibition of proliferation in pig renal epithelial (LLC-$PK_1$) cells [13], human colonic cells HT29 [14] and human keratinocytes [15] depend on sphingoid base accumulation. However, other studies

demonstrated that neoplastic African green monkey kidney cells (COS-7) [16] and primary hepatocytes [17] were resistant to the toxic effects of fumonisin $B_1$ despite sphinganine accumulation.

Ceramide and sphingosine are interconvertable sphingolipid messengers; intensive investigations in past decade have confirmed the role of ceramide and sphingosine in inducing apoptosis. However, there is considerable debate whether the ceramide or sphingosine is the primary inducer of apoptosis, it [18]. In fumonisin $B_1$ sensitive cells the same final pathway may mediate apoptosis by which sphingoid bases affect apoptosis; accumulation of sphingoid bases directly correlates with their cytotoxicity [19]. In HL-60 cells [20, 21] and Hep3B hepatoma cells [22] fumonisin $B_1$ did not affect the apoptosis directly induced by sphingosine or sphinganine indicating that these sphingoid bases could independently produce apoptosis without getting converted to ceramide.

Recent data suggest that sphingoid bases could signal mitochondrial apoptosis by inhibiting the protein kinase Akt, responsible for Bad phosphorylation and hence leading to inhibition of anti-apoptotic protein Bcl-2 by Bad [23, 24,25]. Events leading to apoptosis in fumonisin $B_1$ treated cells have not been characterized, although a role for Bax has been suggested based on the observation that Bcl-2 can protect $p53^{-/-}$ cells from fumonisin $B_1$-induced apoptosis [26].

Levels of free sphingoid bases decrease consistently in fumonisin $B_1$-treated cells between 48 and 72 h indicating the induction of sphingoid base metabolism [19]. Catabolism of sphingoid bases requires two independent and sequential steps. The first step involves phosphorylation of sphingoid bases to their 1-phosphate derivative and is mediated by

sphingosine kinase [27]. Second step involves cleavage of the resulting product by sphingosine-1-phosphate lyase into ethanolamine phosphate and corresponding aldehyde [17]. Due to relative low levels of sphingosine-1-phosphate in cells, it is evident that phosphorylation of sphingosine by sphingosine kinase is the rate-limiting step in this process.

Sphingosine kinase in human tissues is widely expressed with highest levels in adult lung, spleen, kidney, heart, and brain [28]. Activation of sphingosine kinase and formation of sphingosine-1-phosphate are linked to cell growth and survival [29-31]. Diverse external stimuli, particularly growth and survival factors stimulate sphingosine kinase and intracellularly generated sphingosine-1-phosphate has been implicated in their mitogenic and anti-apoptotic effects [32, 33].

In our preliminary experiments, we found that human embryonic kidney (HEK-293) cells were resistant to cytotoxic effects of up to 50 μM fumonisin $B_1$ whereas porcine kidney (LLC-$PK_1$) cells show significant toxic response with 10 μM fumonisin $B_1$ [13, 19]. We hypothesized that the HEK-293 cells are resistant to apoptotic effects of fumonisin $B_1$ due to rapid conversion of accumulate sphinganine or sphingosine to their respective phosphates by sphingosine kinase and the antiapoptotic and proliferative role of the 1-phosphate derivatives. In the present study we tested above hypothesis by employing specific inhibitor of sphingosine kinase, the enzyme that converts sphingoid bases to their respective phosphates. DL-*threo*-dihydrosphingosine was employed as sphingosine kinase inhibitor (SKI).

## 2. **Materials and Methods**

### *2.1. Materials*

Fumonisin $B_1$ (purity>95%) was obtained from Programme on Mycotoxin and Experimental Carcinogenesis (Tygerberg, South Africa). Human Embryonic Kidney cells (HEK-293) were obtained from American type culture collection (CRL-1573, ATCC, Manassas, VA). Dulbecco's Modified Eagle's Medium was purchased from Gibco (Carlsbad, CA). Sphingolipids and DL-*threo*-dihydrosphingosine were procured from Biomol (Plymouth Meeting PA). Annexin V and propidium iodide were purchased from Molecular Probes (Eugene, OR). All other reagents were obtained from Sigma (St. Louis, MO) and were of tissue culture grade.

### *2.2. Preparations of fumonisin $B_1$ and sphingolipid stock solutions*

Fumonisin $B_1$ was dissolved in phosphate-buffered saline (PBS) at 1 mM, and then diluted into growth medium and added to cultures to achieve proper final concentrations. Relatively high concentration of fumonisin $B_1$ was used as the HEK-293 cells are not responsive to it up to 50 μM concentration. Control cultures were treated with similar dilutions of the vehicle but without fumonisin $B_1$. Free sphinganine, free sphingosine, and DL-*threo*-dihydrosphingosine were first dissolved in ethanol to a concentration of 50 mM and then were prepared in 1.0 ml of 1.5 mM fatty acid-free bovine serum albumin (BSA) to get a concentration of 1 mM as previously described [19]. An equal volume of absolute ethanol was added into BSA and processed as above as a BSA vehicle control.

*2.3. Cell Culture and treatment*

HEK-293 cells were grown in Dulbecco's Modified Eagle's Medium containing 100 units/ml penicillin and 10% non-heat-inactivated fetal bovine serum (Atlanta Biologics, Atlanta, GA) in 5% $CO_2$ at 37 °C. Cells were grown in 75 $cm^2$ culture flasks and sub cultured when the cells reached 70–80% confluence. For all experiments cells were seeded in the CD 293 chemically defined media (Gibco, Carlsbad, CA), which was devoid of any active biolipids, containing 0.5% fetal bovine serum, 2 mM glutamine and 5 µg/ml insulin (Sigma, St. Louis, MO). Cells were seeded 18 h before treatment with fumonisin $B_1$, sphingosine, sphinganine or SKI in their 2nd or 3rd passages. Concentrations of each chemical and time points for the treatments were optimized in preliminary trials.

*2.4 Hoechst 33258 and propidium iodide staining*

Apoptotic changes in the nuclear chromatin of cells were evaluated by staining with the DNA binding fluorochrome Hoechst 33258 (bis-benzimide). Cell death was also analysed using propidium iodide (PI), a membrane impermeable dye, which binds to DNA by intercalating between the bases with little or no sequence preference. At the end of treatment, supernatant was removed and 50 µl of 30% glycerol/PBS solution of Hoechst 33258 (8 µg/ml) or PI (1 µg/ml) was added. The plates were read at 346/460 nm or 535/617 nm (excitation/emission) for Hoechst 33258 or PI fluorescence, respectively, using a Spectramax Gemini fluorescent plate reader (Molecular Devices, Irvine, CA).

*2.5. Mitochondrial function assay*

3[4,5-dimethyl thiazolyl-2]2,5-diphenyl tetrazolium bromide (MTT) was employed to observe cell viability. Cells were seeded at $3\times10^4$ cells/well in 96-well plates and treated with the indicated concentration of treatments in total volume of 200 µl. Cells were incubated with addition of 20 µl MTT (5 mg/ml) 4 h before the end of treatment. At the end of treatment, 120 µl of media containing MTT was taken out from each well and 100 µl of 0.02 N HCl-isopropanol (warm) was added to dissolve formazan crystals. The absorbance of each well was measured using a scanning spectrophotometer (Bio-Tek Instrument, INC, Winooski, VT, USA).

*2.6. Determination of intracellular free sphingoid bases*

At the end of 48 h treatment with fumonisin $B_1$, the medium was aspirated, and cells were washed once with ice-cold PBS and then scraped into 1 ml ice-cold PBS. An aliquot (0.1 ml) of cell suspension in PBS was transferred to another tube, spun at 2,000g, 4°C for 5 min. The cells were lysed and stored at -85°C until analysis of total protein by Bradford reagent (Bio-Rad Laboratories, Hercules, CA) using a 96-well plate according to the manufacturer's protocol. Free sphingoid bases were extracted from the remainder of cells by using the modified method described previously [34]. The relative amounts of free sphinganine and sphingosine in base-treated cell extracts were determined by high performance liquid chromatography (HPLC) as described earlier. Sphingoid bases were quantified based on the recovery of a $C_{20}$ sphinganine internal standard. The limit of detection for $C_{20}$ was 26.8 fmol/assay (equivalent to 1 fmol/ g protein).

2.7. Statistical analysis of data

Each experiment was repeated three times with reproducible results consistently. The results expressed as mean ± standard error of combined results from three independent experiments with each performed in triplicates. Differences among treatments were analyzed statistically by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. The level of $p \leq 0.05$ was considered significant for all comparisons.

## 3. **Results**

### *3.1. Fumonisin $B_1$ and SKI decreased the viability of HEK-293 cells when used together*

HEK-293 cells are resistant to cytotoxic effects of fumonisin $B_1$ considering that even 50 µM fumonisin $B_1$ for 72 h could not increase significant LDH release from these cells. (Data not shown). Fumonisin $B_1$ at 25 µM and SKI at 2.5 µM alone had no effect on viability of HEK-293 cells. Cell viability decreased significantly in HEK-293 cell culture, when treated together with 25 µM fumonisin $B_1$ and 2.5 µM SKI for 72 h. (Fig.3 1), determined by MTT assay.

### *3.2. Induction of apoptosis in HEK-293 cells treated with fumonisin $B_1$ and SKI*

Exposure to 25 µM fumonisin $B_1$ alone for 48 h did not induce apoptosis in HEK-293 cells, however treatment with 25 M fumonisin $B_1$ and 2.5 µM SKI together for 48 h resulted in increased apoptosis, measured by the fluorescence of Hoechst 33258 (Fig. 3.2).

Cell death in HEK-293 culture was a combination of apoptosis and necrosis. Cells treated with fumonisin $B_1$ and SKI together had ~20% apoptotic cells and ~ 8% necrotic cells, while in control wells less than 1% cells were undergoing either apoptosis or necrosis. Necrotic cell death was confirmed by nuclear staining with membrane impermeant dye propidium iodide, which is taken up only by necrotic cells as their cell membrane is damaged. To confirm apoptosis, cells were stained with annexin V and Hoechst 33258 dye. Early apoptotic cells stain with Annexin V and Hoechst 33258, which can bind to exposed phosphotidyl serine and fragmented nuclear chromatin respectively but exclude PI as their cell membrane is still intact. Cells treated with fumonisin $B_1$ and SKI together had significantly greater number of cells stained with, annexin V and Hoechst 33258 compared to control and SKI alone (Fig. 3.3a). Visual inspection under Olympus IX71 inverted fluorescence microscope (Olympus America, Melville, NY) confirmed the presence of increased number of cells exhibiting bright green staining of Annexin V on cell surface and blue staining of Hoechst 33258 on nucleus indicating apoptotic cells.

Overlay of Annexin V, Hoechst 33258 and PI staining in fumonisin $B_1$ and SKI treated cells showed predominance of apoptotoic cells stained with only Annexin V and Hoechst 33258 and presence of some necrotic cells which were stained by all three dyes (PI, Annexin V and Hoechst 33258) (Fig. 3.3b).

3.3. Increased accumulation of intracellular sphingoid bases in cells treated with fumonisin $B_1$ and SKI

HEK-293 cells were treated with 25 µM fumonisin $B_1$ and 2.5 µM SKI for 48 h. Treatment of cells together with SKI and fumonisin $B_1$ caused higher elevation of intracellular level of sphinganine compared to fumonisin $B_1$ treatment alone. Fumonisin $B_1$ alone did not increase intracellular level of sphingosine, whereas exposure of cells to fumonisin $B_1$ and SKI together caused significantly higher accumulation of sphingosine (Fig. 3.4)

*3.4. Fumonisin $B_1$ and SKI induced apoptosis was further increased by sphingoid bases*

Sphingoid bases are known to induce apoptosis. We treated HEK-293 cells with 5 µM sphinganine or 5 µM sphingosine alone or in the presence of 25 µM fumonisin $B_1$ and 2.5 µM SKI for 48 h. Apoptosis was measured by recording the fluorescence of Hoechst 33258 at 346/460 nm. Sphingosine when added to cell cultures induced apoptosis by it self. Fumonisin $B_1$ and sphingosine treated cells had more apoptotic cells compared to fumonisin $B_1$ treatment alone. Sphingosine increased the apoptosis induced by the combined treatment of fumonisin $B_1$ and SKI (Fig. 3.5). Sphinganine treatment followed the similar trend and had the synergistic effects with SKI and fumonisin $B_1$ to induce apoptosis except that at 5 µM concentration sphinganine itself could not induce apoptosis (Fig. 3.6).

*3.5. Sphingosine-1-phosphate prevented the apoptosis induced by fumonisin $B_1$ and SKI*

HEK-293 cells were treated with 25 µM fumonisin $B_1$, 2.5 µM SKI and 1, 5 and 10 nM concentrations of sphingosine-1-phosphate for 48 h (Fig. 3.7). Sphingosine-1-phosphate significantly reduced the apoptosis induced by fumonisin $B_1$ and SKI. Apoptosis was measured

by Hoechst 33258 fluorescence at 346/460 nm. A similar trend was shown when sphinganine-1-phosphate (1-100 nM) was used instead of sphingosine-1-phosphate; however, the protection was not statistically significant (data not shown). Direct addition of either sphingosine-1-phosphate or sphinganine-1-phosphate in concentrations greater than 100 nM was cytotoxic in this cell line.

## 4. **Discussion**

We found that HEK-293 cells are resistant to fumonisin $B_1$-induced apoptosis. Results in the current study showed that inhibition of sphingosine kinase enzyme that converts sphingoid bases to their respective 1-phosphates makes HEK-293 cells sensitive to fumonisin $B_1$-induced apoptosis. In HEK-293 cells the rapid formation of sphingoid base-1-phosphate is the putative mechanism of protection from fumonisin $B_1$ toxicity. In most cell lines fumonisin $B_1$ blocks de novo ceramide biosynthesis and causes accumulation of free sphingoid bases but only selected cell lines are sensitive to toxic effects of fumonisin $B_1$ while others are resistant [16, 17]. Accumulation of sphingoid bases has been established as a mechanism for fumonisin $B_1$ induced apoptosis in various types of cells.

For fumonisin $B_1$ the $IC_{50}$ for inhibition of ceramide synthase is 0.1 μM in primary hepatocytes and doses as low as 1 μM caused almost complete inhibition of this enzyme leading to accumulation of sphingoid bases as quickly as in 3 h [11]. Elevation of sphinganine level by almost 20-fold in 24 h treatment with fumonisin $B_1$ is a common finding with most of

the cell lines. Sphingoid bases are toxic to cells [18] and are responsible for fumonisin $B_1$ cytotoxicity [13]. Inhibition of fumonisin $B_1$ cytotoxicity by myriocin (inhibits serine palmitoyltransferase activity) correlated well with this conclusion [14]. Since level of sphingosine depends on the rate of turnover of the complex sphingolipids it either does not change or increases little after fumonisin $B_1$ treatment [11].

It has been a question as why increased levels of sphinganine do not cause toxicity in some cells. Even though sphinganine levels rise significantly within hours with as low as 1 μM fumonisin $B_1$ treatment in sensitive and insensitive cells, high doses of fumonisin $B_1$ and long incubation periods ( > 24 h) are required to produce toxicity even in sensitive cells. Levels of free sphingoid bases decreased consistently in fumonisin $B_1$ treated cells between 48 and 72 h, which indicated temporal sphingoid base metabolism [19]. In J774A.1 cells exogenous sphingoid base initially accumulated and then were rapidly metabolized to their 1-phosphate derivatives; fumonisin $B_1$ caused inhibition of acylation of sphingoid bases and diverted them more towards phosphorylation [19]. About one third of the ethanolamine within phoshatidylethanolamine in J774A.1 cells was derived from long-chain base catabolism when fumonisin $B_1$ was added [35].

The role of sphingoid bases in cell death depends upon the balance between levels of ceramide and sphingosine both of which mediate apoptosis, and sphingosine-1-phosphate that promotes proliferation and survival. For instance, increases in ceramide and sphingosine levels by TNFα and Fas ligand lead to apoptosis of T lymphocytes, where as survival factors like protein kinase C stimulate sphingosine kinase, leading to increased sphingosine-1-phosphate levels, which suppress apoptosis [36]. Recently, the balance of sphingosine-1-

phosphate and sphingosine was reported to have a role in activation of mast cells, where high intracellular concentration of sphingosine inhibited cytokine production by preventing activation of extracellular signal-regulated kinase (ERK) pathway, while high intracellular concentration of sphingosine-1-phosphate activated ERK to stimulate cytokine production [37]. Study by Olivera et al. [38] emphasizes the importance of sphingosine kinase in determining cell fate as expression of sphingosine kinase in HEK-293 cells markedly increased production of sphingosine-1-phosphate, which induced proliferation.

The *threo* enantiomers of sphingosine and dihydrosphingosine were not phosphorylated by sphingosine kinase and were found to be potent competitive inhibitors of platelet sphingosine kinase activity *in vitro* [39]. Substrate and inhibition studies showed that the mixture of DL-*threo*-dihydrosphingosine was more potent and highly specific inhibitor of platelet sphingosine kinase [39]. DL-*threo*-dihydrosphingosine inhibits the production of sphingosine-1-phosphate by competitive inhibition of sphingosine kinase and is useful to investigate the function of sphingosine-1-phosphate in signal transduction processes.

In the current study SKI increased sensitivity of HEK-293 cells to fumonisin $B_1$ toxicity as treatment of these cells with fumonisin $B_1$ and SKI together increased apoptosis; whereas fumonisin $B_1$ alone or inhibitor alone were not cytotoxic. Inhibition of sphingosine kinase by DL-*threo*-dihydrosphingosine prevented conversion of sphingoid bases to their 1- phosphate derivatives and resulted in higher accumulation of sphingosine and sphinganine in fumonisin $B_1$-treated HEK-293 cells. Increased apoptosis with co-incubation of SKI and fumonisin $B_1$ can be explained by the loss of protective role of sphingosine-1-phosphate and toxic effects of

increased levels of sphingoid bases. Sphingoid bases (sphinganine or sphingosine) added to cell cultures induced apoptosis by itself and their effects were potentiated by sphingosine kinase inhibitor and fumonisin $B_1$, supporting our hypothesis.

Sphingosine-1-phosphate prevented the apoptosis caused by sphingosine kinase inhibition, when added to cells treated with fumonisin $B_1$ and SKI. However only very small concentrations of sphingosine-1-phosphate were able to prevent apoptosis and higher concentrations further increased the toxicity of fumonisin $B_1$ and SKI (data not shown). Intracellular concentration of sphingosine-1-phosphate is tightly regulated through its synthesis catalyzed by sphingosine kinase and degradation by sphingosine-1-phosphate lyase and phosphohydrolase. Recently two different kinds of enzymes with sphingosine-1-phosphate phosphohydrolase (SPP) activity, SPP-1 [40] and SPP-2 [41] have been characterized in HEK-293 cells. These enzymes are known to degrade exogenously added sphingosine-1-phosphate in cell cultures to sphingosine and subsequently produce apoptosis [40]. It is possible that in our study, higher concentrations of sphingosine-1-phosphate increased the apoptosis by getting converted to sphingosine by sphingosine-1-phosphate phosphohydrolase. It would be desirable to look at the effect of higher concentrations of sphingosine-1-phosphate after inhibiting the sphingosine-1-phosphate phosphohydrolase enzyme but the lack of specific inhibitor of this enzyme restricts such possibility.

In summary our results indicate that HEK-293 cells are resistant to apoptotic effects of fumonisin $B_1$ due to higher activity of sphingosine kinase, which enhances cell survival by forming sphingosine-1-phosphate. Activity of sphingosine kinase enzyme may have substantial bearing on different responses of fumonisin $B_1$ in other cell types. It is also possible that

fumonisin $B_1$ or SKI sensitized cells to each other by some other mechanisms. However, our findings are applicable to only HEK-293 cells and further investigations are required to determine whether or not this is a general mechanism for resistance to fumonisin $B_1$ in other cells *in vitro* and tissues *in vivo*.

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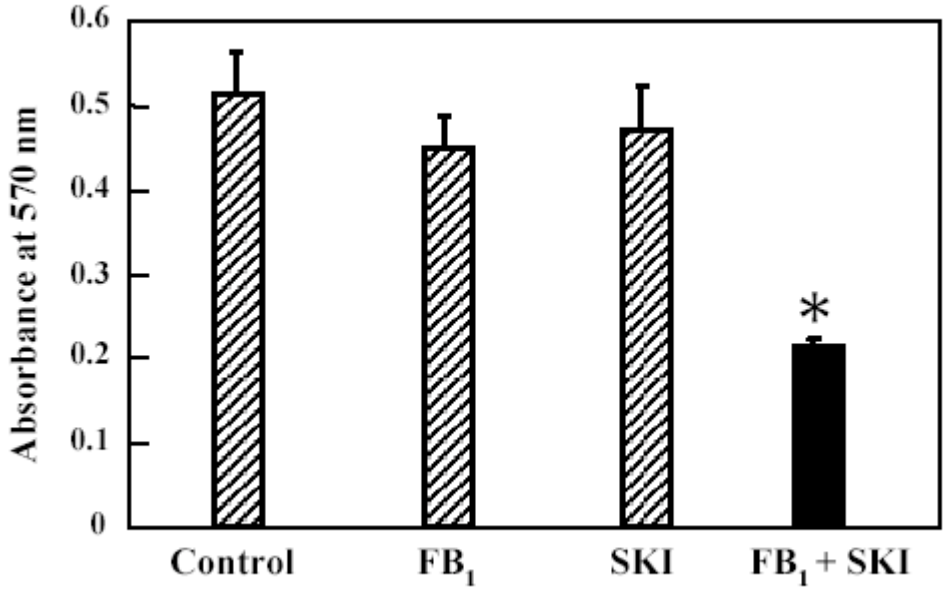


**Fig. 3.1.** HEK-293 cells were treated with 25 μM fumonisin $B_1$ and 2.5 μM SKI for 72 h. Cell viability was determined by MTT assay. Fumonisin $B_1$ and SKI alone were not toxic to HEK-293 cells but together they decreased cell viability significantly. Similar findings were noted in 3 independent experiments. The results expressed as mean ± standard error are combined results from three experiments with each experiment performed in triplicates. Asterisk (*) indicates significantly different value at $p < 0.05$ from the control.

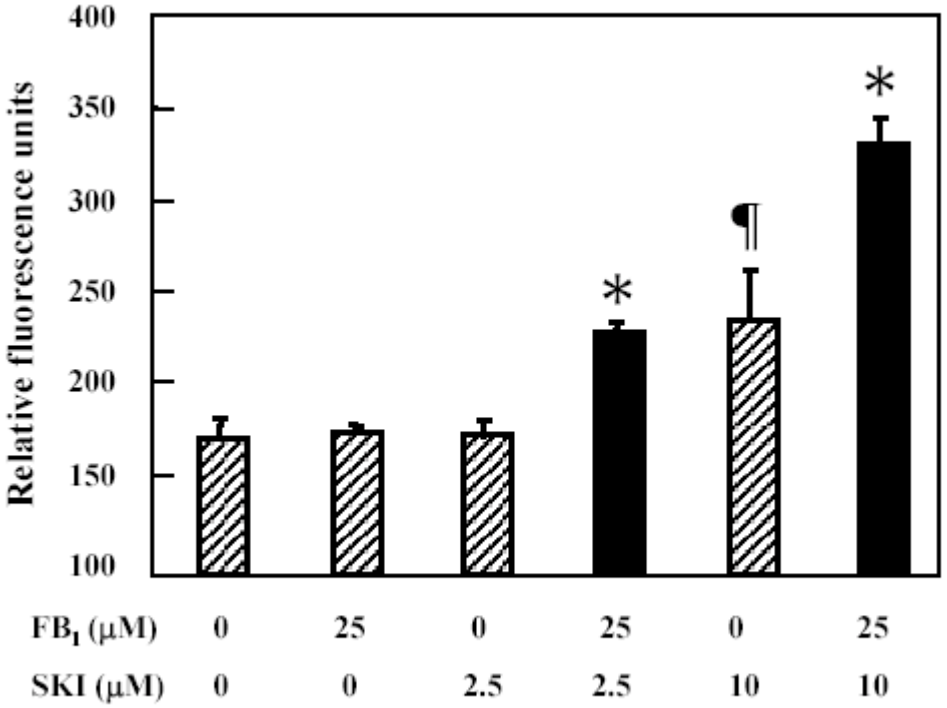


**Fig. 3.2.** HEK-293 cells are resistant to apoptotic effects of fumonisin $B_1$. Exposure to 25 μM fumonisin $B_1$ alone for 48 h did not induce apoptosis in HEK-293 cells whereas, treatment with SKI and fumonisin $B_1$ together for 48 h induced apoptosis. Induction of apoptosis measured by Hoechst 33258 fluorescence at 346/460 nm. Mean ± S.E., n = 9. Asterisk (*) indicates significantly different value at $p < 0.05$ from the fumonisin $B_1$ only treated group. (¶) indicates significantly different value at $p < 0.05$ from the control group (not treated with either fumonisin $B_1$ or SKI).

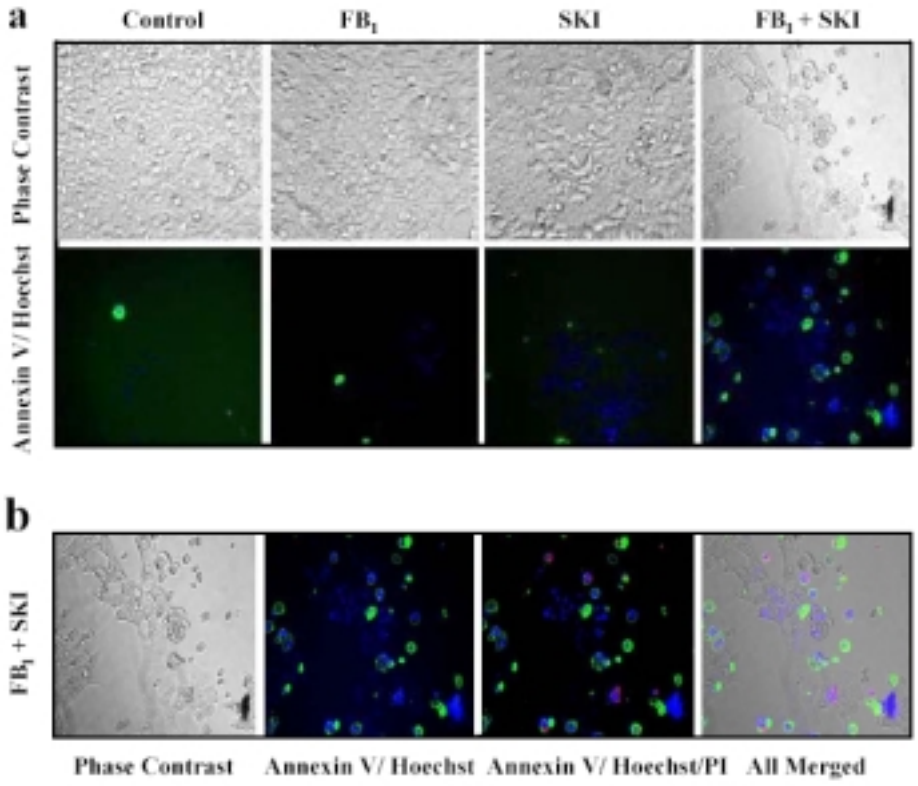


**Fig. 3.3.** (a) HEK-293 cells were treated with 25 μM fumonisin $B_1$ and 2.5 μ SKI for 48 h. Annexin V and Hoechst 33258 staining were used to detect apoptosis. Cells treated with fumonisin $B_1$ and SKI together had significantly greater number of cells stained with Annexin V and Hoechst 33258 compared to control, fumonisin $B_1$ and SKI alone. 3(b). Overlay of few propidium iodide stained (red) necrotic cells with blue (Hoechst 33258) and green (Annexin V) stained apoptotic cells was used to differentiate between necrosis and apoptosis. Similar findings were noted in 3 independent experiments.

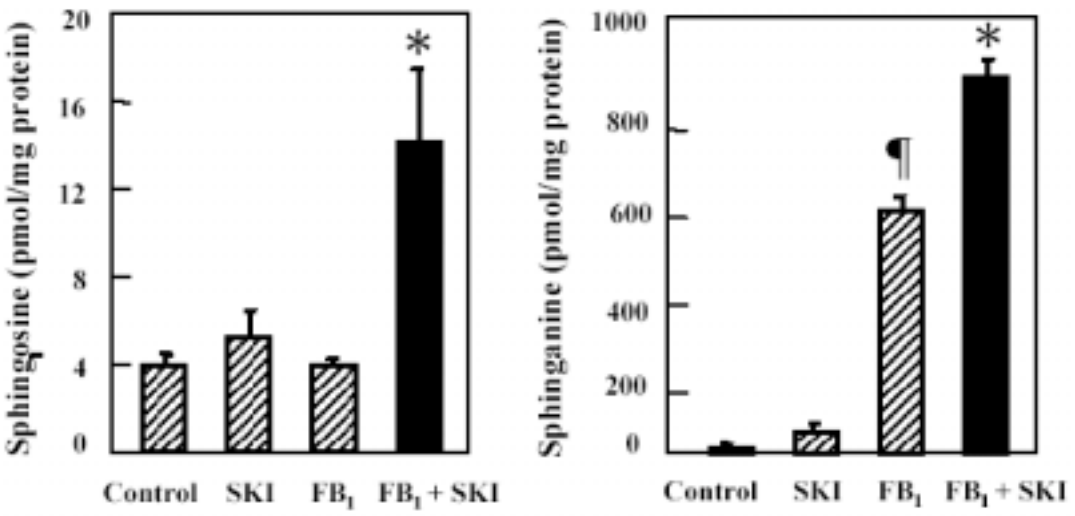


**Fig. 3.4.** HEK-293 cells were treated with 25 μM fumonisin $B_1$ and 2.5 μM SKI for 48 h. Treatment of cells together with SKI and fumonisin $B_1$ caused higher elevation of intracellular level of sphinganine compared to fumonisin $B_1$ treatment alone. Fumonisin $B_1$ alone did not increase intracellular level of sphingosine, whereas exposure of cells to fumonisin $B_1$ and SKI together caused significantly higher accumulation of sphingosine. Mean ± S.E., n = 9. Asterisk (*) indicates significantly different value at $p < 0.05$ from the fumonisin $B_1$ only treated group. (¶) indicates significantly different value at $p < 0.05$ from the control group.

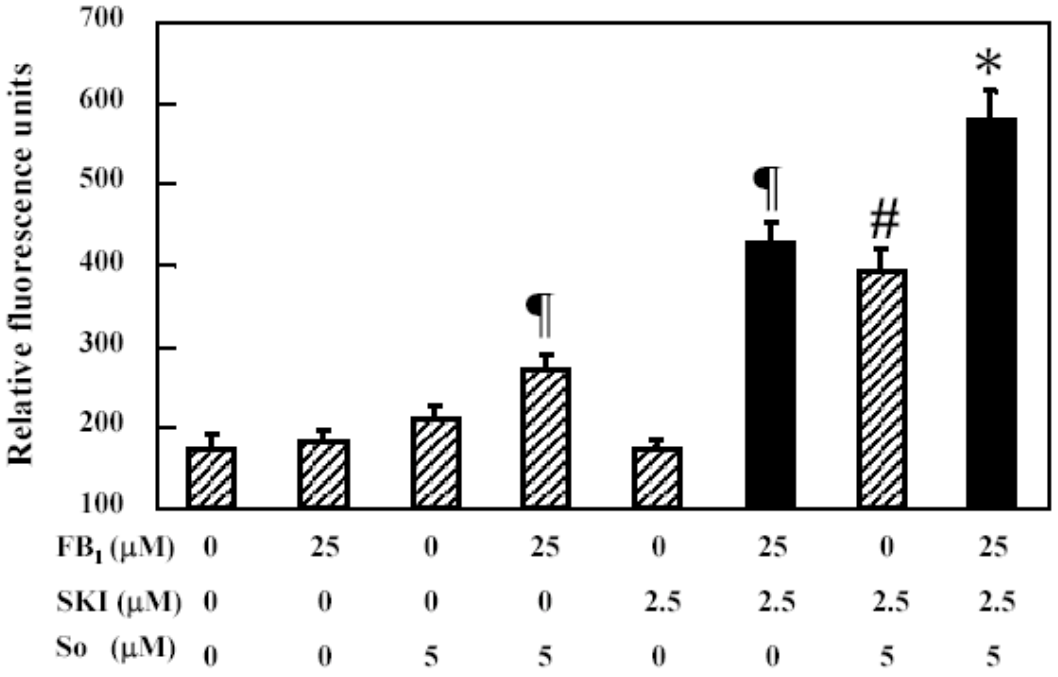


**Fig. 3.5.** HEK-293 cells were treated with 25 μM fumonisin $B_1$, 2.5 μM SKI and 5 μM sphingosine (So) for 48 h. Sphingosine when added to cell cultures induced apoptosis by it self and its effect was potentiated by sphingosine kinase inhibitor and fumonisin $B_1$. Apoptosis was measured by Hoechst 33258 fluorescence at 346/460 nm. Mean ± S.E., n = 9. (¶) indicates significantly different value at $p < 0.05$ from the fumonisin $B_1$-treated group. (#) indicates significantly different value at $p < 0.05$ from the group treated with sphingosine only. Asterisk (*) indicates significantly different value at $p < 0.05$ from the group treated with fumonisin $B_1$ and SKI together.

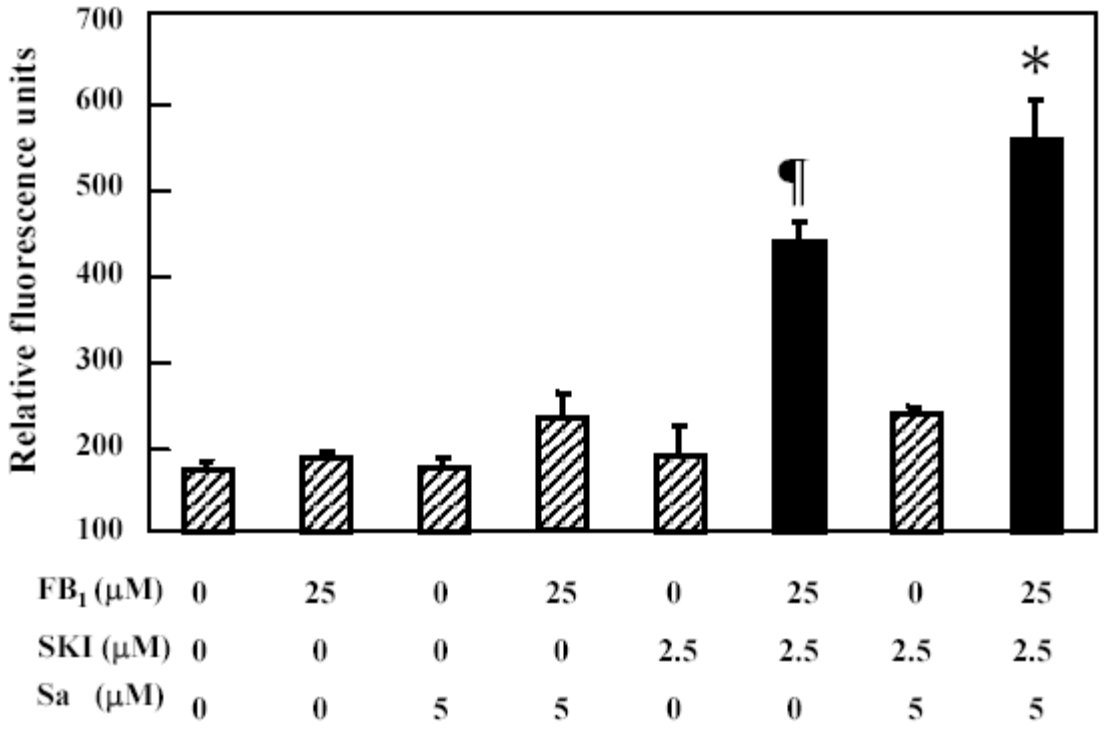


**Fig. 3.6.** HEK-293 cells were treated with 25 μM fumonisin $B_1$, 2.5 μM SKI and 5 μM sphinganine (Sa) for 48 h. Sphinganine also had synergistic effect with SKI and fumonisin $B_1$ in making HEK-293 cells more sensitive to fumonisin $B_1$ apoptosis. Apoptosis was measured by Hoechst 33258 fluorescence at 346/460 nm. Mean ± S.E., n = 9. (¶) indicates significantly different value at $p < 0.05$ from the fumonisin $B_1$ treated group. Asterisk (*) indicates significantly different value at $p < 0.05$ from the group treated with fumonisin $B_1$ and SKI together.

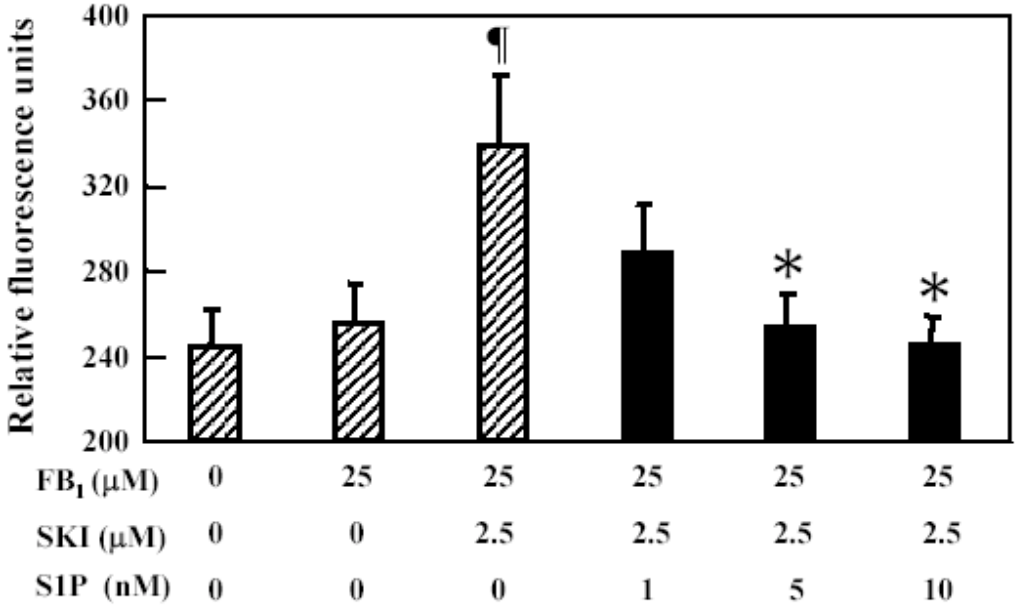


**Fig. 3.7.** HEK-293 cells were treated with 25 μM fumonisin $B_1$, 2.5 μM SKI and 1, 5 and 10 nM sphingosine-1-phosphate (S1P) for 48 h. Sphingosine-1-phosphate prevented the apoptosis induced by fumonisin $B_1$ and SKI together. Apoptosis was measured by Hoechst 33258 fluorescence at 346/460 nm. Mean ± S.E., n = 9. ¶ indicates significantly different value at $p < 0.05$ from the fumonisin $B_1$- treated group. Asterisk (*) indicates significantly different value at $p < 0.05$ from the group treated with fumonisin $B_1$ and SKI together

# CHAPTER 4

# AUGMENTED FUMONISIN $B_1$ TOXICITY IN CO-CULTURES: EVIDENCE FOR CROSSTALK BETWEEN MACROPHAGES AND NONPARANCHYMATOUS LIVER EPITHELIAL CELLS INVOLVING PROINFLAMMATORY CYTOKINES [1]

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[1]Sharma N., He Q., and Sharma R.P. 2004. *Toxicology*. 15;203(1-3):239-51.

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**Abstract**

Fumonisin $B_1$, a common mycotoxin produced by *Fusarium verticillioides* found in corn, causes several fatal animal diseases. Liver and kidney are target organs of fumonisin $B_1$ in laboratory animals, but primary rodent hepatocytes and liver slices were resistant to fumonisin $B_1$ induced cytotoxic effects. We have shown that fumonisin $B_1$ induces expression of tumor necrosis factor (TNF)α, interferon (IFN)γ, and interleukine (IL) 12, in mouse liver. In various models of acute liver injury a positive amplification loop involving TNFα, IFNγ and IL-12 has been implied that involves Kupffer cells (macrophages), hepatic lymphocytes and nonparenchymatous liver epithelial cells (NPECs). In the current study cellular interactions in fumonisin $B_1$ induced toxicity were investigated using co-cultures of murine macrophages (J774A.1) and nonparenchymatous liver epithelial cells (NMuLi). Treatment of the co-cultures with fumonisin $B_1$ produced cytotoxicity whereas either J774A.1 or NMuLi cultures alone showed no response to the mycotoxin. Accumulation of sphinganine occurred to the similar extent in individual cultures as well as co-cultures. Expression of TNFα and IL-12 was increased in co-cultures but not in individual cultures. Transfer of conditioned medium from fumonisin $B_1$ treated J774A.1 cells to NMuLi cultures produced an increase in IFNγ expression in NMuLi cells. Results indicated that macrophages and liver epithelial cells interact in response to fumonisin $B_1$ and potentiate the cytokines expression, which may have implications in making hepatocytes responsive to cytotoxicity of fumonisin $B_1$.

Key words*:* Fumonisin $B_1$; Tumor necrosis factor α; Interferon γ; murine macrophages; Nonparenchymatous liver epithelial cells;

## 1. Introduction

Fumonisins are a group of structurally related mycotoxins produced by *Fusarium verticillioides,* which are commonly found on corn (Riley et al., 1993). Fumonisin $B_1$ is the most abundant naturally occurring fumonisin in contaminated foods and feeds. It is hepatotoxic and nephrotoxic in domestic and laboratory animals and causes equine leukoencephalomalacia and porcine pulmonary edema (Riley et al., 1993). In National Toxicology Program study in Fischer rats and B6C3F mice fumonisin $B_1$ caused renal carcinoma in male rats and hepatic cancer in female mice (Howard et al., 2001). Fumonisin $B_1$ has been identified as cardiotoxic in swine (Constable et al., 2000). An association between human esophageal cancer and fumonisins in areas highly contaminated with these mycotoxins was reported (Sydenham et al., 1990).

Fumonisins are structurally related to sphingoid bases and cause inhibition of ceramide biosynthesis (sphinganine and sphingosine *N*-acyltransferase) leading to accumulation of free sphingoid bases, sphingoid base metabolites, and depletion of more complex sphingolipids (Riley et al., 1996). Studies in pig renal epithelial cells, colonic cells, and human keratinocytes have shown that fumonisin $B_1$ induced apoptosis, necrosis, and inhibition of proliferation are sphinganine-dependent (Yoo et al., 1996; Schmelz et al., 1998; Tolleson et al., 1999). There is a close correlation between increased apoptosis in liver and kidney and the disruption of sphingolipid metabolism (Voss et al., 2002).

It has been demonstrated that fumonisin $B_1$ induces apoptosis in rat kidney and mouse liver (Sharma et al., 1997; Tolleson et al., 1996). Fumonisin $B_1$ also induces apoptosis in several different mammalian cells (Ciacci-Zanella et al., 1999; Wang et al., 1996), where

direct treatment to cells leads to apoptosis. A previous study demonstrated that fumonisin $B_1$ induced cell cycle arrest in the G1 phase in African green monkey kidney cells (CV-1) but CV-1 cells transformed by the simian virus 40 (SV40) large T antigen (COS-7) were unaffected by the same levels of fumonisin $B_1$ (Wang et al., 1996; Ciacci-Zanella et al., 1998). Wang et al. (1991) and Schmelz et al. (1998) showed that concentrations of fumonisin $B_1$ as low as 1 μM caused an almost complete inhibition of ceramide formation by primary hepatocytes, while much higher (>75 μM) concentrations of fumonisin $B_1$ did not induce cytotoxicity.

There is considerable evidence that tumor necrosis factor (TNF) α signaling pathway plays an important role in fumonisin $B_1$ induced toxicity in sensitive cells *in vitro* and *in vivo*. Fumonisin $B_1$ hepatotoxicity was reduced in mice lacking either TNF receptor 1 or 2 (Sharma et al., 2000; Sharma et al., 2001). Interferon knockout mice show reduced hepatotoxicity and TNFα expression in response to fumonisin $B_1$ (Sharma et al., 2003). In the current study we hypothesize that the resistance of primary hepatocytes to the cytotoxic effects of fumonisin $B_1$ may be due to a lack of interactions between the cytokines that are produced by different types of cells in liver.

In various models of acute liver injury a positive amplification loop involving TNFα, IFNγ and IL-12 has been implied and involves Kupffer cells (liver macrophages) hepatic lymphocytes and nonparenchymatous liver epithelial cells (NPECs). We speculated that fumonisin $B_1$ induces localized activation of cytokine network in mouse liver; expression of TNFα, interferon (IFN) γ and interleukine (IL)-12 in mouse liver was increased in response to fumonisin $B_1$ (Bhandari et al, 2002a). A remarkable crosstalk between macrophages and T cells involving TNFα and IFNγ has been suggested using *in vitro* co-culture experiments

(Gantner et al., 1996). Similar crosstalk between macrophages and cell types other than the T cells is also important to amplify the cytokine release in response to various toxins. Interactions of this kind have been reported in several co-culture experiments involving alveolar macrophages and type II pneumocytes (Tao et al., 2002) or microglia and neurons (Hemmer et al., 2001).

In the present study we hereby show that co-cultures of macrophages (J774A.1) and nonparanchymatous liver epithelial cells (NMuLi) are more responsive to fumonisin $B_1$ induced cytotoxicity compared to individual cultures and interaction of the two cell types resulted in increased expression of proinflamatory cytokines in the co-cultures.

## 2. Materials and Methods

### *2.1. Materials*

Fumonisin $B_1$ (purity>98%) was obtained from Programme on Mycotoxin and Experimental Carcinogenesis (Tygerberg, South Africa). Mouse macrophage cells J774A.1 (TIB-67) and NMuLi cells (CRL-1638) were obtained from American type culture collection (Rockville, MD). Delbeco's Modified Eagle's Medium (DMEM) was purchased from Gibco (Carlsbad, CA). Recombinant IFNγ was procured from Biocompare, Inc (South San Francisco, CA) and TNFα from Endogen (Woburn, MA). Anti-TNFα was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-IFNγ was procured from Chemicon

International (Temecula, CA). Annexin V, Hoechst 33258 and propidium iodide were purchased from Molecular Probes (Eugene, OR). All other reagents were obtained from Sigma (St. Louis, MO) and were of tissue culture grade.

*2.2. Cell Culture and Treatment*

Macrophages (J774A.1) and liver epithelial cells (NMuLi) were grown in separate flasks containing DMEM with 10% fetal bovine serum at 37°C and 5% $CO_2$. Early passage clones were mixed in different ratios in 96 or 24 well plates, 16 h before the treatment. For co-culture, ratio of 1 (J774A.1): 7 (NMuLi) [i.e., $5 \times 10^3$ J774A.1 cells and 35 x $10^3$ NMuLi cells] was found to be optimal and used for all experiments. Cells were treated with 10 μM fumonisin $B_1$ for various time points depending on the experiment; the concentration of fumonisin $B_1$ and time periods were optimized in preliminary trials. For experiments involving transfer of supernatant, individual cultures were treated with 10 μM fumonisin $B_1$ and their media was transferred to the other cell types for 6 h to determine cytokine expression, or longer to evaluate toxicity.

*2.3. Determination of Cytotoxicity*

The cytotoxic effect of fumonisin $B_1$ on individual cultures and co-cultures (J774A.1 and NMuLi) was determined by measuring the release of lactate dehydrogenase (LDH) as previously described (He et al., 2002). For the total LDH determination, LDH was released from the cells by adding 1 μl of Triton X-100 (1% final concentration), and incubating for 20-30 min. at 37° C. The change in absorbance of the reaction mixture at 340 nm (an index of

NADH oxidation) was read kinetically at 1 min intervals using a Scanning spectrophotometer (Bio-Tek Instrument, INC, Winooski, VT, USA)

The fluorescent probes annexin V or Hoechst 33258 and propidium iodide (PI) were used to detect the apoptotic and necrotic cells, respectively. Cells were grown and treated in 96 well plates. At the end of treatment, cells were incubated with annexin V (5 μL/100 μL in annexin binding buffer) or Hoechst 33258 (8 μg/ml in 30 % glycerol in PBS) and PI (20 μg/ml) and fluorescence intensity was recorded using Spectramax Gemini fluorescent plate reader (Molecular Devices, Irvine, CA). Propidium iodide fluorescence was detected with excitation 535 nm and emission at 617 nm; excitation at 495 nm and emission at 520 nm detected the fluorescence of annexin V. Hoechst 33258 fluorescence was detected with excitation at 346 nm and emission at 460 nm. Visual documentation of cellular effects was performed using Olympus IX71 inverted microscope (Olympus America, Melville, NY). Digital images were acquired using Magnafire SP digital camera.

*2.4. Immunohistochemistry*

Cells grown in microchambered slides were fixed and permeabilized in cold 1:1 methanol-acetone for 30 min. Primary rat anti-mouse Mac-3 antibody (Pharmingen, San Diego, CA, USA), diluted as 1:1000,was used at 37°C for 2 h followed by biotinylated secondary anti-rat antibody at 37°C for 1 h. Mac-3 antibody reacts with the glycoprotein Mac-3 antigen expressed on mouse mononuclear phagocytes. The slides were incubated with avidin–biotin peroxidase complex for 1 h followed by 3,3'-diaminobenzidine as a substrate.

*2.5. Determination of intracellular free sphingoid bases*

At the end of 24 h or 48 h treatment with fumonisin $B_1$, the medium was aspirated, and cells were washed once with ice-cold PBS and then scraped into 1 ml ice-cold PBS. An aliquot (0.1 ml) of cell suspension in PBS was transferred to another tube, spun at 2,000g, 4°C for 5 min. The cell pellet was lysed and stored at -85°C until analysis of total protein by Bio-Rad Bradford reagent (Bio-Rad Laboratories, Hercules, CA) using 96-well plate according to the manufacturer's protocol. Free sphingoid bases were extracted from the remainder of cells by using the modified method of Yoo et al. (1996). The relative amounts of free sphinganine and sphingosine in base-treated cell extracts were determined by high performance liquid chromatography (HPLC) as described earlier (He et al., 2002). Sphingoid bases were quantified based on the recovery of a $C_{20}$ sphinganine internal standard. The limit of detection for $C_{20}$ was 26.8 fmol/assay (equivalent to 1 fmol/$\mu$g protein).

*2.6. Analysis of cytokine expression*

Total RNA was isolated from cells using TRI® reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNFα, IL-12, IFNγ and β-actin as internal control (He et al., 2001). The amplification products were fractionated on ethidium bromide containing 2% agarose gel and documented using a Kodak DC290 digital camera and digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT). Band intensities (pixel values) for each cytokines were normalized to that of β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

*2.7. Statistical analysis of data*

Each experiment was repeated several times with consistent trends; data from different experiments were pooled. The results are expressed as mean ± standard error (SE) of independent experiments. Differences among treatments were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. The level of $p \leq 0.05$ was considered as significant for all comparison.

## 3. Results

*3.1. Fumonisin $B_1$ induced cell death in mixed cultures while individual cultures were resistant*

Treatment with 10 μM fumonisin $B_1$ for 72 h induced toxicity in co-cultures of mouse macrophage cells (J774A.1) and nonparenchymatous liver epithelial cells (NMuLi), as indicated by increased lactate dehydrogenase (LDH) release (Fig. 4.1). Individual cells alone were unresponsive and showed significantly less LDH release compared to co cultures with the same treatment. Preliminary studies indicated that concentrations of fumonisin $B_1$ up to 50 μM were not toxic to either J774A.1 or NMuLi cells but were cytotoxic in co-cultures (data not shown). The effects were time and concentration dependent (not illustrated).

*3.2. Induction of apoptosis in co-cultures exposed to Fumonisin $B_1$*

Treatment of co-culture with 10 μM fumonisin $B_1$ for 24 h resulted in increased apoptosis, which was measured by recording the fluorescence of Annexin V dye at 495/520 nm

(Fig. 4.1). Cell death in co-culture was confirmed by nuclear staining with membrane impermeant dye propidium iodide (PI), which is taken up only by necrotic cells as their cell membrane is damaged. Fumonisin $B_1$ treated co-culture had significantly greater number of PI stained cells as compared to control (Fig. 4.2A). We determined apoptosis by Annexin V staining in fumonisin $B_1$ treated co-cultures. Visual inspection under fluorescence microscope confirmed the presence of increased number of cells exhibiting bright green staining of Annexin V on cell surface indicating apoptotic cells (Fig. 4.2A). Early apoptotic cells with exposed phosphotidyl serine and intact cell membrane bind annexin V but exclude PI. Overlay of annexin V and PI stained cells showed predominance of annexin V stained cells after 24 h of fumonisin $B_1$ treatment.

Immunohistochemical staining of macrophages with surface marker Mac-3 in the co-culture showed that fumonisin $B_1$ treatment did not change the number of macrophages while reduced the density of cells not stained with Mac-3 marker i.e. nonparenchymatous liver epithelial cells (Fig. 4.2B).

*3.3. Increase in cytotoxicity is not related to sphingoid base accumulation*

Intracellular levels of sphinganine were increased to similar extent in individual cell types and co-culture upon treatment with 10 μM fumonisin $B_1$ for 24 h or 48 h. Sphingosine levels decreased significantly in NMuLi cells and co-culture treated with 10 μM fumonisin $B_1$ for 24 h or 48 h while remained unchanged in J774A.1 cells (Fig. 4.3).

*3.4. Potentiation of cytokine expression in Fumonisin $B_1$ treated co-culture*

In fumonisin $B_1$ sensitive cell lines or *in vivo* experiments, increased production of proinflammatory cytokines has been implicated in fumonisin $B_1$ toxicity. To evaluate the role of cytokines, we measured the expression of TNFα, IL-12, and IFNγ in co-cultures and individual cell types treated with 10 μM fumonisin $B_1$. NMuLi cells had little expression of TNFα. None of the cytokines expression changed in individual cultures; co-cultures had increased expression of TNFα after 24 h treatment with fumonisin $B_1$ (Fig. 4.4). There was no change in expression of IFNγ and IL-12 in co-culture treated with fumonisin $B_1$ for 24 h but expression increased significantly after 48 h incubation with fumonisin $B_1$ (Fig. 4.4).

*3.5. Fumonisin cytotoxicity in co-cultures is reduced by anti TNFα and anti IFNγ*

Treatment of co-culture with neutralizing antibodies for either TNFα or IFNγ decreased the fumonisin $B_1$-induced apoptosis. Co-cultures were treated with 10 μM fumonisin $B_1$ in the presence of neutralizing antibodies for TNFα (1μg/ml) or IFNγ (1μg/ml) for 24 h (Fig. 4.5).

*3.6. Transfer of medium produced alterations in cytokine expression similar to co-cultures*

Interactions of epithelial cells and macrophages have been shown to be mediated by soluble mediators such as cytokines. We investigated possibility of such interaction between NMuLi and J774A.1 cells. Both cells were treated with 10 μM fumonisin $B_1$ for 24 h. The supernatant from NMuLi cells was transferred to untreated J774A.1 cells and that of J774A.1 cells to untreated NMuLi cells. After 6 h of incubation with the preconditioned media TNFα expression was examined in J774A.1 cells and IFNγ expression in NMuLi cells. We

observed, increase in IFNγ expression in NMuLi cells after media transfer while change of media did not alter the expression of TNFα in J774A.1 cells (Fig. 4.6).

*3.7. Effect of transfer of conditioned supernatant from Fumonisin $B_1$ treated co-culture to individual cultures*

Co-cultures were treated for 24, 36 and 48 h with 10 μM fumonisin $B_1$ and their conditioned media was transferred to individual cultures to evaluate the effect of soluble mediators of crosstalk on cell viability. LDH release was measured from individual cultures at the same time, 48 h after the transfer of supernatant. Supernatant from cultures that was conditioned for 24 h in co-culture did not induce any increase in LDH release from NMuLi cells. Preconditioning of 36 and 48 h in treated co-cultures induced increased LDH release from NMuLi cells (Fig. 4.7). There was no significant change in LDH release when media preconditioned in treated co-cultures were transferred to J774A.1 cells (Fig. 4.7).

*3.8. Recombinant TNFα and IFNγ induce expression of each other in NMuLi and J774A.1 cells*

In order to confirm whether individual cells in co-culture respond to cytokines produced by the other cells, we treated J774A.1 cells with 15 ng/ml recombinant IFNγ (Hemmer et al., 2001) and NMuLi cells with 10 ng/ml recombinant TNFα (Johnson et al., 2003). Treatment of J774A.1 cells with IFNγ for 6 h produced increased expression of TNFα (Fig. 4.8). Similarly treatment of NMuLi cells with TNFα for 6 h produced increased expression of IFNγ (Fig. 4.8).

*3.9. Replacement with fresh medium did not prevent cell death in co-culture*

In order to investigate if the effect of fumonisin $B_1$ induced signaling lasted until cell death, the fumonisin $B_1$ containing media was replaced with fresh media with no fumonisin $B_1$. Release of LDH was measured at 72 h after the first addition of fumonisin $B_1$. Cytotoxicity of fumonisin $B_1$ in co-culture was prevented when fumonisin $B_1$ containing media was replaced with fresh media at 24 h. Replacing with fresh media after fumonisin $B_1$ treatment for 36 or 48 h did not prevent cell death in co-culture as evident by increased LDH release from these cells at 72 h (Fig. 4.9).

## 4. Discussion

Liver and kidney are primary target organs of fumonisin $B_1$ in rodents. However primary hepatocyte cultures (Wang et al., 1991) and liver slices (Norred et al., 1996) are quite resistant to fumonisin $B_1$-induced cytotoxic effects. In primary rat hepatocytes, higher concentrations of fumonisin $B_1$ were required to elicit lactate dehydrogenase (LDH) release (van der Westhuizen et al., 1998). We found that mouse macrophage cells (J774A.1) and liver nonparenchymatous epithelial cells (NMuLi) are also resistant to fumonisin $B_1$ toxicity when cultured separately but become responsive when grown in co-culture at concentrations as low as 10 μM. Cytotoxicity in treated co-culture was evident by lactate dehydrogenase release.

This suggested the possibility of interactions between these two cell types that amplify their response to fumonisin $B_1$.

In agreement with previous studies in fumonisin $B_1$ sensitive cell lines (Tolleson et al., 1999), we also found that apoptosis is the primary mode of cell death in fumonisin $B_1$ treated co-cultures. Although accumulation of sphingoid bases has been established as a mechanism for fumonisin $B_1$ induced apoptosis in various cells, in current study results showed that accumulation of sphinganine was not sufficient to cause apoptosis. Intracellular levels of sphingoid bases changed to similar extent in co-cultures and individual cell types.

We observed that TNFα, IL-12 and IFNγ expressions were higher in co-cultures compared to individual cell lines after fumonisin $B_1$ treatment. Cytotoxicity observed in the current study was due to potentiation of cytokines as neutralizing antibodies of TNFα and IFNγ partially inhibited the fumonisin $B_1$-induced apoptosis. There is considerable evidence that cytokine signaling pathways play an important role in fumonisin $B_1$ induced toxicity *in vitro* and *in vivo*. Jones et al. (2001) reported that fumonisin $B_1$ toxicity was effectively prevented by inhibition of caspases, which are downstream of TNFα cellular signaling. Fumonisin $B_1$ toxicity was reduced in mice lacking either TNFα receptor (TNFR) 1 or TNFR 2 (Sharma et al., 2000; Sharma et al., 2001).

Fumonisin $B_1$ treatment causes an increased expression of TNFα, IFNγ and IL-12 in liver; cells involved in TNFα production were identified as Kupffer cell (Bhandari et al., 2002b). Previous studies showed that TNFα and IL-12 produced by the Kupffer cells in liver

could act on natural killer (NK) cells or Th 1 cells to produce IFNγ (Gantner et al., 1996). This IFNγ can then further activate the Kupffer cells to produce more TNFα and IL-12 eventually producing more IFNγ from the Th1 cells, thus generating a positive feedback loop between the two cell types (Bhandari et al., 2002b). The two cytokines, TNFα and IFNγ, have synergistic effects in producing liver damage (Ozmen et al., 1994).

Our laboratory has previously demonstrated that fumonisin $B_1$ treatment increases TNFα production from J774A.1 cells (Dugyala et al., 1998). In the present study we found that transfer of supernatant from fumonisin $B_1$ treated J774A.1 cells, leads to increased expression of IFNγ in NMuLi cells. This correlated well with our hypothesis that TNFα production from macrophages (J774A.1) can increase IFNγ production from liver epithelial cells (NMuLi). This is also evident by our findings that increase in IFNγ expression in co-culture is delayed until 48 h; the effect was expected considering that accumulation of TNFα in the medium from J774A.1 cells would require about 24 h to be synthesized and secreted in the medium. Transfer of medium from fumonisin $B_1$ treated epithelial cells to macrophage cells did not increase TNFα expression, indicating that fumonisin $B_1$ has no direct effect on IFNγ expression in epithelial cells only TNFα produced from macrophages stimulates them to produce IFNγ.

To confirm that J774A.1 cells respond to IFNγ, we treated them with recombinant IFNγ, providing the anticipated increased expression of TNFα in J774A.1 cells. Similarly treatment of NMuLi cells with recombinant TNFα resulted in increased expression of IFNγ, supporting our hypothesis that TNFα produced from macrophages stimulated NMuLi cells in co-culture to

produce IFNγ. Transfer of supernatant from fumonisin $B_1$ treated co-cultures to liver epithelial cells induced toxicity in the latter, which suggested that cross talk involved soluble mediators. A preconditioning of 36 h was required for the observed toxicity, indicating that toxic factor in co-cultures requires that period to accumulate in concentrations sufficient enough to induce toxicity. In a similar experiment transfer of supernatant from fumonisin $B_1$ treated co-cultures to macrophage cells did not induce cytotoxicity in macrophages. It indicated that death of epithelial cells might be responsible for the observed cytotoxicity in co-culture; however, it needs to be verified by direct immunohistochemistry.

Persistent exposure to fumonisin $B_1$ is not necessary for cytotoxicity as replacement with fresh medium in place of fumonisin $B_1$ containing medium after 36 h and 48 h did not prevent cell death in co-cultures. These results suggest that presence of fumonisin $B_1$ is dispensable after 36 h. A persistent inhibition of the ceramide synthase may have occurred despite removal of fumonisin $B_1$ from the medium. Alternatively, production of some cytotoxic factor might have triggered an irreversible pathway leading to cell death without fumonisin $B_1$.

Recent studies have demonstrated that apart from hepatocyte multipotential NPEC system generates differentiated lineages needed for liver regeneration under conditions where hepatocytes are unable to respond or are functionally compromised (Thorgeirsson, 1996; Fausto, 2000). NMuLi cells were shown to exhibit characters of such nonparenchymal bipotential progenitor cells. Sections of hepatocyte growth factor treated NMuLi colonies resembled ductules proliferating in liver damage caused by hepatotoxins and biliary tract obstruction (Johnson et al., 1993). NMuLi cells produce a variety of tumors when inoculated

in subcutaneous sites in newborn mice (Anderson et al., 1979) and show selective tumorigenesis when transfected with hepatocyte growth factor (Johnson et al., 1995). This further suggested the potential of NmuLi cells as stem cells to differentiate into hepatocytes and biliary epithelial cells.

Role of NPECs in liver regeneration following fumonisin $B_1$ hepatotoxicity seems conceivable as NPECs are the major regenerating cells in other models, where hepatocyte proliferation is impaired or slowed (Thorgeirsson, 1996). Fumonisin $B_1$ inhibits hepatocyte proliferation in rats fed fumonisin $B_1$ before or after partial hepatectomy (Gelderblom et al., 1994). Fumonisin $B_1$ also inhibits epidermal growth factor induced DNA synthesis in primary rat hepatocytes (Gelderblom et al., 1995). NPECs were noted as oval cell proliferation inside regenerating nodules in rats fed fumonisin $B_1$ containing diet (Lemmer et al., 1999; 2004). Our results indicated that fumonisin $B_1$ is toxic to NPECs; therefore lack of regeneration in response to fumonisin $B_1$ may contribute to observed hepatotoxicity of fumonisin $B_1$ *in vivo*.

In summary our results indicate that macrophage and NPECs interact in co-culture in response to fumonisin $B_1$ and potentiate expression of various cytokines, which may have implications in making hepatocytes more responsive to fumonisin $B_1$-induced cytotoxicity.

**Acknowledgement**

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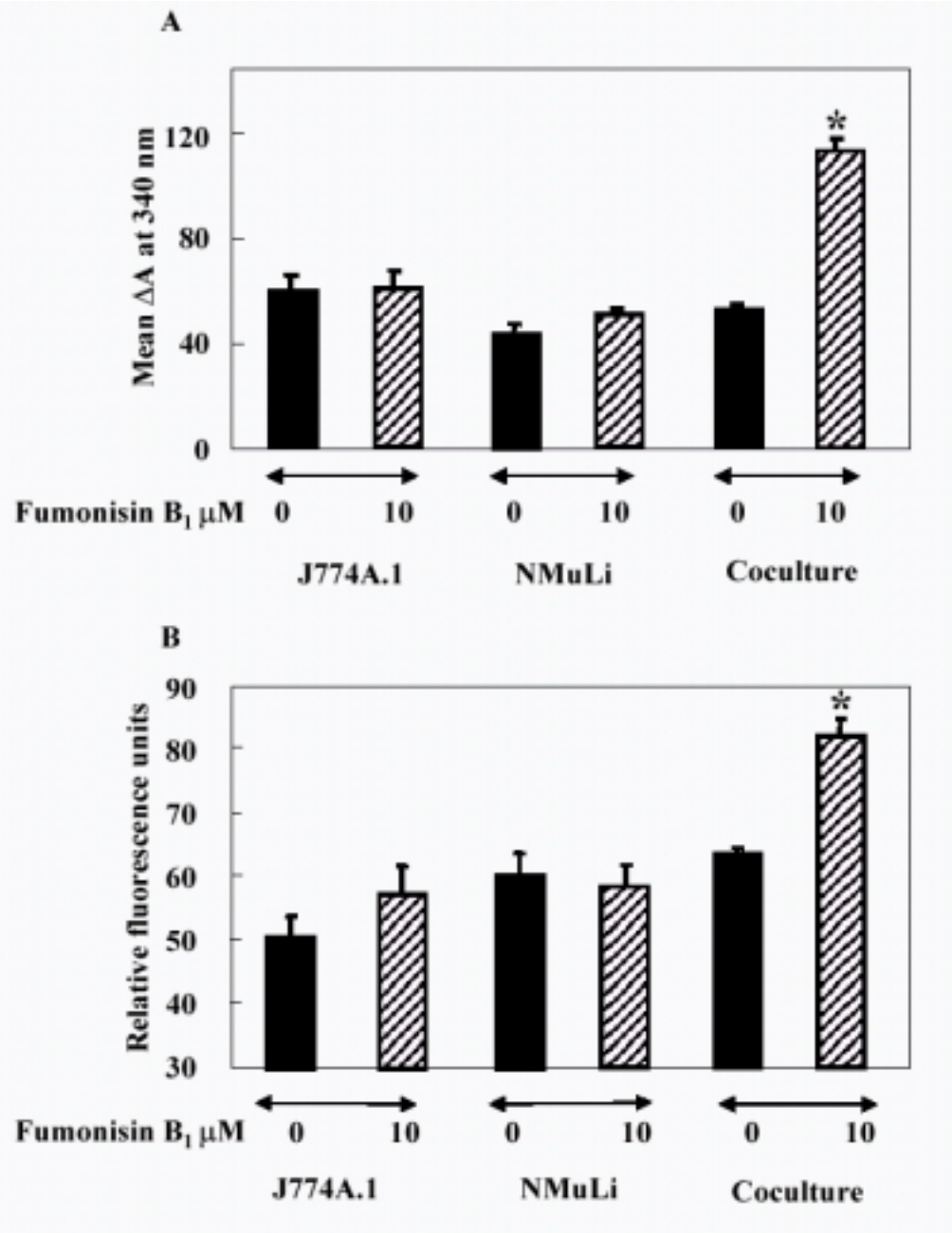


**Fig. 4.1.** (A) Treatment of individual cultures and co-culture (J774A.1 and NMuLi) with 10 μM fumonisin $B_1$ for 72 h resulted in higher lactate dehydrogenase (LDH) release from co-culture, compared to individual cultures. For Individual cultures, cells were plated in 96 well

plates at $4 \times 10^4$ cell/well. For co-culture, cells were mixed in a ratio of 1 (J774A.1) : 7 (NMuLi) i.e. $5 \times 10^3$ J774A.1 cells and 35 x $10^3$ NMuLi cells. At the end of treatment LDH release was measured separately in supernatant and cells, lysed with 1% triton X-100 (for determination of total LDH release). Data are expressed as percentage of total LDH released from cells. (B) Fumonisin $B_1$ induced apoptosis in co-culture. After treatment with 10 μM fumonisin $B_1$ for 24 h, cells were incubated with annexin V for 15 min and fluorescence intensity was recorded by fluorescent plate reader at 495/520 nm. Relative fluorescence units are arbitrarily defined based on fluorescence recorded at 495/520 nm. Mean ± S.E. of 3 independent experiments. Asterisk (*) indicates significantly different value ($p < 0.05$) from the control.

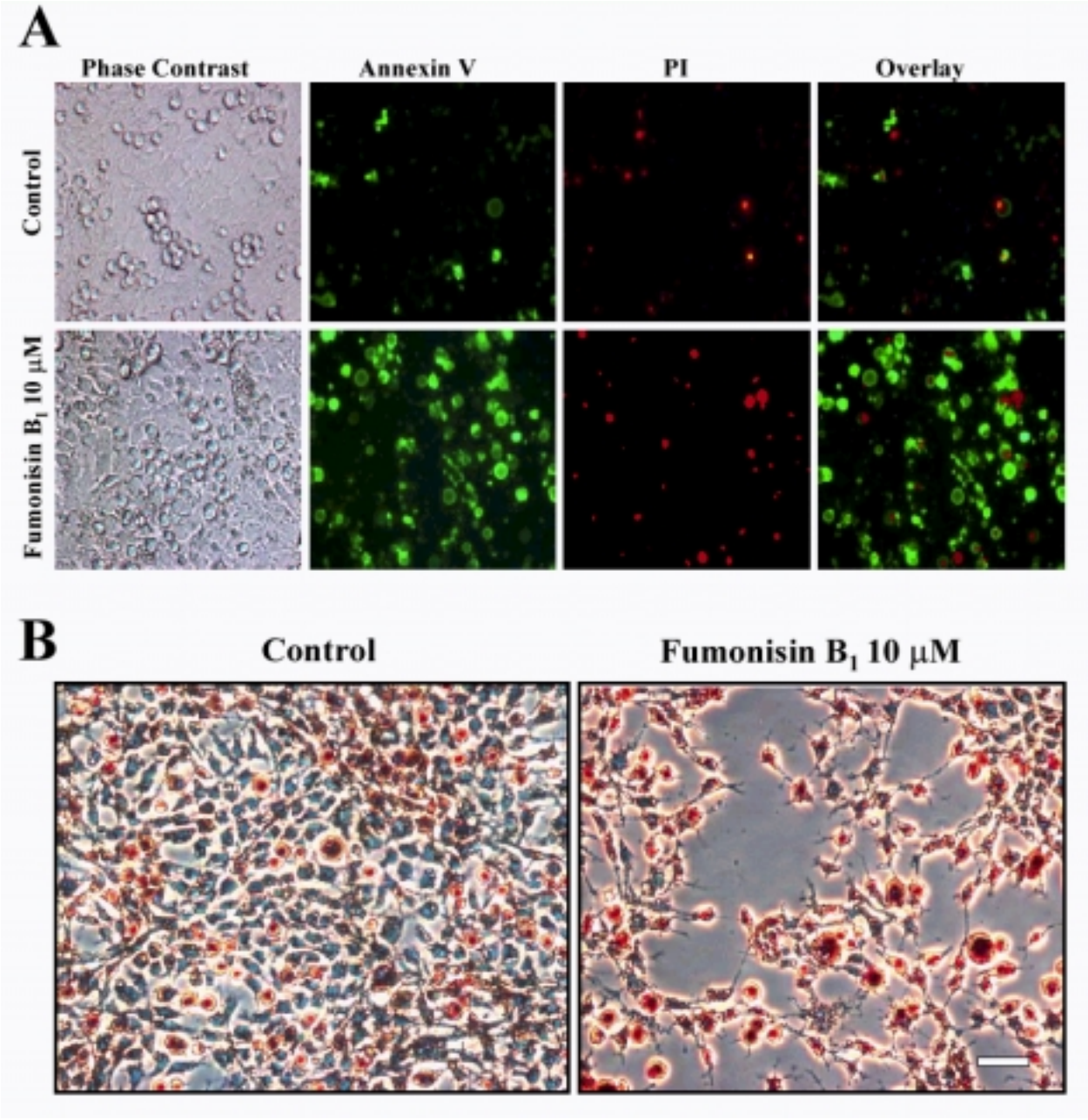


**Fig. 4.2.** Fumonisin $B_1$ induced cell death in co-culture. (A) Co-culture was treated with 10 μM fumonisin $B_1$ for 24 h and examined using annexin V or propidium iodide under phase contrast or fluorescence microscope. Annexin V binds with exposed phosphotidyl serine on

the surface of apoptotic cells and imparts cell surface a bright green staining while PI binds with chromatin of necrotic cells and stains cell nucleus red. Overlay of same fields of annexin V and PI stained cells shows predominance of apoptotic cells, which are stained green on surface and lack red nuclear staining. Representative fields are illustrated. (B) Fumonisin $B_1$-induced cell death in co-cultures treated with 10 μM fumonisin $B_1$ for 48 h involved predominantly nonparenchymatous epithelial cells (NMuLi). Macrophages in the co-culture were stained brown by Mac-3 antibody, which specifically binds with Mac-3 surface antigen on macrophages. In control culture the NMuLi cells are visualized as light blue hematoxylin-stained cells; these cells are largely absent in the treated culture. Bar on lower left represents 50 μm.

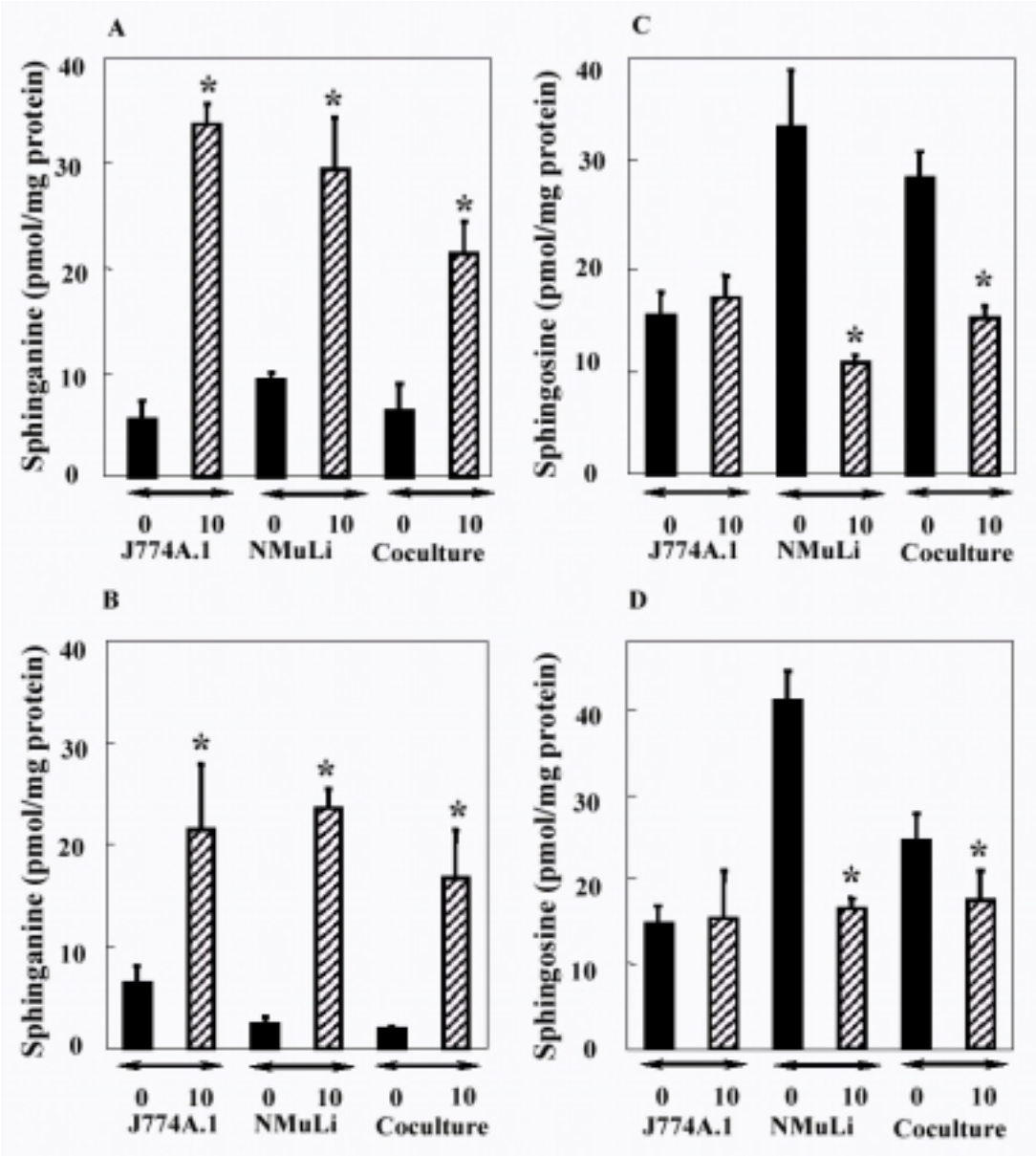


**Fig. 4.3.** Concentrations of free sphinganine and sphingosine after exposure of J774A.1, NMuLi and co-culture to 10 μM fumonisin $B_1$ for 24 h (A and C) or 48 h (B and D). Mean ± SE (n = 3). Asterisk (*) indicates significantly different value ($p < 0.05$) from the concurrent control.

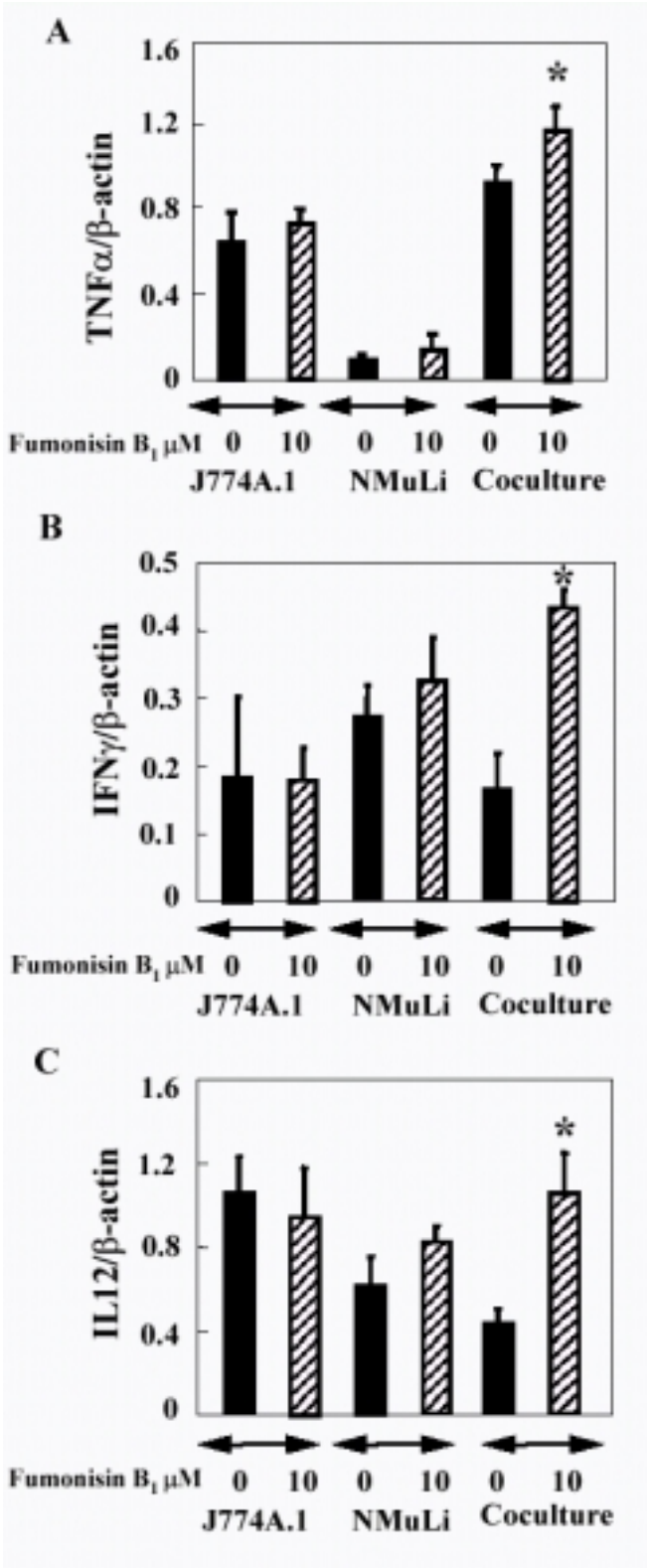
A
TNFα/β-actin
1.6
1.2
0.8
0.4
0
*
Fumonisin B1 μM 0 10 0 10 0 10
J774A.1 NMuLi Coculture
B
IFNγ/β-actin
0.5
0.4
0.3
0.2
0.1
0
*
Fumonisin B1 μM 0 10 0 10 0 10
J774A.1 NMuLi Coculture
C
IL12/β-actin
1.6
1.2
0.8
0.4
0
*
Fumonisin B1 μM 0 10 0 10 0 10
J774A.1 NMuLi Coculture

**Fig. 4.4.** Effect of fumonisin $B_1$ on expressions of TNFα (A), IFNγ (B) and IL-12 (C) in individual and co-cultures. In co-culture, cells were mixed in a ratio of 1 (J774A.1) : 7 (NMuLi) cells. All cultures were incubated for 16 h for conditioning with the media before treatment with 10 μM fumonisin $B_1$. Expression of TNFα was measured after treatment with fumonisin $B_1$ for 24 h and that of IFNγ and IL-12 after treatment for 48 h. Expression of specific mRNA was quantified by RT-PCR and normalized to that of β-actin in the same sample. Mean ± SE of 3 independent experiments. Asterisk (*) indicates significant difference ($p < 0.05$) from the control.

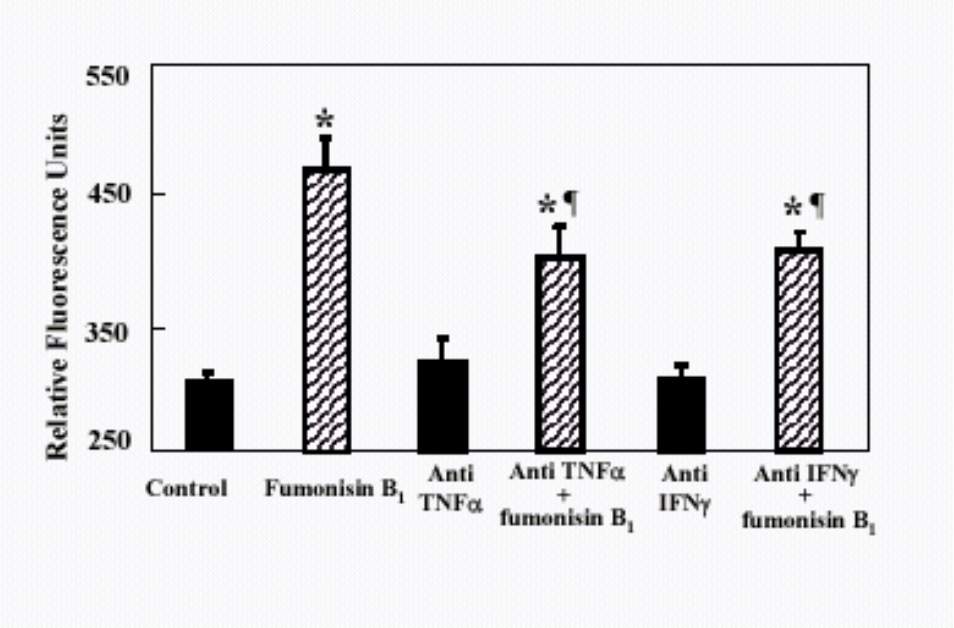


**Fig. 4.5.** Inhibition of cytotoxicity by anti-TNFα and anti-IFNγ antibodies. Co-cultures were treated with 10 μM fumonisin $B_1$ in the presence of neutralizing antibodies for TNFα (1 μg/ml) or IFNγ (1μg/ml) for 24 h. At the end of treatment, cells were incubated with Hoechst 33258 dye for 10 min and fluorescence intensity was recorded by fluorescent plate reader at 346/460 nm. Relative arbitrarily fluorescence units are indicated at excitation/emission of 346/460 nm. Mean ± SE of 3 independent experiments. * indicates significantly different value ($p < 0.05$) from the control; ¶ represents significant ($p = < 0.05$) difference from the fumonisin $B_1$-treated culture with no antibody.

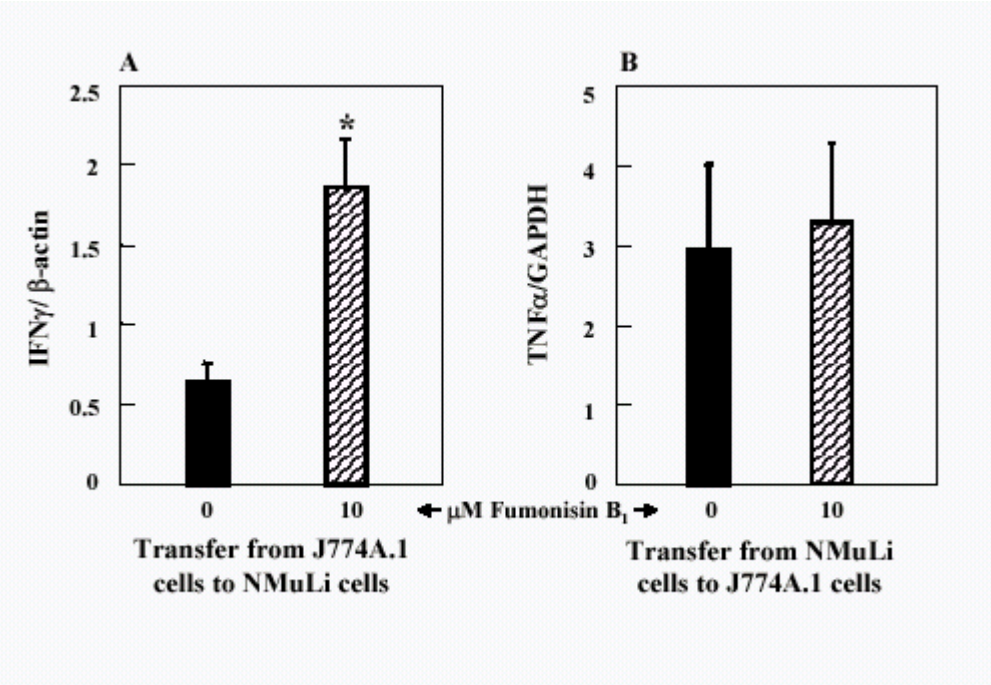


**Fig. 4.6.** Effect of transfer of preconditioned supernatant from J774A.1 cells to NMuLi cells (A) and NMuLi cells to J774A.1 cells (B) on expressions of IFNγ and TNFα respectively. J774A.1 or NMuLi cells (5 x $10^5$ /well) were grown and treated with 10 μM fumonisin $B_1$ in 6 well plates for 24 h before transferring their supernatant to the other untreated cells. After 6 h incubation of cells in preconditioned supernatant, total RNA was extracted and mRNA levels were quantified by RT-PCR. Mean ± SE of 3 independent experiments. Asterisk (*) indicates significantly different value ($p < 0.05$) from the control.

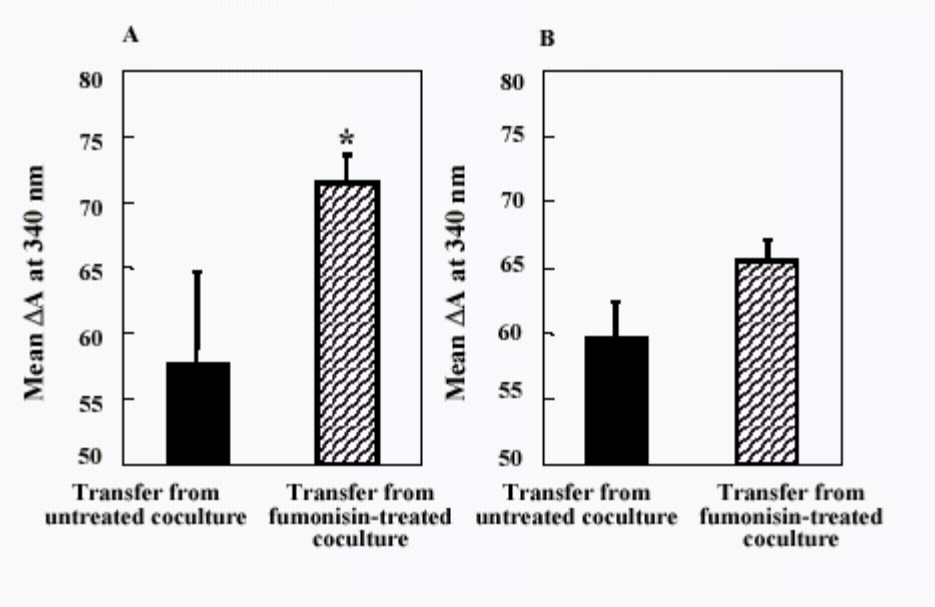


**Fig. 4.7.** Effect of transfer of conditioned supernatant from fumonisin $B_1$ treated co-culture to individual cultures. (A) Supernatant of 10 μM fumonisin $B_1$ treated or untreated co-culture was transferred to NMuLi cultures after conditioning for 36 h in co-culture. LDH released in the medium from NMuLi cells was measured 48 h after the transfer. (B) Supernatant of 10 μM fumonisin $B_1$ treated or untreated co-culture was transferred to J774A.1 cultures after conditioning for 48 h in co-culture. LDH released in the medium from J774A.1 cells was measured 48 h after the transfer. The relative LDH activity was calculated as mean of change in absorbance (ΔA) at 340 nm. Mean ± SE (n = 3). Asterisk (*) indicates significantly different value ($p < 0.05$) from the control.

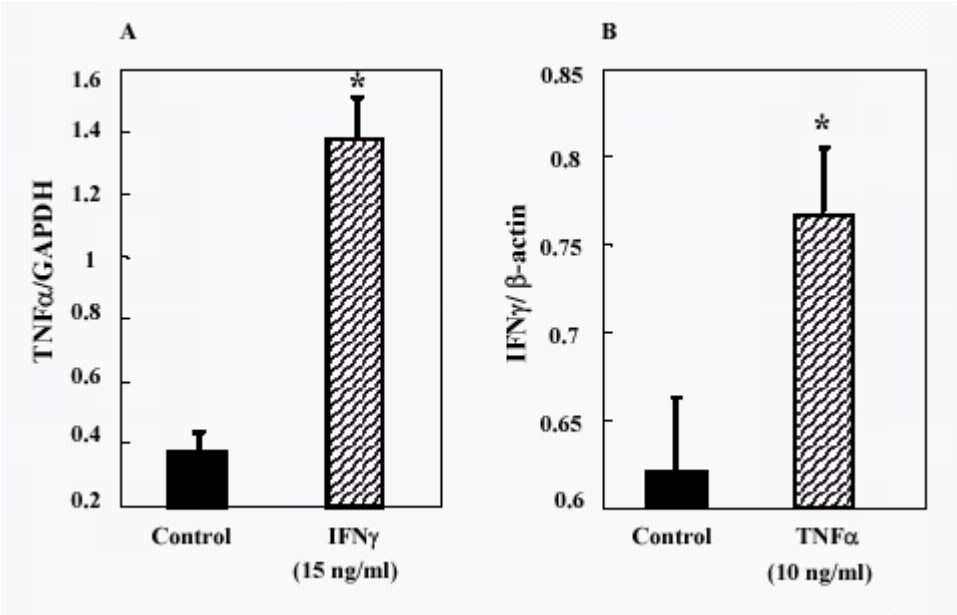


**Fig. 4.8.** (A) To confirm that IFNγ produced from NMuLi cells can stimulate J774A.1 cells to augment expression of TNFα, J774A.1 cells were treated with 15 ng/ml recombinant IFNγ for 6 hr. The mRNA level of TNFα were quantified by RT-PCR and normalized against GAPDH. (B) NMuLi cells were treated with 10 ng /ml recombinant TNFα for 6 h and mRNA levels of IFNγ were quantified by RT-PCR and normalized against β actin. Mean ± SE (n = 3). Asterisk (*) indicates significantly different value ($p < 0.05$) from the control.

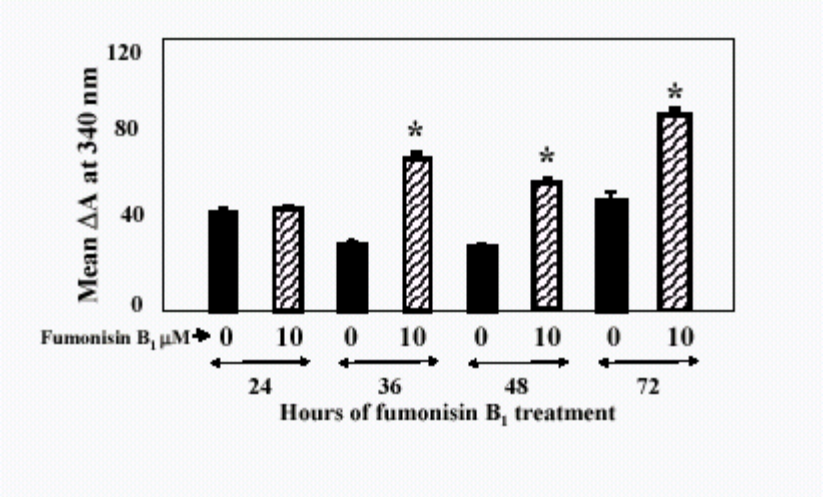


**Fig. 4.9.** Effect of replacing fumonisin $B_1$ treated media with fresh media (without fumonisin $B_1$). Co-culture was treated with 10 μM fumonisin $B_1$ and it's medium was replaced with fresh medium (without fumonisin $B_1$) 24, 36 and 48 h after the fumonisin $B_1$ treatment. LDH released in the replaced fresh medium and in the medium without replacement was measured 72 h after the beginning of fumonisin $B_1$ treatment. The relative LDH activity was calculated as mean of change in absorbance (ΔA) at 340 nm. Mean ± SE (n = 3). Asterisk (*) indicates significant difference ($p < 0.05$) from the control.

# CHAPTER 5

# TUMOR NECROSIS FACTOR α-MEDIATED ACTIVATION OF C-JUN NH2-TERMINAL KINASE AS A MECHANISM FOR FUMONISIN $B_1$-INDUCED APOPTOSIS IN MURINE PRIMARY HEPATOCYTES [1]

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[1]Sharma N., Suzuki H., He Q., and Sharma R.P. 2005. To be submitted to *Journal of Biochemical and Molecular Toxicology*

**ABSTRACT**

Fumonisin $B_1$ is a mycotoxin produced by *Fusarium verticillioides*, frequently associated with corn. It produces hepatic or renal damage in most animal species. Fumonisin $B_1$ perturbs sphingolipid metabolism by inhibiting ceramide synthase. Our previous studies indicated that fumonisin $B_1$ caused localized activation of cytokines in liver produced by macrophages and other cell types that modulate fumonisin $B_1$-induced hepatic apoptosis in mice. The role of tumor necrosis factor α (TNFα) in fumonisin $B_1$-mediated hepatocyte apoptosis has been established; however, not much is known about the downstream events leading to apoptosis. In the current study, fumonisin $B_1$ induced apoptosis in primary cultures of mouse liver cells. In agreement with previous reports, fumonisin $B_1$ caused accumulation of sphingoid bases and led to increase in TNFα expression. Phosphorylated and total c-Jun NH2-terminal kinase (JNK) activities were increased after 24 h fumonisin $B_1$ treatment. JNK inhibitor (SP600125) and anti-TNFα reduced the apoptosis induced by fumonisin $B_1$. The role of JNK signaling in fumonisin $B_1$ induced apoptosis is downstream of TNFα production, as fumonisin $B_1$ mediated activation of JNK was reduced by the presence of anti TNFα in the medium, whereas presence of JNK inhibitor did not change the fumonisin $B_1$ induced TNFα expression. Results of this study imply that generation of fumonisin $B_1$-induced TNFα results in modulation of mitogen activated protein kinases, particularly of JNK and provides a possible mechanism for apoptosis in murine hepatocytes.

Key words*:* Fumonisin $B_1$; hepatotoxicity; c-Jun NH2-terminal kinase; tumor necrosis factor α; mitogen activated protein kinase

## INTRODUCTION

Fumonisins are a group of structurally related mycotoxins produced by *Fusarium verticillioides* and related species, which are commonly found on corn [1]. Fumonisin $B_1$ is the most abundant fumonisin in naturally contaminated foods and feeds. Liver and kidney are the targets of fumonisin $B_1$ toxicity in most domestic and experimental animals [2,3,4]. It causes equine leukoencephalomalacia and porcine pulmonary edema [5]. It is hepatocarcinogenic in male BD IX rats [6], nephrocarcinogen in male F344/N rats and has been associated with human esophageal cancer in southern Africa [7] and primary liver cancer in China [8]. In mouse embryo cultures, fumonisin $B_1$ has been shown to produce embryotoxicity and neural tube defects [9]. It has been suggested that high incidences of neural tube defects occur in some regions of the world (Guatemala, South Africa, and China), where consumption of fumonisin in diet is high [10].

Fumonisin $B_1$ is structurally similar to free sphingoid bases, i.e., sphinganine and sphingosine and is a potent inhibitor of ceramide synthase (sphinganine and sphingosine-N-acyl transferase), a key enzyme responsible for acylation of sphinganine in the *de novo* synthesis of sphingolipids and the reacylation of sphingosine [11,12]. The inhibition of ceramide synthase by fumonisin $B_1$ results in apoptosis caused by accumulation of free sphingoid bases (sphinganine and sphingosine), increased proliferation caused by increased sphingosine-1-phosphate, or/and decreased ceramide and altered lipid raft function due to disruption of complex sphingolipids synthesis/transport [13]. Studies in pig renal epithelial cells have shown that fumonisin $B_1$-induced apoptosis and necrosis are sphinganine-dependent [14]. There is a

close correlation between increased apoptosis in liver and kidney and the disruption of sphingolipid metabolism [15].

Different cell lines respond differently to fumonisin $B_1$ treatment. Diverse cellular responses credited to fumonisin $B_1$ exposure include apoptosis, proliferation and cell cycle arrest. Fumonisin $B_1$ induces apoptosis in various cell lines such as HT29 cells (human colonic cell line) [16], and porcine renal epithelial (LLC-PK1) cells [17], while murine or human leukemia cells (U937), human embryonic kidney cells (HEK-293) do not undergo apoptosis, when treated with fumonisin $B_1$ [18,19,20]. Primary rat hepatocytes and liver slices [21] are quite resistant to fumonisin $B_1$-induced apoptosis. Fumonisin $B_1$ induced cell cycle arrest in the G1 phase in African green monkey kidney cells (CV-1) but CV-1 cells transformed by the simian virus 40 (SV40) large T antigen (COS-7) are unaffected by the same levels of fumonisin $B_1$ [22].

We have demonstrated the role of tumor necrosis factor α (TNFα) in fumonisin $B_1$-induced toxic responses in Balb/c mice [2]. Increased TNFα release was documented in lipopolysaccharide-stimulated macrophages from mice treated with different doses of fumonisin $B_1$. Mouse macrophages also produced TNFα when cultured in the presence of fumonisin $B_1$; acute hematologic effects of fumonisin $B_1$ were neutralized after pretreatment of mice with anti-TNFα [2]. Increase of proinflammatory cytokines from immune cells has been implicated in fumonisin $B_1$-induced hepatotoxicity. Depletion of Kupffer cells by gadolinium chloride attenuated fumonisin $B_1$ induced hepatotoxicity [23]. A positive amplification loop involving TNFα and interferon γ (IFNγ) has been implicated in hepatotoxicity that involves Kupffer cells, hepatic lymphocytes and nonparenchymatous liver epithelial cells [24]. Deletion of

tumor necrosis factor receptors 1 and 2 and IFNγ genes reduced fumonisin $B_1$-induced hepatotoxicity in mice [25,26,27]. The hepatotoxicity in mice in response to fumonisin $B_1$ was abolished after pretreatment of animals with antibody against Thy-1.2 surface antigen of mature T lymphocytes [unpublished].

Taken together, these studies have demonstrated that fumonisin $B_1$ can induce apoptosis *in vitro*, in different cultured cell lines and *in vivo* in liver and kidney of rodents, where production of pro inflammatory cytokines such as TNFα and IFNγ is an important mediator of apoptosis. Although role of TNFα and IFNγ in fumonisin $B_1$-mediated apoptosis of mouse hepatocytes has been established, not much is known about the downstream signaling events leading to cytotoxicity. In this study, we hypothesized that fumonisin $B_1$ induced apoptosis involves TNFα and downstream signaling events such as mitogen activated protein kinases (MAPKs) that lead to cytotoxic outcome in murine hepatocytes. The role of MAPKs in fumonisin $B_1$ toxicity is not well explored [28,29,30]. Our previous studies in porcine renal cells (LLC-PK-1) indicated differential effects of fumonisin $B_1$ on MAPKs involving selective activation of c-Jun NH2-terminal kinase (JNK).

## MATERIALS AND METHODS

### Chemicals

Six to eight week old male C57BL/6 mice weighing 20 to 30 g were obtained from Jackson Laboratory (Bar Harbor, Maine). Fumonisin $B_1$ (purity $> 98\%$) was obtained from Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC, Tygerberg, South

Africa). Antibodies specific for the total and phosphorylated forms of JNK (p54/46) were obtained from Cell Signaling (Beverly, MA). Specific JNK inhibitor (SP600125) was purchased from Calbiochem (La Jolla, CA). Anti-TNFα was procured from Santa Cruz Biotechnology (Santa Cruz, CA). Annexin V and Hoechst 33258 dyes were purchased from Molecular Probes (Eugene, OR). All other reagents were obtained from Sigma (St. Louis, MO) and were of tissue culture grade.

**Isolation of parenchymatous liver cells**

Livers from 6-8 week old male C57BL/6 mice were perfused through the portal vein with 10 ml of enzyme solution (0.05% collagenase dissolved in calcium free Hanks solution). Liver tissue was then cut into slices and placed in to each of three 50 ml Erlenmeyer flasks containing 10 ml of the enzyme solution [31]. The flasks were incubated at 37°C in an atmosphere of 95% $O_2$ and 5% $CO_2$ for 70 min, with constant shaking. After incubation, contents of the flasks were filtered through a single layer of nylon mesh of 61 μm pore size (Sefar America, Depew, NY). Calcium free Hanks solution was added to a final filtrate volume of 40 ml. The resultant cell suspension was centrifuged (50 × *g* for 2 min), hepatocyte pellet was washed and cultured in Williams's medium supplemented with 10% fetal calf serum (heat inactivated), 10 μg/ml insulin, 0.1 mM hydrocortisone, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The non-parenchymal cells (NPCs) were separated from the supernatant by centrifugation (1400 × *g* for 6 min) and cultured in Williams's medium. For co-culture, hepatocytes and NPCs were mixed together in 7:1 proportion as in the original cell preparation. Cultures were maintained at 37°C in a humidified atmosphere

and were allowed to grow overnight (15 h) prior to the treatment. The concentration of fumonisin $B_1$ and time periods for the experiments were optimized in preliminary trials. The concentration used for JNK inhibitor (SP 600125) was 20 μM, added 30 min prior to fumonisin $B_1$ treatment.

**Determination of apoptosis**

The apoptotic effect of fumonisin $B_1$ on mice primary hepatocytes was determined by measuring the fluorescence intensity of Hoechst 33258 using Spectramax Gemini fluorescent plate reader (Molecular Devices, Irvine, CA). Cells were grown and treated in 96 well plates. At the end of treatment, cells were incubated with annexin V (5 μL/100 μL in annexin binding buffer) or Hoechst 33258 (8 μg/ml in 30 % glycerol in phosphate buffered saline). Hoechst 33258 fluorescence was detected at excitation/emission of 346/460 nm and that of annexin V at excitation/emission of 495 /520 nm. Visual documentation of cellular effects was performed using Olympus IX71 inverted microscope (Olympus America, Melville, NY). Digital images were acquired using Olympus Magnafire digital camera.

**Analyses of sphingolipids**

Levels of free sphinganine and sphingosine in hepatic tissues were determined after base treatment of lipid extracts by high performance liquid chromatography according to the methods reported previously [15]. The concentration of sphingoid bases, sphinganine and sphingosine, was quantified based on the recovery of the $C_{20}$-sphinganine (D-erythro-$C_{20}$-dihydro-sphingosine; Matreya, Pleasant Gap, PA) as the internal standard.

**Western blot analysis of JNK**

Levels of total and phosphorylated JNK were determined using antibodies for total and phospho specific JNK, as described previously [32]. Cells were grown at $1.5\times10^6$ cells/well in 6-well microplates and treated with 20 μM fumonisin $B_1$ for 24 h. Following treatment, cells were washed with phosphate buffered saline (PBS) and cell lysates were prepared by scraping in 100 μl of lysis buffer [20 mM Tris–HCl (pH 8.0), 1 mM sodium orthovanadate, 10%glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethyl-enediaminetetraacetate (EDTA), 1%Triton X-100, 50 mM -glycerolphosphate, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin]. Twenty micrograms of protein, determined by Bradford assay, was separated electrophoretically using a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose paper followed by antibody staining. Equal loading and transfer of total protein was verified with the reversible Ponceau S stain (Sigma) dye. Immunodetection was performed using enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Piscataway, NJ).

**Estimation of the presence of Thy-1.2 bearing T cells in parenchymatous cultures**

Primary hepatocytes were grown in sterile four-chambered collagen coated slides (Nunc International, Naperville, IL, USA). Cells were fixed in 1:1 cold methanol: acetone for 30 min, washed gently in PBS and blocked in PBS containing 10% horse serum for 20 minutes. Monoclonal anti-mouse fluorescein conjugated Thy-1.2 (CD90.2) antibody (Southern Biotech, Birmingham, AL) diluted (0.3 μg/$10^6$ cells) in PBS containing 10% horse serum was applied to the slides and incubated for 30 min. Slides were washed, mounted and viewed with an

Olympus IX71 inverted fluorescence microscope (Olympus America, Melville, NY). Images from microscope were recorded by Olympus digital camera using Olympus MagnaFire software.

**Analysis of gene expression**

Total RNA was isolated from cells using TRI® reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNFα and β-actin as internal control [33]. The amplification products were fractionated on ethidium bromide containing 2% agarose gel and documented using a Kodak DC290 digital camera and digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT).

**Statistical analysis**

The studies were repeated 2-3 times, each time with 3-4 replicates, with similar results. Data from these experiments were analyzed by two-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Results are presented as mean ± standard error (SE). All tests were performed using an SAS computer program (SAS Institute, Cary, NC). The level of $p \leq 0.05$ was considered statistically significant.

**RESULTS**

**Fumonisin $B_1$ induced apoptosis in primary cultures of mice hepatocytes**

Treatment of various cultures with 20 μM fumonisin $B_1$ for 48 h resulted in increased apoptosis in the primary culture of mice hepatocytes, measured by recording the fluorescence

of Hoechst 33258 dye (Fig. 5.1). Isolated hepatocytes, NPCs or co-culture were equally sensitive to fumonisin $B_1$ induced apoptosis. To confirm apoptosis, cells were visualized under Olympus IX71 inverted fluorescence microscope (Olympus America, Melville, NY), which confirmed the presence of increased number of apoptotic cells exhibiting bright green staining of Annexin V upon treatment with fumonisin $B_1$ (Fig. 5.2). To detect the presence of NPCs in the primary culture, cells were stained with fluorescein conjugated Thy-1.2 (CD90.2) antibody (Southern Biotech, Birmingham, AL), which indicated that Thy 1.2 surface antigen bearing mature T cells were isolated with the parenchymatous cells (Fig. 5.3).

**Fumonisin $B_1$-induced accumulation of sphingolipids**

Intracellular levels of sphinganine and sphingosine were increased in hepatocytes exposed to 20 μM fumonisin $B_1$ for 24 h. Over the 48 h incubation period with fumonisin $B_1$, sphingosine and sphinganine concentrations decreased compared to 24 h fumonisin $B_1$ treatment (Figure 4).

**Role of activated JNK in fumonisin $B_1$ induced apoptosis**

To examine the relationship between fumonisin $B_1$ induced JNK activation and apoptosis, we employed the JNK inhibitor anthra-pyrazolone (SP600125) [34]. Primary hepatocyte cultures were incubated with different doses of fumonisin $B_1$ in the absence or presence of 20 μM SP600125 and assayed for apoptosis by measuring the fluorescence of Hoechst 33258. Hoechst 33258 fluorescence was similar in SP600125-treated or control cultures, but it significantly decreased in cultures, treated with 10 μM or higher concentrations of fumonisin $B_1$ in the presence of SP600125 compared to cultures treated with fumonisin $B_1$ only (Fig. 5.5).

**Activation of JNK in fumonisin $B_1$ treated primary hepatocytes**

To investigate whether fumonisin $B_1$ alters activity of phosphorylated JNK and total JNK, the cell lysates were examined by Western blots. In mice primary hepatocyte cultures, the levels of phosphorylated forms of p46 (JNK1) and p 54 (JNK2) increased after 24 h treatment with 20 μM fumonisin $B_1$ (Fig. 5.6). Total JNK levels also increased upon treatment with fumonisin $B_1$ for 24 h (Fig. 5.6).

**Fumonisin $B_1$ induced apoptosis is inhibited by anti-TNFα**

In fumonisin $B_1$ sensitive cell lines or *in vivo* experiments, increased production of TNFα and IFNγ has been implicated in fumonisin $B_1$ toxicity. To evaluate the role of these cytokines in fumonisin $B_1$ mediated apoptosis in primary hepatocyte culture; we measured the induction of apoptosis in the presence of neutralizing antibodies for TNFα and IFNγ. Results indicated that elevation of Hoechst 33258 fluorescence by fumonisin $B_1$ was reduced in cultures treated with anti-TNFα and but not by anti-IFNγ alone. The combination of anti-TNFα and IFNγ did not provide additional protection (Fig. 5.7).

**Inhibition of JNK activation by anti-TNFα**

To delineate the role of JNK in fumonisin $B_1$-activated signaling pathways, we examined the phosphorylation JNK in primary hepatocyte cultures in the presence of anti-TNFα. The immunoblot with phosphorylated form of JNK specific antibody revealed that presence of anti TNFα significantly reduced fumonisin $B_1$-induced JNK activation (Fig. 5.8). Similarly, the fumonisin $B_1$ -mediated increase in total JNK was also inhibited by anti-TNFα but not

significantly and the total JNK in the presence of anti-TNFα was not significantly different between control and fumonisin $B_1$-treated cultures (Fig. 5.8).

**JNK inhibitor does not influence TNFα expression**

To establish that JNK modulation was downstream of TNFα induction by fumonisin $B_1$, the relationship of JNK activation and TNFα production was examined by measuring the expression of TNFα in primary hepatocyte culture in the presence of 20 μM JNK inhibitor SP600125. Expression of TNFα was increased with fumonisin $B_1$ treatment of primary hepatocyte culture for 24 h and inhibition of JNK did not change such induction in TNFα expression suggesting that production of TNFα is not a consequence of JNK activation (Fig. 5.9).

## DISCUSSION

Fumonisin $B_1$ causes *in vivo* hepatotoxicity in rats and mice. We previously reported that mice injected with different doses of fumonisin $B_1$ exhibited dose related apoptosis in hepatocytes [3]. Hepatopathy in mice in another study was described as presence of small, round to slightly ovoid cells separated from neighboring cells with condensed chromatin; features consistent with apoptosis [4]. Increase in the activity of hepatic enzymes, a marker for hepatotoxicity has been a regular feature of fumonisin $B_1$ toxicity in mice but not in rats [35]. Liver is the primary site of fumonisin $B_1$ toxicity in male mice, whereas in rats nephrotoxicity appears to predominate [3].

In this study, we found that fumonisin $B_1$ induces apoptosis in primary culture of mouse liver cells, which is consistent with other *in vivo* studies. Earlier we reported that fumonisin $B_1$ is more toxic to non-parenchymatous cells in a coculture of macrophage and non-parenchymatous epithelial cells (NPECs) due to interaction of different cytokines produced by them [33]. In this study the ability of fumonisin $B_1$ to induce apoptosis in isolated hepatocytes, NPCs or their coculture was similar. However, the separation procedure does not allow removal of lymphocytes from the hepatocytes as we noted the presence of lymphocytes in hepatocyte cultures and no additive effect on cytotoxicity of fumonisin $B_1$ in co-culture further confirms that lymphocytes may be integral for fumonisin $B_1$-mediated apoptosis in parenchymal cells.

There is increasing evidence that sphingolipids play a key role in fumonisin $B_1$ toxicity by modulating numerous intracellular pathways controlling differentiation, growth, and apoptosis [36]. Fumonisin $B_1$ induced hepatotoxicity in several strains of mice or with different doses of fumonisin $B_1$, has been shown to correlate well with intracellular accumulation of sphinganine and sphingosine [15]. In this study, fumonisin $B_1$ induced accumulation of sphinganine and sphingosine in primary mice heaptocyte culture during 24 hr treatment. After fumonisin $B_1$ treatment for 48 h, levels of sphingosine decreased to the baseline level i.e. same as in control group and level of spinganine also decreased compared to the level at 24 h fumonisin $B_1$ treatment. Decrease in free sphingoid bases observed in fumonisin $B_1$-treated cells between 48 and 72 h was also reported earlier and may indicate the induction of sphinganine metabolism or down regulation of the *de novo* pathway [14].

Fumonisin $B_1$ exposure of primary hepatocytes for 24 h resulted in increased TNFα expression, which was consistent with our earlier studies, where peritoneal macrophages isolated from fumonisin $B_1$ treated mice produced increasing levels of TNFα when stimulated with bacterial lipopolysaccharide *in vitro*, and a macrophage cell line (J774A.1) treated with fumonisin $B_1$ also produced higher levels of TNFα after 24 h [2]. There is considerable evidence that TNFα signaling pathways play an important role in fumonisin $B_1$-induced hepatotoxicity. Fumonisin $B_1$ toxicity was reduced in mice lacking either TNFα receptor (TNFR) 1 or TNFR 2 [25,26]. The acute hematological effects of fumonisin $B_1$ were neutralized after pretreatment of mice with anti- TNFα [2]. It was reported that fumonisin $B_1$ toxicity was effectively prevented by inhibition of caspases, which are downstream of TNFα cellular signaling [37].

JNK is a member of the mitogen-activated protein (MAP) kinase family and is activated by a variety of extracellular stimuli through JNK kinases and multiple MAP kinase kinase kinases [38]. Activated JNK, phosphorylates and activates c-Jun, a component of the transcription factor AP-1 and many other targets as well [38]. Our results showed that fumonisin $B_1$ stimulated JNK activity as phosphorylated and total JNK both were increased in primary hepatocyte cultures after 24 h exposure of fumonisin $B_1$. SP600125, a specific JNK inhibitor decreased the extent of apoptosis by fumonisin $B_1$ indicating that fumonisin $B_1$ mediated activation of JNK was at least partly responsible for the induction of apoptosis in primary hepatocyte cultures.

There are only few reports indicating the role of MAPKs in fumonisin $B_1$ induced toxicity in mammalian cells [28,29,30]. A differential activation of JNK but not of ERK or p38 was reported on LLC-PK1 cells by fumonisin $B_1$ [32]. Recently, a decrease in ERK has been reported in LLC-PK1 cells at 24 h [29]. In our other studies, we have noticed the induction of both total and phosphorylated JNK in livers of fumonisin $B_1$-treated mice (unpublished data).

Fumonisin $B_1$ stimulated JNK activity is downstream of TNFα production, as fumonisin $B_1$ mediated activation of JNK was reduced by the presence of anti TNFα in the medium whereas presence of JNK inhibitor did not change the fumonisin $B_1$ induced TNFα expression in primary hepatocyte culture. The role of JNK activation in mediating the TNF receptor signaling is uncertain, ranging from being protective, irrelevant or pro-apoptotic, which depends on the stimuli and cell types [39]. However, there is strong evidence indicating that JNK activation could increase TNFα-induced apoptosis in hepatocytes [40,41]. JNK-mediated apoptosis in hepatocytes was shown to involve mitochondrial permeability transition, release of cytochrome c and activation of caspase-3, -8 and -7, suggesting that JNK acts upstream of the mitochondria [40]. A recent report provided strong evidence that JNK activation in TNFα signaling involves release of Smac/DIABLO from the mitochondria, but not cytochrome c [42]. The released Smac/DIABLO then disrupts the TRAF2-cIAP1 complex, relieving the inhibition imposed by TRAF2-cIAP1 on caspase 8 activation and induction of apoptosis [42]. TRAF2 also mediates the activation of the JNK pathway, as shown by JNK inhibition by TRAF2Δ and in TRAF2 (−/−) mice [43].

Increases in hepatocellular oxidation stress such as those that occur with hepatic overexpression of cytochrome *P*-450 isoforms promoted TNFα mediated apoptosis in hepatocytes through increased activity of c-Jun $NH_2$-terminal kinase [44]. Overexpression of CYP2E1 sensitized hepatocyte to TNFα-mediated cell death despite increased levels of nuclear factor-κB transcriptional activity and was associated with increased lipid peroxidation and glutathione(GSH) depletion [44]. Fumonisin $B_1$ was reported to increase induction of cytochrome p450 isoforms and cause peroxidation of membrane lipids in isolated rat liver nuclei as well as GSH depletion of in pig kidney cells [45,46,47]. GSH depletion is known to activate JNK through redox inhibition of glutathione S-transferase Pi, which normally binds to and inhibits stress kinases [48].

To our knowledge this is the first report that fumonisin $B_1$ can induce apoptosis in hepatocytes by involving JNK activation. TNFα production is upstream of JNK signaling and is responsible for fumonisin $B_1$-induced apoptosis in mice primary hepatocytes.

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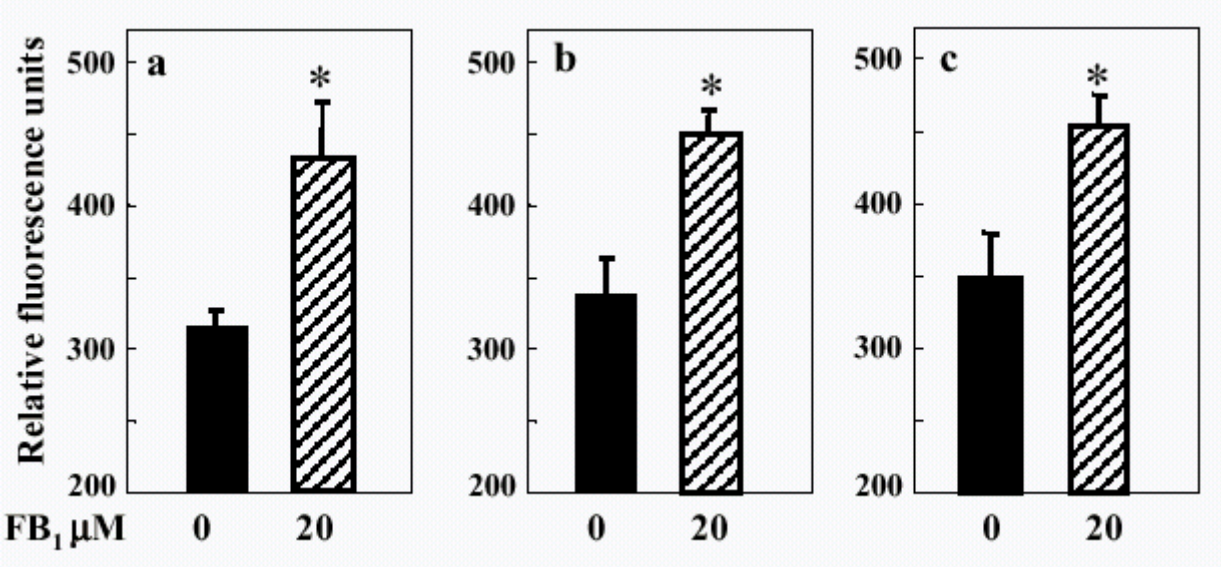


**Fig. 5.1** Fumonisin $B_1$ induced apoptosis in primary culture of liver cells. Treatment of isolated hepatocytes (a), nonparenchymal cells (b) and co-culture of hepatocytes and NPCs with 20 μM fumonisin $B_1$ for 48 h resulted in increased apoptosis compared to untreated cultures. For individual cultures, cells were plated in 96 well plates at $4 \times 10^4$ cells/well. For co-culture, cells were mixed in a ratio of 1 (NPCs) : 7 (hepatocytes) i.e. $5 \times 10^3$ NPCs and 35 x $10^3$ hepatocytes. At the end of treatment, cells were incubated with Hoechst 33258 dye for 10 min and fluorescence intensity was recorded by microplate spectrofluorometer at 346/460 nm. Relative fluorescence units are arbitrarily defined based on fluorescence recorded at 346/460 nm. Similar findings were noted in 3 independent experiments. Results are expressed as mean ± S.E. of 3 independent experiments. Asterisks on bars indicate significantly different value ($p < 0.05$) from the control.

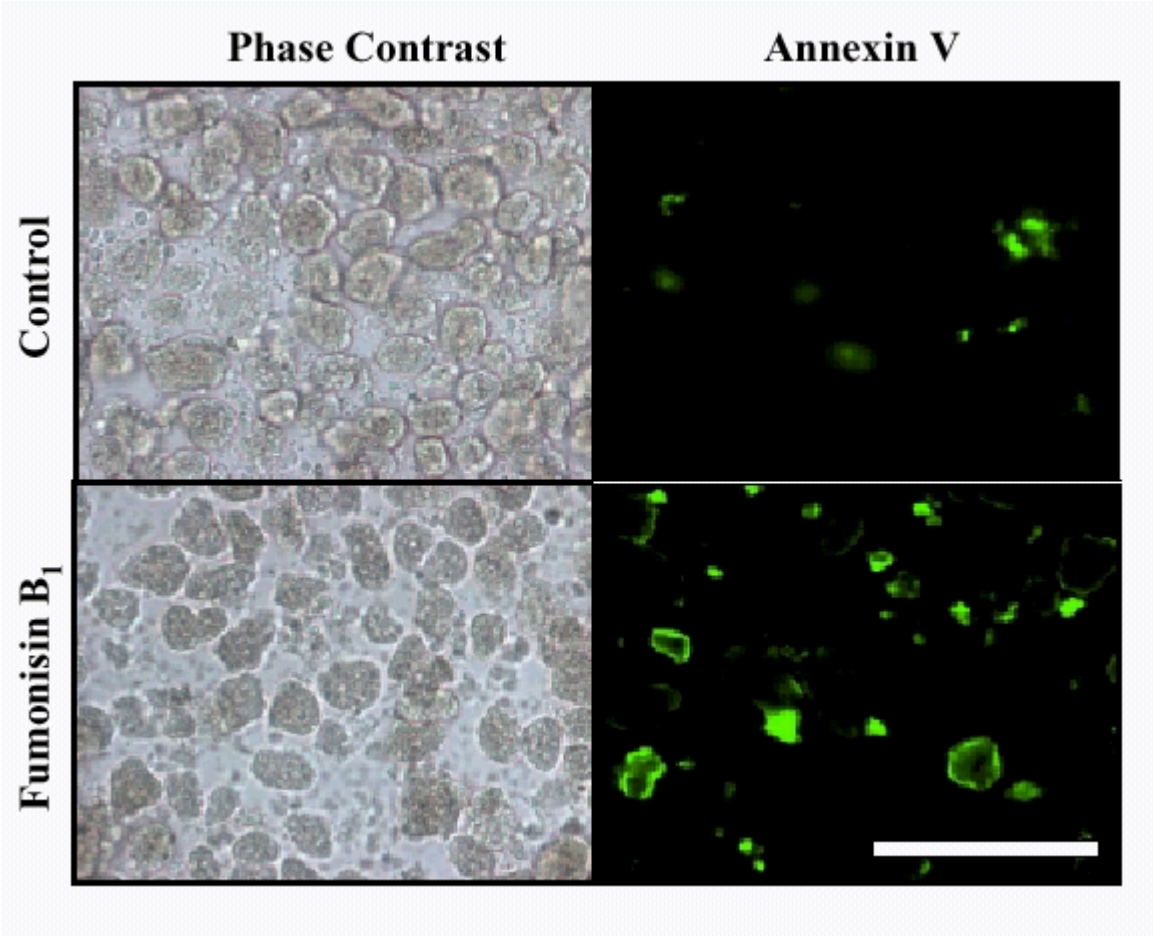


**Fig. 5.2** Primary hepatocyte cultures were treated with 20 μM fumonisin $B_1$ for 48 h and examined using annexin V dye under phase contrast or fluorescence microscope. Annexin V binds with exposed phosphatidyl serine on the surface of apoptotic cells and imparts on the cell surface a bright green staining. Hepatocyte culture treated with fumonisin $B_1$ had greater number of cells stained with Annexin V compared to control. Bar on lower left represents 10 μm.

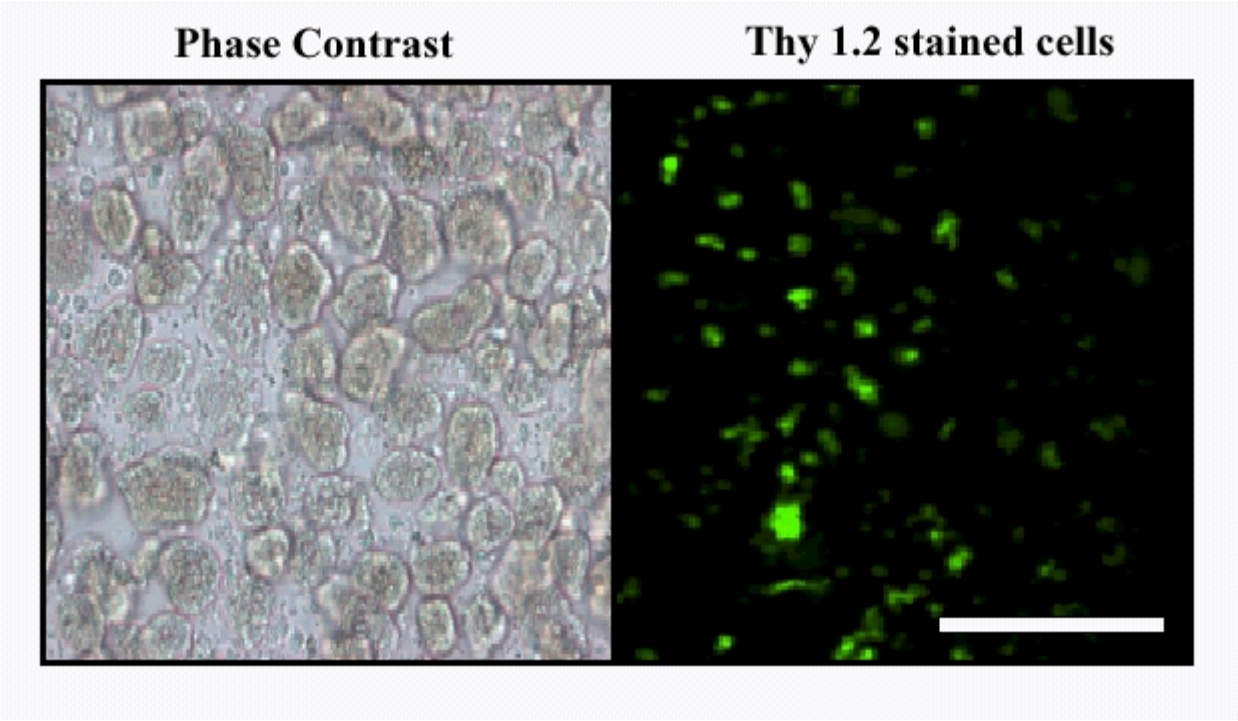


**Fig. 5.3** Culture of primary liver cells was stained by fluorescene-conjugated antibody against mice Thy 1.2 antigen on the surface of mature T cells. Green stained Thy 1.2 bearing cells in the right panel confirms the presence of mature T cells in the liver parenchymatous cell culture. Bar on lower left represents 10 μm.

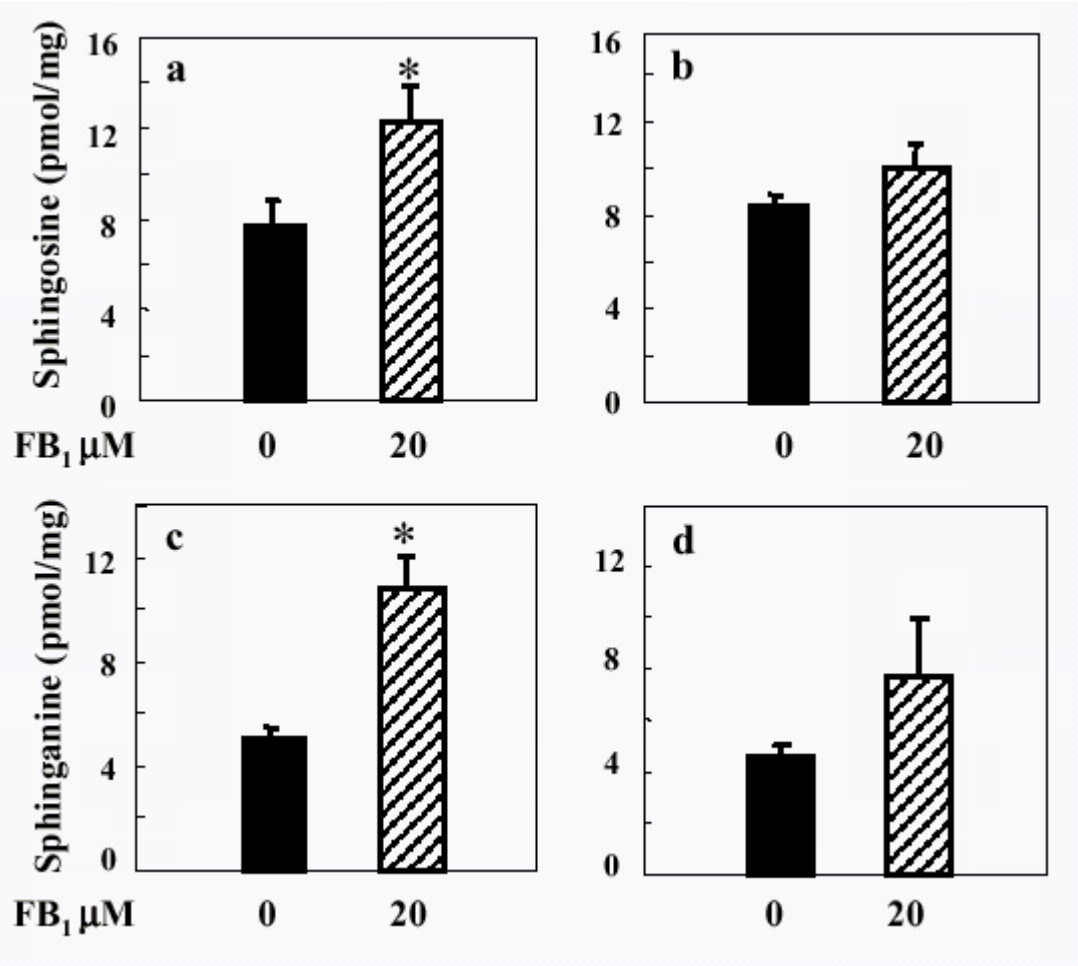


**Fig. 5.4** Concentrations of free sphinganine and sphingosine after exposure of hepatocytes to 20 μM fumonisin $B_1$ for 24 h (A and C) or 48 h (B and D). Mean ± SE (n = 3). Asterisks on bars indicate significantly different value ($p < 0.05$) from the concurrent control.

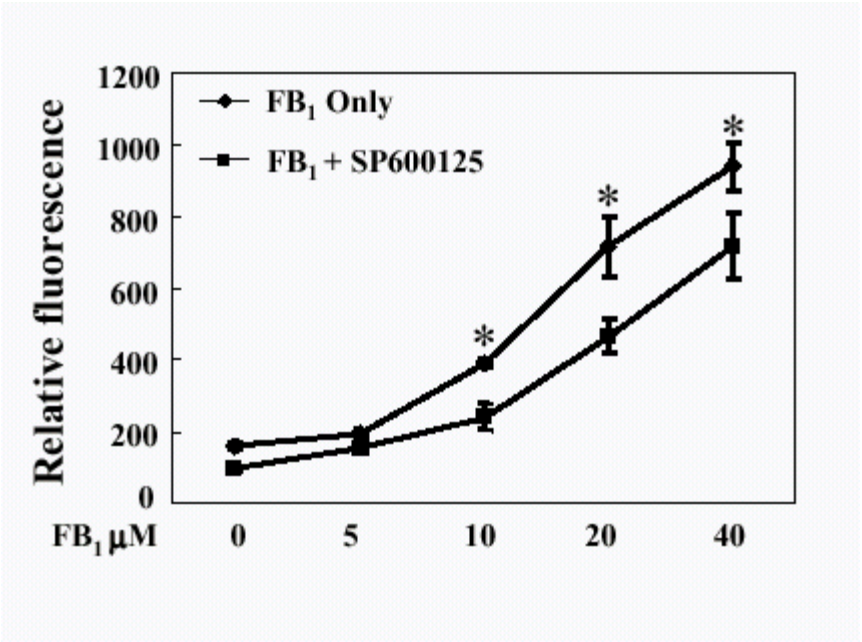


**Fig. 5.5** Inhibition of fumonisin $B_1$ mediated apoptosis by JNK inhibitor (SP600125). Hepatocytes were pre-treated with 20 µM SP600125 for 30 min and then exposed to different concentrations of fumonisin $B_1$ for 48 h. The intensity of Hoechst 33258 fluorescence after fumonisin $B_1$ exposure was read by microplate spectrofluorometer. Relative arbitrarily fluorescence units are indicated at excitation/emission of 346/460 nm. Mean ± SE of 3 independent experiments. Asterisks on bars indicate a significant difference from the control ($p < 0.05$).

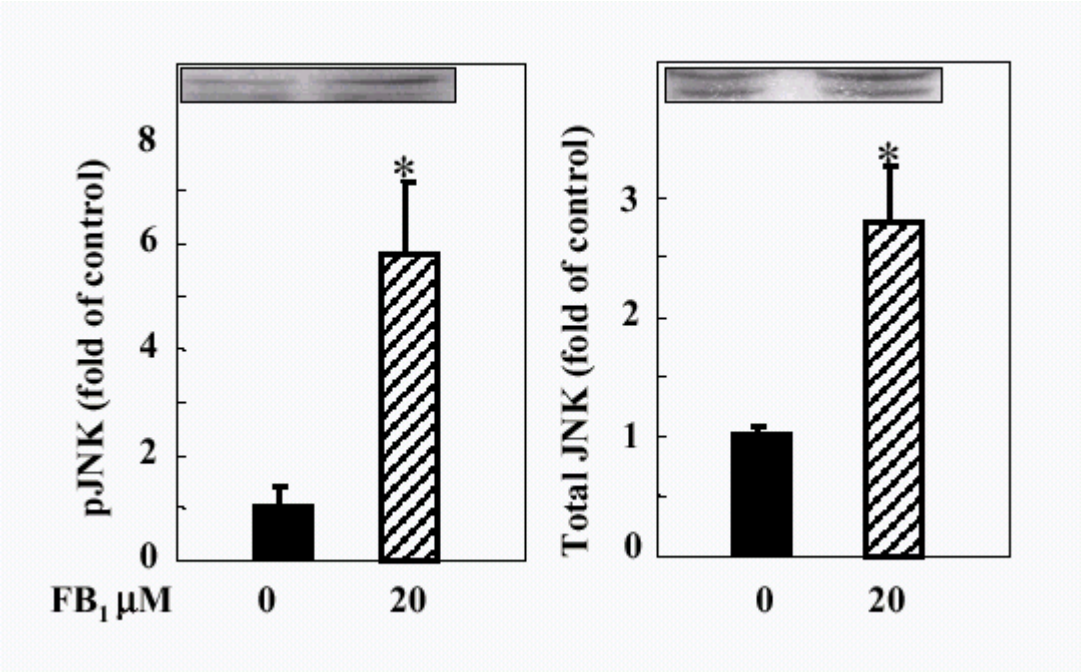


**Fig. 5.6** Effect of fumonisin $B_1$ treatment on phosphorylation of JNK and expression of total JNK. Primary culture of hepatocytes were exposed to 20 μM fumonisin $B_1$ for 24 h. Cell lysates were analyzed by western blot to detect the p-JNK and total JNK (p54 and p46). Mean ± SE (n = 3). Asterisks on bars indicate a significant difference from the control $p < 0.05$.

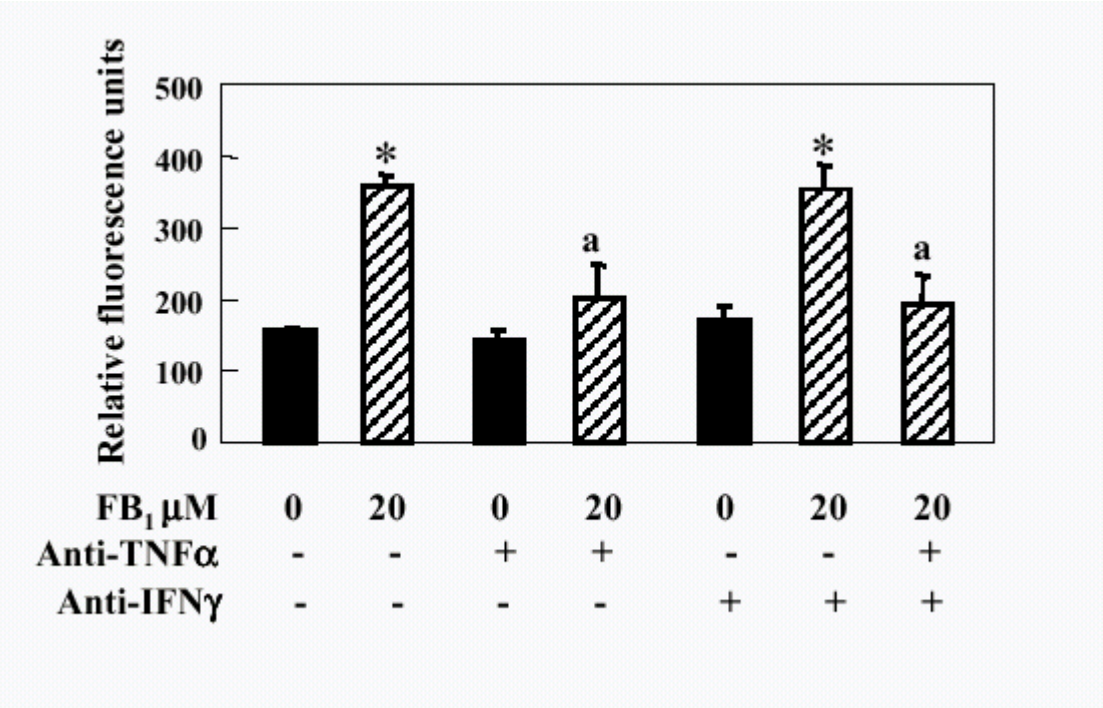


**Fig. 5.7** Effect of anti-TNFα and anti-IFNγ on fumonisin $B_1$ mediated apoptosis. Hepatocytes were treated with 20 µM fumonisin $B_1$ in the presence of neutralizing antibodies for TNFα (1 µg/ml) or/and IFNγ (1µg/ml) for 48 h. At the end of treatment, cells were incubated with Hoechst 33258 dye for 10 min and fluorescence intensity was recorded by fluorescent plate reader at 346/460 nm. Relative arbitrarily fluorescence units are indicated at excitation/emission of 346/460 nm. Mean ± SE of 3 independent experiments. Asterisks on bars indicates significantly different value ($p < 0.05$) from the control. Letter "a" on bars indicates a significant decrease from the fumonisin $B_1$ treated group ($p < 0.05$).

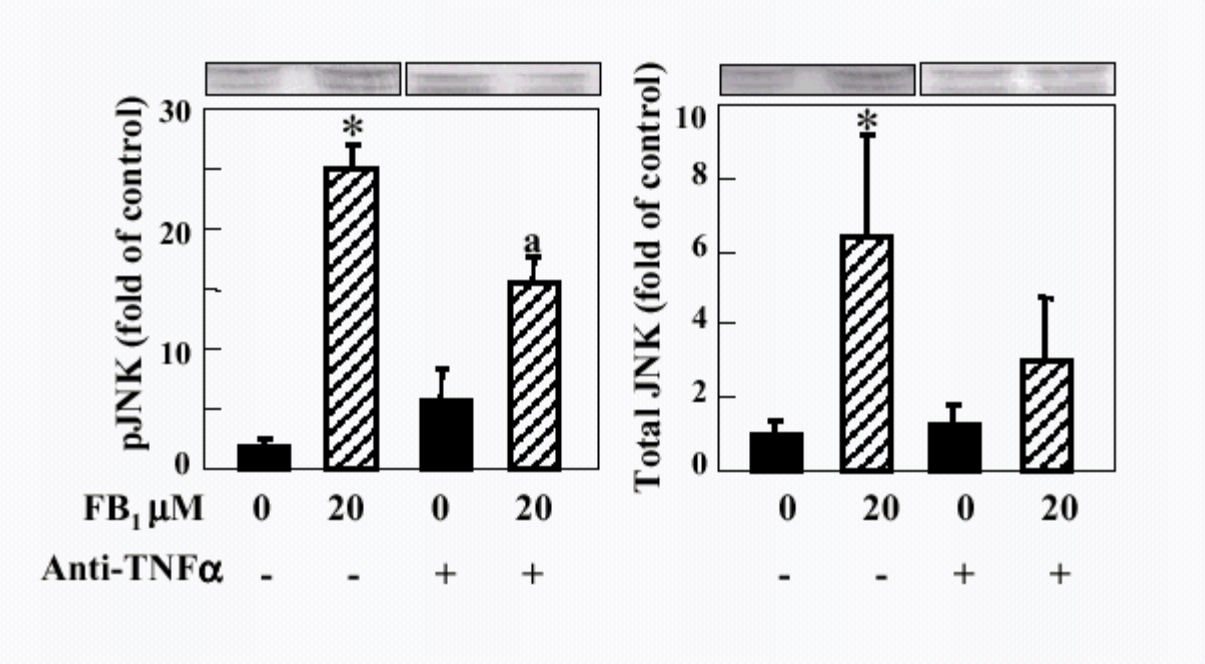


**Fig. 5.8** Effect of fumonisin $B_1$ treatment on phosphorylation of JNK and expression of total JNK in the presence of neutralizing antibodies for TNFα. Primary culture of hepatocytes were exposed to 20 μM fumonisin $B_1$ and TNFα (1 μg/ml) for 24 h. Cell lysates were analyzed by western blot to detect the p-JNK and total JNK (p54 and p46). Mean ± SE (n = 3). Asterisks on bars indicate a significant difference from the control $p < 0.05$.

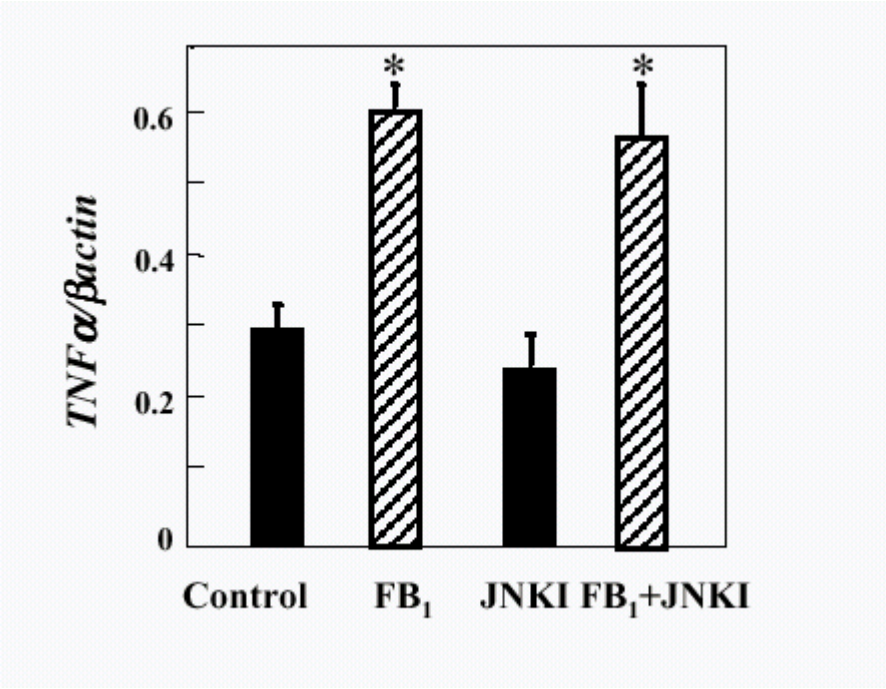


**Fig. 5.9** Effect of JNK inhibitor (JNKI) on fumonisin $B_1$ induced expression of TNFα. Cultures of primary hepatocytes were treated with 20 μM fumonisin $B_1$ in the presence of 20 μM SP600125 for 24 h. Expression of specific mRNA was quantified by RT-PCR and normalized to that of β-actin in the same sample. Mean ± SE of 3 independent experiments. Asterisks on bars indicates significantly different value ($p < 0.05$) from the control.

# CHAPTER 6

# AMELIORATION OF FUMONISIN $B_1$ HEPATOTOXICITY IN MICE BY DEPLETION OF T CELLS WITH ANTI-THY1.2[1]

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[1]Sharma N., He Q., and Sharma R.P. 2005. Submitted to *Toxicological Sciences*

**ABSTRACT**

Fumonisin $B_1$ is a mycotoxin produced by *Fusarium verticillioides*, frequently associated with corn. It produces hepatic or renal damage in most animal species. Our previous studies indicated that fumonisin $B_1$ caused localized activation of cytokines in liver. In the current study, male athymic nude mice and their wild type counterparts (WT) with or without depletion of T cells were treated subcutaneously with fumonisin $B_1$ at 2.25 mg/kg/day for 5 days and sampled 24 h after the last injection. Depletion of T cells in WT was achieved by a single intravenous injection of 50 μg monoclonal antibody against Thy-1.2 surface antigen of mature peripheral T lymphocytes 24 h before the first fumonisin $B_1$ treatment. The fumonisin $B_1$-induced elevation in activities of circulating liver enzymes were not different between WT and nude mice, while depletion of T cells nearly abolished fumonisin $B_1$-mediated liver toxicity, indicated both by concentrations of circulating liver enzymes and enumeration of apoptotic hepatocytes. Since all the fumonisin $B_1$-treated groups had similar extent of hepatic sphinganine accumulation, the reduced toxicity of fumonisin $B_1$ in T cell-depleted mice was unrelated to its effect on sphinganine accumulation. Expression of fumonisin $B_1$-induced tumor necrosis factor α and interleukin-1 was observed in nude and WT mice but not in T cell-depleted mice. This study suggested that T cells and corresponding proinflammatory cytokines have a vital role in mediating fumonisin $B_1$-induced hepatic toxicity.

Key words*:* Fumonisin $B_1$; hepatotoxicity; CD90.2 T cells; tumor necrosis factor α; interferon γ; anti-Thy 1.2

## INTRODUCTION

Fumonisin $B_1$ is a mycotoxin produced by *Fusarium verticillioides* and related species, commonly found in corn. The toxicity of fumonisin $B_1$ has been described in a number of farm and laboratory animals and depends on different factors such as animal species, gender, age, dose and route of administration (Diaz and Boermans, 1994). It has been established as a cause of equine leukoencephalomalacia and porcine pulmonary edema. Fumonisin $B_1$ has been shown to be hepatotoxic and nephrotoxic in mice and rats. The National Toxicology Program study reported renal carcinoma in male Fischer rats and hepatic cancer in female B6C3F mice (Howard *et al.*, 2001). In the Transkei region of South Africa where incidence of esophageal cancer is high, an association between fumonisin $B_1$ consumption and esophageal cancer was reported (Sydenham *et al.*, 1990). International Agency for Research on Cancer (1992) classified fumonisin $B_1$ as a Group 2B carcinogen, i.e., possibly carcinogenic to humans.

Fumonisin $B_1$ is structurally similar to sphingoid bases and causes inhibition of ceramide synthase (sphinganine or sphingosine *N*-acyltransferase) leading to accumulation of free sphingoid bases, sphingoid base metabolites, and depletion of more complex sphingolipids (Riley *et al.*, 1996). Studies in pig renal epithelial cells (LLC-$PK_1$) and human colonic cells (HT29) have shown that fumonisin $B_1$-induced apoptosis, necrosis, and inhibition of proliferation are sphinganine dependent (Merrill *et al.*, 2001). There is a close association between increased apoptosis in liver and kidney and the disruption of sphingolipid metabolism (Tsunoda *et al.*, 1998).

Fumonisin $B_1$ treatment caused an increased expression of tumor necrosis factor α (TNFα), interferon γ (IFNγ) and interleukin (IL)-12, in liver, where cells involved in TNF production were identified as Kupffer cells (Bhandari *et al.*, 2002). Increase of proinflammatory cytokines from immune cells has been implicated in fumonisin $B_1$-induced hepatotoxicity. Depletion of Kupffer cells by gadolinium chloride attenuated fumonisin $B_1$ induced hepatotoxicity (He *et al.*, 2005). Deletion of tumor necrosis factor receptors 1 and 2 and interferon γ genes reduced fumonisin $B_1$-induced hepatotoxicity in mice (Sharma *et al.*, 2001, 2003).

The role of inflammatory cytokines in the initiation and development of liver toxicity has been well documented. Liver associated immune cells such as lymphocytes, Kupffer cells and their cytokine products; especially TNF and IFN are involved in liver injury during alcoholic hepatitis (Batey and Wang, 2002). TNFα and IL-1α are responsible for certain pathological manifestation of acetaminophen induced hepatotoxicity in mice (Blazka *et al.*, 1995). IFNγ and TNFα released from T lymphocytes and macrophages, respectively, are involved in concanavalin A (Con A)-induced hepatitis and in liver toxicity in response to repeated challenges of lipopolysaccharide (Wolf *et al.*, 2001).

Reports on the effects of fumonisin $B_1$ on the immune system are conflicting. Fumonisin- $B_1$ was found to be both immunosuppressive and immunostimulatory in BALB/c mice, depending on the dose administered and induced an antigenic response (Martinova and Merrill, 1995). Fumonisin $B_1$ was shown to alter the expression of the CD3 receptor, which is

integral to T lymphocyte activation. Gavage studies in mice indicated no changes in myeloid, erythroid or other cell types in bone marrow or total circulating immunoglobulin levels (Bondy *et al.*, 1997). Recently Taranu *et al*. (2005) reported that ingestion of fumonisin $B_1$ alters the Th1/Th2 balance leading to reduced vaccinal antibody response in pigs.

Despite many reports confirming the role of proinflammatory cytokines in fumonisin $B_1$ induced liver damage, little is known about the contribution of T cells in this regard. We hypothesized that fumonisin $B_1$ hepatotoxicity in mice will be compromised by depletion of T cells. Athymic nude mice are deficient in T cells; therefore in the current study we employed nude mice and their immunocompetent wild-type counterparts in this investigation. Depletion of T cells in wild-type mice was accomplished by injecting monoclonal antibodies directed against Thy-1.2 surface antigens; this approach has become an established method to investigate the T cell functions in mice (Opitz *et al.*, 1982). The objective of this study was to investigate the role of T cells in fumonisin $B_1$-induced hepatotoxicity in immunocompetent mice by depleting T cells and comparing it with immunodeficient athymic nude mice.

## MATERIALS AND METHODS

### *Animals*

Seven-week-old male athymic nude mice (Hsd:*nu*/*nu*, stock #6904M) and their wild-type (WT) counterparts (Hsd:nu/+, stock #7004M) were obtained from Harlan Laboratories

(Indianapolis, IN). The nu/+ mice are heterozygous between nude and normal mice and are fully immunocompetent; these were used as controls (henceforth referred to as WT) as our aim was to compare their response to fumonisin $B_1$ with corresponding immunodeficient nude mice. The mice were acclimated for 1 week before dosing under environmental conditions at 23°C and 65% relative humidity with a 12-h light/dark cycle. Animals had free access to fumonisin free feed and water at their own discretion. All experiments were conducted according to the Public Health Service Policy on Humane Care and Use of Laboratory Animals and protocols were approved by the Institutional Animal Care and Use Committee.

*Treatments*

Animals were randomly divided in four groups of WT mice and two groups of nude mice (5 mice per group). Two groups of WT mice received intravenous injections of 50 μg monoclonal anti-mouse Thy-1.2 (CD90.2) antibody (Southern Biotech, Birmingham, AL) per mouse 24 h before the first fumonisin $B_1$ treatment; these mice from now on are referred as T cell-depleted (TCD) mice. Dose of antibody to achieve depletion of T cells was selected based on previous studies and modified for the purity of the product and route of administration (Opitz *et al.*, 1982; Tiegs *et al.*, 1992). One group each of WT, TCD and nude mice were treated with five daily subcutaneous injections of either phosphate-buffered saline (PBS) or 2.25 mg/kg/day of fumonisin $B_1$ (> 98% purity, from Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, South Africa) in PBS. The dose of fumonisin $B_1$ and duration of treatment was based on our previous studies in which it produced a consistent hepatotoxic damage in male mice (Sharma *et al.*, 2001, 2003).

Twenty four hours after the fifth and final injection, mice were euthanized by halothane and blood was drawn by heart puncture in heparinized tubes for hematological evaluation; plasma was isolated for determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Activities of plasma ALT and AST were determined using a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN).

Total white and red cells in blood were counted using an electronic counter (Coulter Electronics Inc., Hialeah, FL). Liver, kidney and spleen were removed, weighed, and aliquots of liver were fixed immediately in neutral 10% formalin or quickly frozen in liquid nitrogen and stored at –85°C until analysis.

*Determination of apoptosis in liver*

Liver sections (5 μm) were prepared from formalin-fixed paraffinized liver samples and subjected to terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) with a peroxidase-based *in situ* Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN) as described previously (Sharma *et al.*, 2003). The stained apoptotic cells were normalized to the unit area.

*Detection of Thy-1.2 bearing T cells in liver*

Frozen sections (5 μM) were thawed in the presence of 10% normal horse serum in PBS and blocked for 1 h. Sections were incubated overnight with 1:100 dilution of anti-mouse Thy-1.2 antibody in a humidified chamber at 4°C, then washed with PBS and incubated with peroxidase-labeled anti-mouse immunoglobulin G for 1 h. After washing with PBS, the

sections were stained with 3,3-diaminobenzidine and counterstained with hematoxylin and eosin.

*Analyses of sphingolipids*

Levels of free sphinganine and sphingosine in hepatic tissues were determined after base treatment of lipid extracts by high performance liquid chromatography according to the methods reported previously (Tsunoda *et al*. 1998). The concentration of sphingoid bases, sphinganine and sphingosine, was quantified based on the recovery of the $C_{20}$-sphinganine (D-erythro-$C_{20}$-dihydro-sphingosine; Matreya, Pleasant Gap, PA) as the internal standard.

*RNA isolation and ribonuclease protection assay (RPA)*

Total RNA from liver tissue was isolated using TRI® reagent (Molecular Research Center, Cincinnati, OH) and used for RPA using RiboQuant TM RPA starter kit (BD Biosciences, San Diego, CA), as described earlier (Sharma *et al.*, 2003). High specific activity $\alpha^{32}$P-UTP-labeled anti-sense RNA probes were synthesized using T7 RNA polymerase in vitro transcription kit according to the manufacturer's protocol (BD Biosciences, San Diego, CA). The synthesized probes were hybridized with 50 μg RNA overnight at 65°C and digested with RNase A and RNase T1, followed by treatment with protease K. The RNase-protected products were purified and resolved on gels containing 5% polyacrylamide/7 M urea. The $\alpha^{32}$P labeled bands were exposed to an FX Imaging Screen K-HD ® (Bio-Rad Laboratories, Hercules, CA) for 6–24 h and scanned by Bio-Rad Molecular Imager ® FX. The relative gene expression was digitized using the Quantity one® software (Bio-Rad Laboratories)

*Statistics*

Data from these experiments were analyzed by two-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Results are presented as mean ± standard error (SE). All tests were performed using an SAS computer program (SAS Institute, Cary, NC). The level of $p \leq 0.05$ was considered statistically significant.

## RESULTS

*Depletion of T cells reduced $FB_1$-induced hepatotoxicity*

After 5 days of treatment with fumonisin $B_1$, body and kidney weight were significantly reduced in WT mice and liver weight significantly increased in nude mice; in all other groups there were no significant differences in body, liver kidney or spleen weights elated to fumonisin $B_1$ treatment (Table. 6.1). No differences were observed for food and water intake in any of the experimental groups (data not shown). Fumonisin $B_1$ treatment of WT mice caused a marked increase in plasma ALT and AST activities, 32-fold and 14-fold, respectively, compared to corresponding saline-treated WT group (Fig. 6.1). The fumonisin $B_1$-induced elevation of ALT and AST activity was not observed in TCD mice. Plasma ALT and AST activities increased in fumonisin $B_1$-treated nude mice to a similar extent as in WT mice, indicating that nude mice had no protection against fumonisin $B_1$ hepatotoxicity.

*Depletion of T cells diminished fumonisin $B_1$-induced hepatocyte apoptosis*

Histological observations were consistent with plasma ALT and AST findings, i.e., after treatment with fumonisin $B_1$ the WT mice had a number of TUNEL-positive cells in liver (Fig. 6.2). No apoptotic cells were seen in any of the saline-treated groups. The occurrence of TUNEL-positive cells was not observed in the TCD mice (pretreated with anti-Thy-1.2 antibody). The athymic nude mice were susceptible to hepatotoxic effects of fumonisin $B_1$ similar to WT mice, as evident by the presence of apoptotic cells after fumonisin $B_1$ treatment.

*Injection of antibody against Thy1.2 antigen depleted T cells in WT mice*

Treatment of WT mice with the monoclonal Thy-1.2 antibody resulted in reduction of peripheral leukocyte count to nearly half of the WT mice, measured 7 days after the intravenous injection of the antibody (Fig. 6.3a). Athymic nude mice also had a lower white blood count compared to WT mice. No effect of antibody injection or fumonisin $B_1$ treatment was observed on peripheral red blood cell counts in any group (data not presented). Since athymic nude mice were sensitive to fumonisin $B_1$ hepatotoxicity, we examined the presence of T cells in their livers by immunochemical staining of Thy-1.2 antigen bearing cells. Results indicated moderate staining of T cells in WT or nude mice but no staining in TCD mice, further confirming a nearly total depletion of T cells by anti-Thy-1.2 antibody, and suggesting the presence of residual Thy-1.2 antigen-bearing cells in livers of nude mice (Fig. 6.3b-d).

*Fumonisin $B_1$-induced accumulation of free sphingoid bases is not affected by T cell depletion*.

Concentrations of the free sphingoid bases, sphingosine and sphinganine, in liver with or without the fumonisin $B_1$ treatment of different groups are shown in Fig. 6.4. Fumonisin $B_1$

treatment increased the levels of free sphinganine and sphingosine to the same extent in livers of WT or TCD mice. The concentration of sphinganine in the liver of fumonisin $B_1$-treated nude mice was somewhat higher than that observed in fumonisin $B_1$-treated WT mice; however, the difference was not statistically significant (Fig. 6.4).

*T cell depletion decreased the expression of proinflammatory cytokines after fumonisin $B_1$ treatment*

It has been established that fumonisin $B_1$ causes localized activation of proinflammatory cytokines in mice liver produced by Kupffer cells, T cells, NK cells or epithelial cells. In the current study fumonisin $B_1$ induced the expression of cytokines produced by helper T (Th1) lymphocytes such as IL-2, IFN$\gamma$, lymphotoxin $\beta$ (LT-$\beta$) in both WT and nude mice (Fig. 6.5). The cytokines produced by Kupffer cells or natural killer T cells, e.g., TNF$\alpha$, IL-1$\alpha$, IL-1$\beta$, IL-6 and interleukin-1 receptor antagonist (IL-1Ra), were also increased in these groups (Figs 6.5, 6.6). Such effect of fumonisin $B_1$-induced expression of cytokines produced by both T cells and Kupffer cells was not observed in TCD mice (Fig. 6.5 and 6.6).

## DISCUSSION

Our results showed that in vivo depletion of T cells by antibodies directed against the Thy-1.2 surface antigen of T lymphocytes protected mice from fumonisin $B_1$-induced liver damage. Induction of an immune deficient state in experimental animals by anti-lymphocyte

antibodies has been intensively studied. Opitz *et al*. (1982) showed that within 8 h after administration of Thy-1.2 antibody helper function of T cells in vivo was decreased and reached to undetectable level within 24 h. No Thy-1.2 bearing cells could be detected in the peripheral lymphoid organs by fluorescein-labeled antibody 24 h later. Antibody-coated Thy 1.2 bearing cells are rapidly phagocytized by macrophages and eliminated from tissues and circulation. Despite the fact that the anti-Thy 1.2 antibody induced a transient and complete immune deficient state, the animals tolerated this treatment without any clinical signs of adverse effects.

Fumonisin $B_1$-induced hepatotoxicity was not altered in athymic nude mice. This can be explained by the observation that residual functional T cells were present in livers of nude mice and their livers showed induced expression of proinflammatory cytokines after fumonisin $B_1$ treatment. Immunochemical evaluation employing anti-Thy-1.2 antibody confirmed the presence of T cells in the liver of nude mice. Others have also demonstrated the presence of functional T lymphocytes in athymic nude mice (Ikehara *et al*. 1984; Shimamura *et al*. 1997).

Participation of T cell in liver cell destruction is a common mechanism as shown for Con A (Tiegs *et al*. 1992) and Pseudomonas exotoxin (Schumann *et al*. 1998). In these models activation of T lymphocytes followed by Kupffer cell activation and subsequent induction of cytokines are critical factors in the development of hepatotoxicity. Lymphocytes can damage hepatocytes either by releasing TNF-related apoptosis-inducing ligand (TRAIL), as well as Fas ligand or by inducing production of proinflammatory cytokines. The Fas ligand is expressed in activated T cells and induces apoptosis in Fas-bearing hepatocytes, as described for alcohol-induced hepatitis (Batey and Wang, 2002) and hepatitis caused by hepatitis B virus (Kondo *et*

*al*. 1997). Development of Con A-induced hepatitis was reduced significantly in IFNγ knockout mice, suggesting a central role for cytokines produced by activated T cells (Streetz *et al*. 2001b).

Consistent with our previous studies (Bhandari and Sharma, 2002) we observed that expression of various proinflammatory genes such as TNF , IFNγ, IL-lβ, IL-1 -, IL-6, and LT-β, were higher in fumonisin $B_1$ treated WT or nude mice compared to saline-treated animals. There is considerable evidence that cytokine signaling pathways play an important role in fumonisin $B_1$-induced hepatotoxicity in vitro and in vivo. Fumonisin $B_1$ toxicity was reduced in mice lacking either TNF receptor (TNFR) 1 or TNFR 2 (Sharma *et al.,* 2001). Jones *et al*. (2001) reported that fumonisin $B_1$ toxicity was effectively prevented by inhibition of caspases, which are downstream of TNF cellular signaling. Interaction of different types of cells in liver may amplify the cytokine production in response to fumonisin $B_1$ as reported by Bhandari *et al*. (2002) after treatment of mice with fumonisin $B_1$, and also demonstrated by interactions of macrophages and nonparenchymal epithelial cells in vitro (Sharma *et al*. 2004). The ultimate extent of liver injury in fumonisin $B_1$ hepatotoxicity may therefore involve the hepatic cytokine network rather than a single cytokine.

Several studies have demonstrated that fumonisin $B_1$ alters immune function in vivo by altering the production of the pro-inflammatory mediators. Nitric oxide (NO) production by peritoneal macrophages from mice fed with fumonisin $B_1$ and challenged with *Trypanosoma cruzi* was significantly higher than by peritoneal macrophages from control mice (Dresden *et al*. 2002). Fumonisin $B_1$ stimulated not only the basal production of NO by rat splenic macrophages but significantly potentiated the Con A, IL-2 and anti-T cell receptor induced

proliferation of lymphocytes (Dombrink-Kurtzman *et al.*, 2000). The immunoregulatory cytokines IL-2, IFNγ, and TNFα constitute the type 1 (Th1) cytokine response, whereas the cytokines IL-4, IL-5, IL-6, IL-8, IL-10, and IL-13 constitute the type 2 (Th2) cytokine response (Abbas *et al*. 1996). Taranu *et al*. (2005) have shown that fumonisin $B_1$ can alter the Th1/Th2 balance in pigs towards activation of Th1 cytokines such as IFN and reduction of Th2 cytokines such as IL-4.

At microscopic level minimal or absent inflammatory response has been reported in fumonisin $B_1$-treated mice livers; however, it is possible that cytokines released by activated T cells in the liver sinusoid may reach Kupffer cells in the periportal area and surrounding hepatocytes by diffusion through fenestrae without involving the infiltration of immune cells. Liver sinusoidal endothelial cells pose a barrier for T cells and widespread T cell infiltration into the parenchyma is only observed in acute (viral) hepatitis. Streetz *et al*. (2001) showed that in Con A induced hepatitis TNF and IFNγ production does not contribute to influx of immune activated cells into the liver. It appears that activation of helper T cells is predominantly responsible for the induction of cytokine network as depletion of Kupffer cells by gadolinium chloride did not prevent fumonisin $B_1$-induced induction of various inflammatory cytokines (He *et al.*, 2005).

The role of cytokines in toxicant-induced hepatic injury has been established (Luster *et al.*, 2001). TNFα induced apoptosis primarily depends on the recruitment of a complex of adapter proteins, including TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD) leading to the further activation of various caspases (Baud and Karin, 2001). Fas, TNF-related apoptosis-inducing ligand (TRAIL) and TNF receptors

(TNFR) can initiate cell death by two alternative pathways, one relying on caspase-8 and the other dependent on the kinase receptor interacting protein (RIP, Holler *et al*. 2000). IFNγ induced apoptosis is mediated by interferon regulatory factor-1 either directly or by inducing the transcription of IL-1β converting enzyme (Streetz *et al*. 2001).

There is increasing evidence that sphingolipids play a key role in fumonisin $B_1$ toxicity by modulating numerous intracellular pathways controlling differentiation, growth, and apoptosis (Merrill *et al*. 2001). Fumonisin $B_1$ induced hepatotoxicity in several strains of mice or with different doses of fumonisin $B_1$ has been shown to correlate well with intracellular accumulation of sphinganine and sphingosine (Tsunoda *et al*. 1998). In our study, sphinganine and sphingosine accumulated to the similar extent in control and TCD mice, which seems to indicate that, their accumulation is not related to the observed protection from fumonisin $B_1$-induced hepatotoxicity in TCD mice. It is likely that accumulation of sphingolipids is responsible for activation of T cells leading to the production of various cytokines. Since T cells are only a minor part of the cellular components of liver, their depletion may not result in any significant change in the overall liver sphingolipid concentration.

Fumonisin $B_1$ increased the accumulation of intracellular sphingosine 1-phosphate (S1P) by phosphorylation of sphingosine by sphingosine kinase (Smith and Merrill, 1995). Higher concentrations of intracellularly produced S1P increase IFNγ and IL-2 secretion in peripheral blood lymphocytes (Payne *et al*. 2004). Physiological concentrations of extracellular S1P are required for optimal activity of T cells (Graeler and Goetzl, 2002) and increase the response of T cells to chemokines by binding to the S1P receptor on T cells (Wang *et al*. 2004). Liver-associated lymphocyte population is very diverse and includes heterogeneous population of

cells such as $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells and a thymus independent natural killer (NK) cells (Doherty *et al*. 1999). Unlike Con A induced hepatotoxicity, fumonisin $B_1$-induced hepatotoxicity does not depend on NK cells as deletion of NK cells by induced mutation did not influence the fumonisin $B_1$-induced hepatotoxicity (Unpublished data).

Results obtained in this study indicate that T cell activation followed by production of proinflammatory cytokines is an important mechanism for fumonisin $B_1$-induced hepatotoxicity in mice.

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**Table 6.1.** Changes in body and organ weights after fumonisin $B_1$ ($FB_1$) treatment in mice

| Treatment | Body weights | | Relative Liver weight (% of body weight) | Relative kidney weight (% of body weight) | Relative spleen weight (% of body weight) |
|---|---|---|---|---|---|
| | Before treatment | After treatment | | | |
| WT, saline-treated | 31.2±0.9 | 32.9±0.9 | 4.31±0.25 | 1.52±0.05 | 0.29±0.01 |
| WT, $FB_1$-treated | 28.6±1.5 | 28.4±1.7* | 4.66±0.21 | 1.33±0.05* | 0.30±0.02 |
| TCD, saline-treated | 28.7±0.7 | 30.3±0.8 | 4.46±0.13 | 1.56±0.06 | 0.26±0.02 |
| TCD, $FB_1$-treated | 30.1±1.5 | 31.2±1.6 | 4.62±0.16 | 1.45±0.08 | 0.27±0.02 |
| Nude, saline-treated | 25.0±1.0 | 26.6±0.7 | 4.73±0.12 | 1.46±0.06 | 0.35±0.01 |
| Nude, $FB_1$-treated | 25.7±0.7 | 27.0±0.9 | 5.53±0.21* | 1.35±0.04 | 0.31±0.02 |

*Significant difference at $p < 0.05$, compared to the respective control (saline treatment).

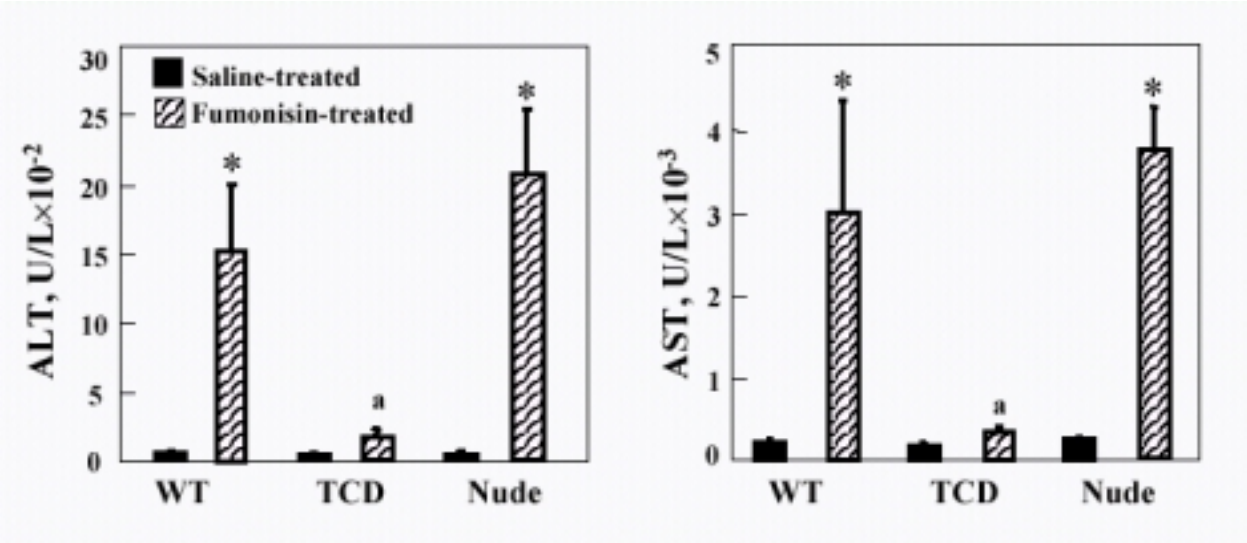


**Fig. 6.1.** Activities of circulating liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma of WT, TCD and nude mice after treatment with fumonisin $B_1$. Results are expressed as mean ± standard error, (n = 5). Asterisks on bars indicate a significant difference from the corresponding saline treated group at $p < 0.05$. The letter "a" on bar indicates significant difference ($p < 0.05$) between the fumonisin $B_1$-treated WT and TCD groups.

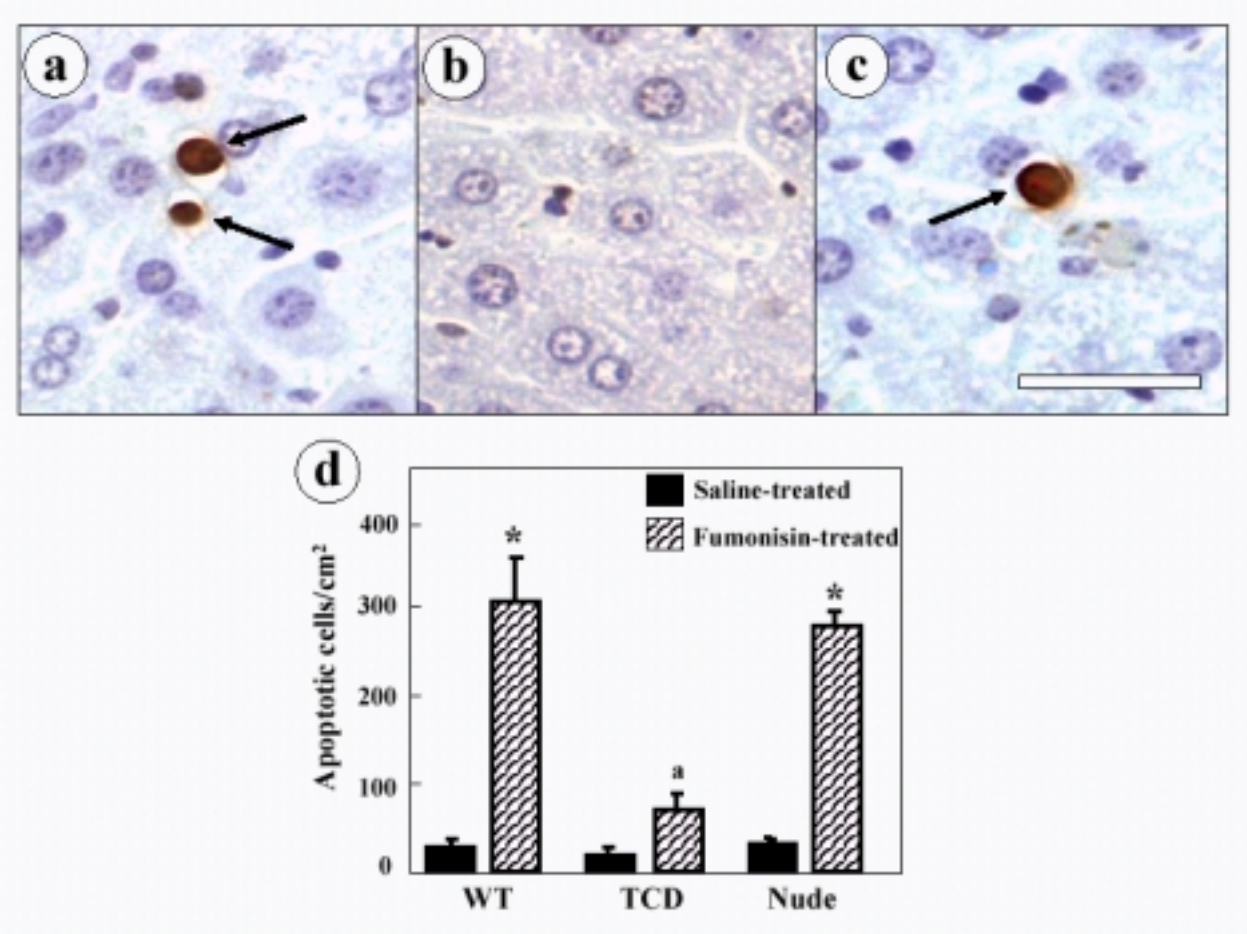


**Fig. 6.2.** Enumeration of apoptotic cells in liver by TUNEL assay. (a) Liver from fumonisin $B_1$-WT mouse. (b) Liver from a fumonisin $B_1$-treated TCD mouse. (c) Liver from fumonisin $B_1$-treated nude mouse. Bar in the lower right in "c" equals 10 μm. Arrows indicate TUNEL positive nuclei in apoptotic cells. (d) The number of TUNEL-positive cells in tissue sections (mean ± standard error, $n = 5$). The asterisk (*) indicates a significant difference from the corresponding saline-treated group ($p < 0.05$). The letter "a" on the bar indicates a significant difference ($p < 0.05$) between the fumonisin $B_1$-treated WT and TCD groups.

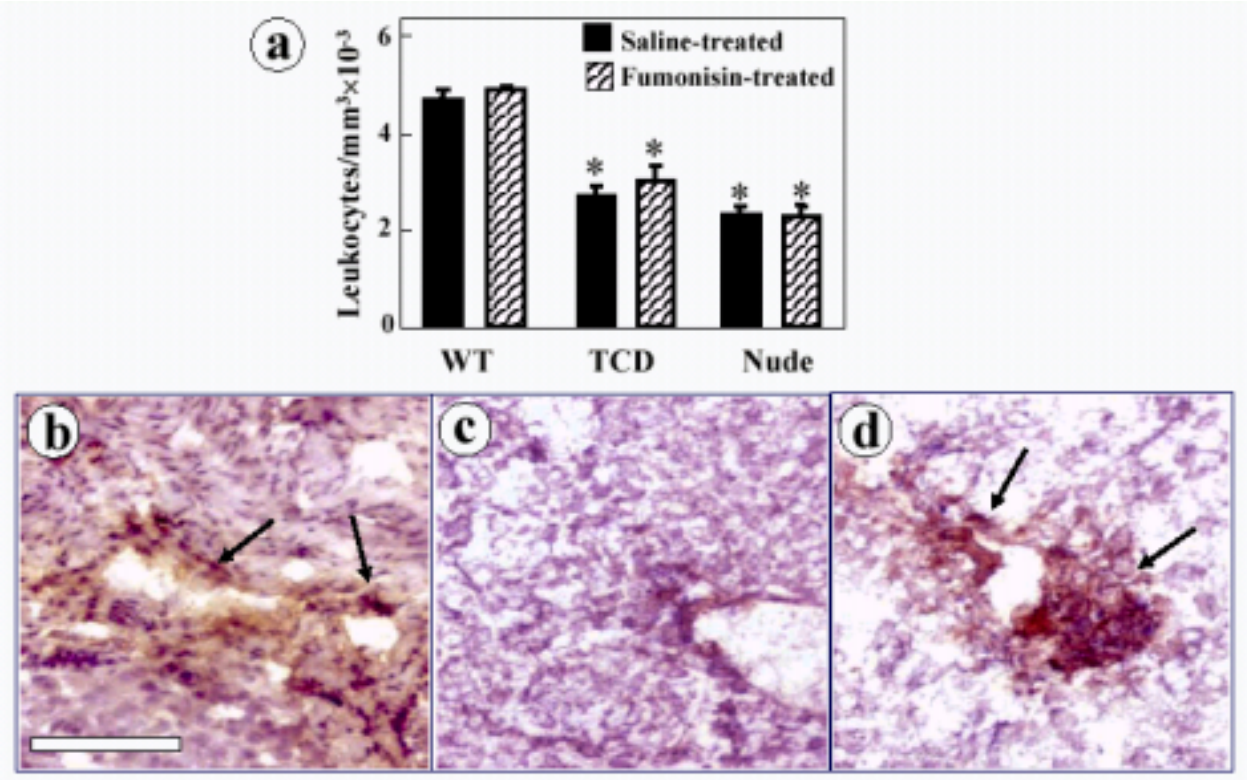


**Fig. 6.3.** (a) Peripheral white blood cell count in different groups of mice after 5 days of saline or fumonisin $B_1$ treatment; mean ± standard error (n = 3). The * indicates a significant difference from the similarly treated WT group ($p < 0.05$). (b) Immunohistochemical staining of frozen mouse liver section with anti Thy 1.2 antibody showing presence of Thy 1.2 antigen bearing T cells in the periportal regions of liver in WT. (c) No stained areas were observed in TCD mice. (d) Occurrence of T cells in nude mice (d). The bar in the lower right in "b" represents 25 μm.

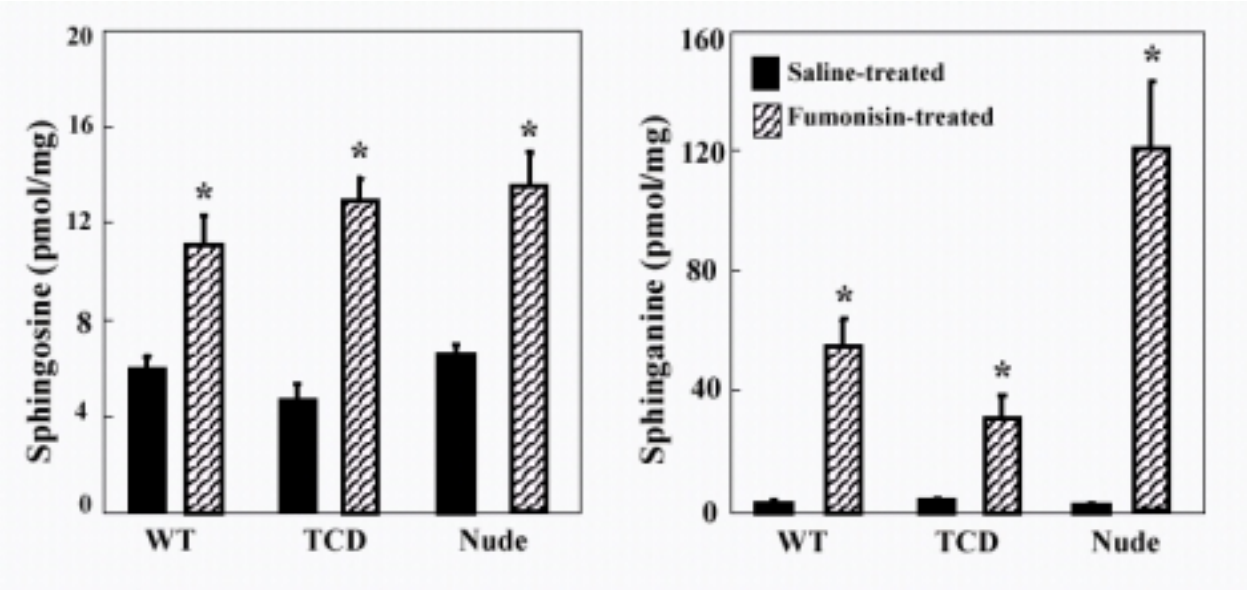


**Fig. 6.4.** Free sphingosine and sphinganine levels in the liver of saline-treated or fumonisin $B_1$-treated mice. Results are expressed as mean ± standard error, (n = 5). Asterisk on bars indicates a significant difference from the corresponding saline-treated group ($p < 0.05$).

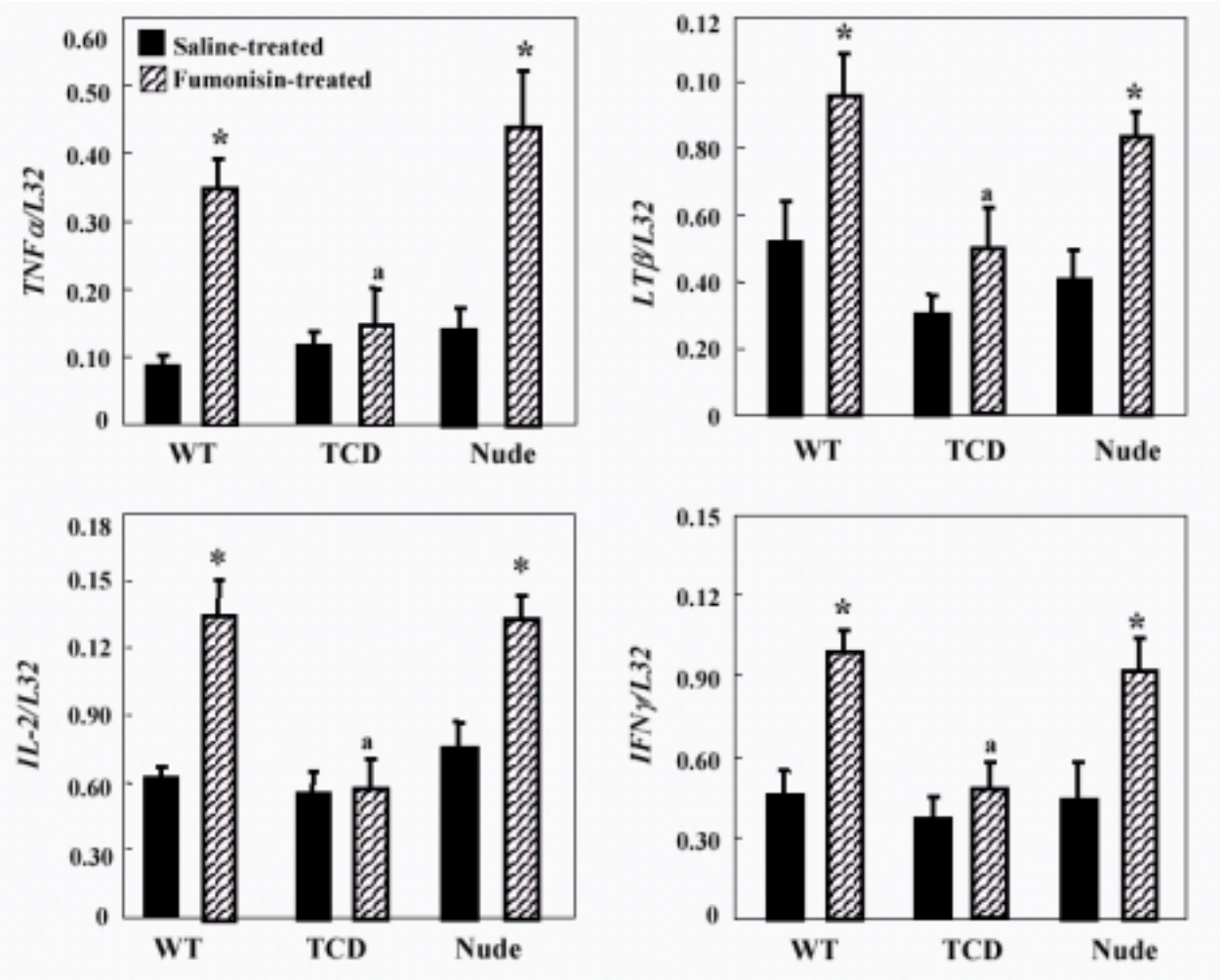


**Fig. 6.5.** Fumonisin $B_1$ induced alteration in gene expression of selected inflammatory cytokines. The relative mRNA expression was normalized against *L-32*, a house-keeping gene. Mean ± standard error, (n = 5). Asterisks on bars indicate a significant difference from the saline treated group of same strain $p < 0.05$. Letter "a" on bars indicates a significant decrease from the similarly treated WT ($p < 0.05$).

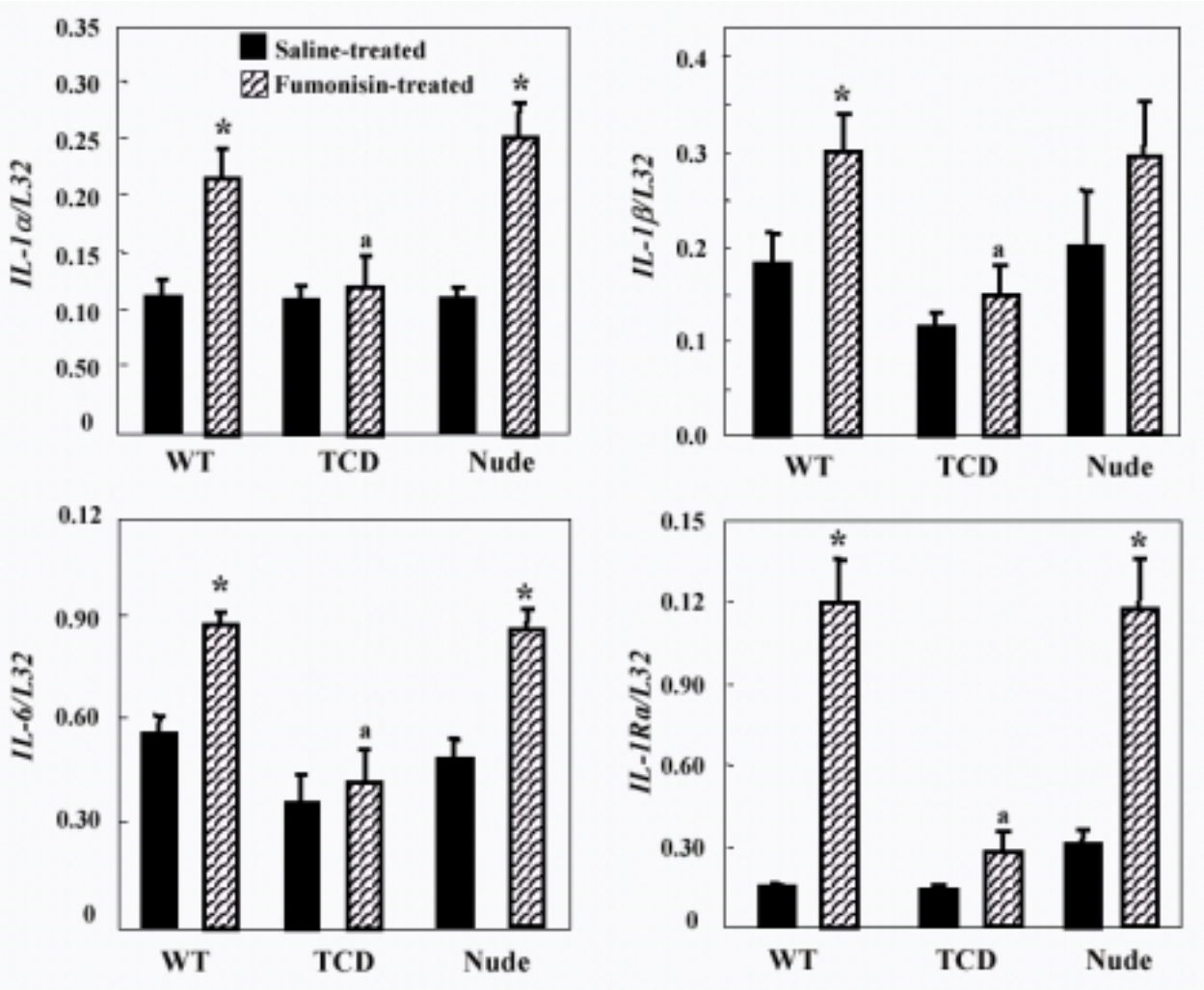


**Fig. 6.6.** Fumonisin $B_1$ induced alteration in gene expression of selected inflammatory cytokines. The relative mRNA expression was normalized against *L32*, a house-keeping gene. Mean ± standard error, (n = 5). Asterisks on bar indicate a significant difference from the saline treated group of same strain ($p < 0.05$). Letter "a" on bars indicates a significant decrease from the similarly treated WT ($p < 0.05$).

# CHAPTER 7

# SUMMARY AND CONCLUSIONS

Fumonisin $B_1$ ($FB_1$), produced by *Fusarium verticillioides*, is a common mycotoxin found on corn and corn-based foods. It produces species, organ and gender-specific toxicity, including equine leukoencephalomalacia, porcine pulmonary edema renal damage in some animals and hepatic damage in all and is hepato and nephrocarcinogenic in rodents. Fumonisin $B_1$ perturbs sphingolipid metabolism by inhibiting ceramide synthase. Physiological consequences possible after ceramide synthase inhibition include apoptosis caused by accumulation of free sphingoid bases (sphinganine and sphingosine), increased proliferation caused by increased sphingosine-1-phosphate, or/and decreased ceramide and altered lipid raft function due to disruption of complex sphingolipids synthesis/transport. Fumonisin $B_1$ induces expression of tumor necrosis factor (TNF)$\alpha$ and other cytokines *in vivo* and *in vitro*. Fumonisin $B_1$ induces apoptosis in some cells whereas other cells are resistant to its apoptotic effects. Various sphingolipids and cytokines are important cell signaling factors, which may be important in rendering some cells resistant to fumonisin $B_1$ induced apoptosis.

The objectives of the present study were to (1) evaluate the role of sphingosine kinase, the enzyme that converts sphingoid bases to their respective phosphates, in rendering some cells resistant to fumonisin $B_1$-induced apoptosis, (2) Investigate the effect of interaction of macrophages (J774A.1) and nonparanchymatous liver epithelial cells (NMuLi) in a co-culture on fumonisin $B_1$-induced apoptosis, (3) evaluate the role of TNF$\alpha$ and mitogen activated protein kinases in fumonisin $B_1$-induced apoptosis in murine primary hepatocytes, (4) examine the fumonisin $B_1$-induced hepatotoxicity in mice depleted of T cells.

In the first study, we demonstrated using specific inhibitor of sphingosine kinase, the enzyme that converts sphingoid bases to their respective phosphates that HEK-293 cells were resistant to apoptotic effects of fumonisin $B_1$ due to rapid conversion of accumulate sphinganine or sphingosine to their respective phosphates by sphingosine kinase. Higher activity of sphingosine kinase enhances cell survival by forming sphingosine-1-phosphate, which has anti-apoptotic and pro survival properties. Activity of sphingosine kinase enzyme may have substantial bearing on different responses of fumonisin $B_1$ in other cell types also. However, our findings are applicable to only HEK-293 cells and further investigations are required to determine whether or not this is a general mechanism for resistance to fumonisin $B_1$ in other cells *in vitro* and tissues *in vivo*.

The second study evaluated the effect of fumonisin $B_1$ on co-cultures of macrophages (J774A.1) and nonparanchymatous liver epithelial cells (NPECs). Co-cultures were more responsive to fumonisin $B_1$ induced cytotoxicity compared to individual cultures and interaction of the two cell types resulted in increased expression of proinflamatory cytokines in the co-cultures. Our results indicated that macrophage and NPECs interact in co-culture in response to fumonisin $B_1$ and potentiate expression of various cytokines, which may have implications in making hepatocytes more responsive to fumonisin $B_1$-induced cytotoxicity.

In the third study, we investigated the role of TNFα and downstream signaling events such as mitogen activated protein kinases (MAPKs) in fumonisin $B_1$ induced apoptosis in murine hepatocytes. Fumonisin $B_1$ induced apoptosis in hepatocytes by involving JNK activation, which could be prevented to some extent by JNK inhibitor and anti-TNFα. TNFα production is upstream of JNK signaling as JNK activation was inhibited by anti-TNFα,

whereas inhibition of JNK had no effect on TNFα expression. This study provides evidence for tumor necrosis factor α-mediated activation of c-Jun NH2-terminal kinase as a mechanism for fumonisin $B_1$-induced apoptosis in murine primary hepatocytes.

The final study determined the role of T cells in fumonisin $B_1$-induced apoptosis in mice liver. Athymic nude mice are deficient in T cells; therefore, we employed nude mice and their immunocompetent wild-type counterparts in this study. Depletion of T cells in wild-type mice was accomplished by injecting monoclonal antibodies directed against Thy-1.2 surface antigens; which is an established method to investigate the T cell functions in mice. Fumonisin $B_1$-induced elevation in activities of circulating liver enzymes were not different between WT and nude mice, while depletion of T cells nearly abolished fumonisin $B_1$-mediated liver toxicity, indicated both by concentrations of circulating liver enzymes and enumeration of apoptotic hepatocytes. Expression of fumonisin $B_1$-induced tumor necrosis factor α and other proinflammatory cytokines was observed in nude and wild type mice but not in T cell-depleted mice. Results obtained in this study indicated that T cell activation followed by production of proinflammatory cytokines is an important mechanism for fumonisin $B_1$-induced hepatotoxicity in mice.

All together, the data presented in this dissertation indicate that sphingolipid metabolism and lack of interaction of cytokines may be responsible for observed resistance to apoptotic effects of fumonisin $B_1$ in various cultured cells or *in vivo*.