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Review

FUMONISIN B_1: A NEUROTOXIC MYCOTOXIN*

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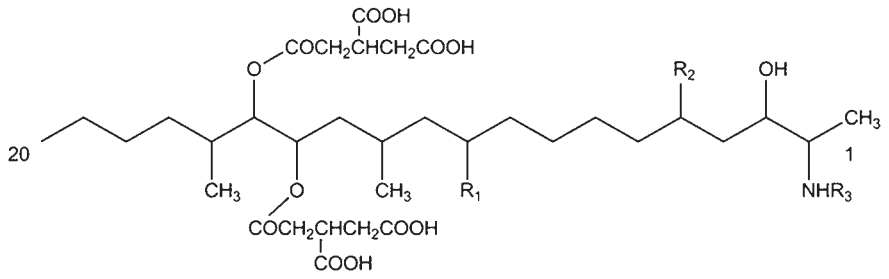
Fumonisin B_1 (FB_1) is a mycotoxin produced by *Fusarium* spp. moulds that contaminate crop, predominantly maize, all around the world. More than 15 types of fumonisins have been indentified so far, but FB_1 is the most abundant and toxicologically the most significant one. FB_1 has a wide range of toxic effects, depending on animal species. In horses FB_1 causes equine leukoencephalomalacia (ELEM), in pigs pulmonary oedema and in experimental rodents nephrotoxicity and hepatotoxicity. In humans exposure to FB_1 is linked with higher incidence of primary liver cancer and oesophageal cancer, which are frequent in certain regions of the world (such as Transkei region in South Africa) where maize is staple food. The occurrence of neural tube defect in children in some countries of Central America (such as Mexico and Honduras) is connected with the consumption of FB_1-contaminated maize-based food. However, possible involvement of FB_1 in the development of human diseases is not clear. Nevertheless, the International Agency for Research on Cancer (IARC) has classified FB_1 as a possible carcinogen to humans (group 2B).
FB_1 is a causative agent of ELEM, a brain disorder in equines, indicating that brain is a target organ of FB_1 toxicity. Several studies on experimental animals or on cell cultures of neural origin have established that FB_1 has a neurodegenerative potential, although the mechanism of its neurotoxicity is still vague. The aim of this article is to give an overview of available literature on FB_1 neurotoxicity and involved mechanisms, and to offer a new perspective for future studies.

KEY WORDS: *equine leukoencephalomalacia, neural tube defect, neurotransmitters, sphingolipids, sphingoid bases, oxidative stress, mitochondria*

Fumonisins are a group of mycotoxins produced by *Fusarium* spp. moulds, mainly by *Fusarium verticilloides* (formerly *F. moniliforme*) mould (1, 2). Fumonisins were first isolated and chemically characterised by South African scientists in 1988 (3). More than 15 types of fumonisins have been identified so far (Figure 1). The most abundant and the most toxic fumonisin is fumonisin B_1 (FB_1) (1). As contaminants, fumonisins are found throughout the world in various crops, but predominately in maize (reviewed by Sorriano and Dragacci, ref. 4).

Consumption of FB_1-contaminated feed causes pulmonary oedema in pigs and equine leukoencephalomalacia (ELEM) in horses (1). To experimental animals (rats and mice) FB_1 is nephrotoxic and hepatotoxic. Chronic dietary exposure of rats and mice of both sexes to FB_1 resulted in significant increases in kidney tumours in male BD IX rats and liver tumours in male F344 rats and female $B6C3F_1$ mice, which suggests that FB_1 toxicity is sex-, strain-, and species-dependent (5,6). As for FB_1 pharmacokinetics, it is poorly absorbed from the gastrointestinal tract, rapidly eliminated from plasma, and not extensively accumulated in tissue, although low amounts are found in the liver and kidney (for

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	FA_1	FA_2	FB_1	FB_2	FB_3	FB_4	FC_1
R_1	OH	H	OH	H	OH	H	OH
R_2	OH	OH	OH	OH	H	H	OH
R_3	CH_2CO	CH_2CO	H	H	H	H	H

Figure 1 *Structural formula of several fumonisins depending on substituent (R_1, R_2 and R_3): fumonisin A_1 (FA_1), fumonisin A_2 (FA_2), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), fumonisin B_4 (FB_4), and fumonisin C_1 (FC_1).*

review see ref. 2). The effect of FB_1 on human health is uncertain. There is a record of only one outbreak of foodborne disease connected with exposure to FB_1 (7). In 1995, due to unseasonal rains in the Deccan plateau in India, consumption of damaged maize and sorghum led to an outbreak characterised by abdominal pain, borborygmi, and diarrhoea. A rapid epidemiological survey of food samples collected from the affected households revealed higher levels of *Fusarium* spp. and FB_1 compared to food samples collected from unaffected households. Additionally, diarrhoea was reproduced in one-day-old cockerels fed with contaminated grains from the affected households. In certain regions of the world such as the Transkei region in South Africa and Linxian region in China, where maize is staple food, higher incidence of oesophageal cancer and primary liver cancer were connected with dietary exposure to FB_1 (1). However, epidemiological data are still lacking and a direct causal role for this toxin in the aetiology of such tumours has not been established (1, 8). FB_1 has also been implicated in the aetiology of neural tube defect (NTD) in parts of Texas, Mexico, Guatemala, and South Africa. Studies on mouse embryos in culture and on pregnant mice have provided evidence that FB_1 has the potential to produce embryotoxicity and NTD (9, 10). In a case-control study conducted on Mexican American women in Texas, maternal exposure to FB_1 (assessed as level of FB_1 in tortillas consumed by mothers and level of biomarker of FB_1 exposure in maternal serum) was associated with increased incidence of NTD in children (11). This established an association between exposure to FB_1-contaminated food and NTD, but further investigations are required to establish the connection between FB_1 and NTD (12, 13). On the basis of the available toxicological evidence, International Agency for Research on Cancer (IARC) has classified FB_1 in group 2B as a possible carcinogen to humans (14).

Although FB_1 has a wide range of biological effects, brain is undoubtedly a target organ of FB_1 toxicity. For the last three decades, extensive studies on FB_1 toxicity have been conducted, but only a few have looked into its neurodegenerative potential. Moreover, the underlying mechanism of FB_1 toxicity as well as neurotoxicity is still not known. Therefore, the aim of this article is to give an overview of available literature on the neurotoxic potential and studied mechanisms of FB_1 neurotoxicity, and offer a new perspective for future studies.

NEUROTOXIC POTENTIAL OF FB_1

ELEM is a neurotoxic syndrome with high mortality in equines, associated with FB_1-contaminated feed (15, 16, for recent review see ref 2). The disease is characterised by a sudden development of nervous signs such as aimless circling, head pressing, paresis, ataxia, blindness, and depression, and concurrently by changes in the liver (15). The primary pathological feature of ELEM is focal necrosis of cerebral white

matter, and histopathological findings show rarefied white matter with pyknotic nuclei and eosinophilic cytoplasm (15). FB_1 was confirmed as causative agent of ELEM experimentally on horses, ponies, and donkeys fed with naturally fumonisin-contaminated feed or administered pure FB_1 (17-20). When FB_1 was injected to a horse (*i.v.*, 0.125 mg kg^{-1} b.w. a day for 7 days, totalling 276 mg of FB_1) lesions characteristic of ELEM were found in the brain (18). The same was observed in a study in which two horses received pure FB_1 orally (1.25 mg kg^{-1} to 4 mg kg^{-1} b.w. for 21 days, and 1 mg kg^{-1} to 4 mg kg^{-1} b.w. for 20 days) (19). Ross et al. (16), determined the level of FB_1 in 45 feed samples collected from different parts of the USA where an outbreak of ELEM was recorded. Most samples associated with ELEM had FB_1 level above 10 ppm, while none of the control samples contained FB_1 above 8 ppm. This points to the concentration of 10 mg kg^{-1} of FB_1 as the threshold that can induce ELEM (16). As the level of FB_1 contamination required to induce ELEM is low, it is not surprising that this equine neurotoxic disease has been reported worldwide (2). However, as only several horses on the same farm are usually affected by ELEM, this suggests that in addition to the level of dietary contamination, other factors are involved in its development such as length of exposure, individual susceptibility, and previous exposure to FB_1 (2).

Experiments on animals

Only a few animal studies have reported FB_1 effects on the central nervous system (CNS), mainly because there are no suitable laboratory animal models to study ELEM (21). Rabbits gavaged with purified FB_1 (1.75 mg kg^{-1} b.w. for 9 and 13 days) showed CNS changes similar to ELEM, such as focal small haemorrhages in cerebral white matter (22). A hyperaemia was observed in the brain of adult female Fischer rats, but these animals were fed with FB_1 (1 mg kg^{-1} diet, for 17 days) together with *Ustilago maydis,* a facultative biotrophic basidiomycete, another well-known contaminant of maize (23). In a study on developing rats, multiple subcutaneous dosing with FB_1 (0.4 mg kg^{-1} or 0.8 mg kg^{-1} b.w. a day, from postnatal day 3 to 12) significantly reduced body and brain weights and caused hypomyelination (24). The same group of authors demonstrated that FB_1 crosses the blood-brain barrier in developing rats after a single subcutaneous FB_1 dose (8 mg kg^{-1} b.w.) (25). However, the level of FB_1 in the brain was low, and at low-dose treatment (0.8 mg kg^{-1} b.w.) no FB_1 was found in the brain. Authors suggested that this was probably due to low lipid solubility and a large molecular weight of FB_1. In a study of FB_1 toxicity in fish brain (26), one-year-old carps (*Cyprinus carpio* L.) were receiving FB_1 in feed (100 mg kg^{-1} diet or 10 mg kg^{-1} diet) for 42 days. Both FB_1-exposed groups showed slower body-weight gain and histopathological changes such as brain oedema, increase in the number of degenerative cells, and accumulation of inflammatory cells. FB_1 was again found to cross the blood-brain barrier in young carp, confirming the findings of Kwon et al. (25).

Experiments on cell cultures

Several *in vitro* studies on cell cultures of CNS origin demonstrated that FB_1 caused changes such as disruption of axonal outgrow and myelination, and these changes were observed before FB_1 induced cell death. However, it is not clear which cell types in the brain tissue are more sensitive to FB_1 and are the likely target for the initial degenerative effect of FB_1.

In a study of Harel and Futerman (27), although FB_1 treatment (10 μmol L^{-1}, 24 h) of primary rat hippocampal neurons inhibited axonal growth, FB_1 was not toxic to hippocampal neurons, since the number of dead cells was not altered even after treatment with 40 μmol L^{-1} of FB_1 for 48 h.

A study on brain cell cultures (28) aggregated from foetal rat telencephalon exposed to FB_1 (3 μmol L^{-1} to 40 μmol L^{-1}) for 10 days showed that FB_1 selectively affected glial cells. It disturbed myelin formation and deposition and delayed oligodendrocyte maturation, but was not generally cytotoxic. This absence of FB_1 cytotoxicity to glial cells, assessed as DNA or protein content, was also observed in a study on primary cultures of rat cerebrum containing astrocytes and oligodendrocytes treated with FB_1 (0.5 μmol L^{-1} to 75 μmol L^{-1}) for 5 or 10 days (29). Even the highest FB_1 concentration (75 μmol L^{-1}) and continuous exposure of glial cultures for 5 days did not result in any FB_1-associated changes in protein or DNA level. That FB_1 is not cytotoxic to cells of glial origin was confirmed by a study of Galvano et al. (30), where no change in cell viability was observed in rat primary astrocytes after FB_1 treatment (50 μmol L^{-1}, up to 6 days).

Nevertheless, FB_1 seems able to induce cell death in the cells of glial origin. In rat C6 glioblastoma cells treated with FB_1 (3 μmol L^{-1} to 54 μmol L^{-1}) for 24 h, 9 μmol L^{-1} of FB_1 decreased viability to about 65 % (31). Dose-dependence was not observed and viability

was never lower than 50 %, although higher concentrations of FB_1 (30 μmol L^{-1} to 50 μmol L^{-1}) were used. The same group of authors observed a similar effect of FB_1 on cell viability in the same cell line (C6 glioblastoma cells) (32). In another study (33) on human U-118MG glioblastoma cells, FB_1 treatment (0.1 μmol L^{-1} to 100 μmol L^{-1}, 12 h to 144 h) decreased cell viability to about 73 % but only at the concentration of 100 μmol L^{-1} and after 72 h and 144 h of incubation. Earlier time points or lower concentrations of FB_1 did not induce cell death.

Stockmann-Juvala et al. (34) showed that 48-hour exposure to 100 μmol L^{-1} of FB_1 decreased cell viability in human SH-SY5Y neuroblastoma and mouse GT1-7 hypothalamic cells to about 80 %, and viability decreased further to about 70 % with longer exposure time (72 h and 144 h). However, contrary to Mobio et al. (31), even at the concentration of 100 μmol L^{-1} and exposure time of 144 h, FB_1 did not induce cell death in C6 glioblastoma cells. In addition, Stockmann-Juvala et al. suggest that cells of neural origin may be more sensitive to FB_1 than glial cells. However, Osuchowski and Sharma (21) came up with different findings on two cell types of neural origin - murine N2A neuroblastoma and mouse primary cortical neurons - and on two cell lines of glial origin - murine BV-2 microglia and mouse primary astrocytes. FB_1 (up to 50 μmol L^{-1} for 4 days or 8 days) induced cell death in the cells of glial origin, but not in the cells of neuronal origin. Cytotoxicity was observed in murine BV-2 microglia cells as soon as after four days of treatment with 20 μmol L^{-1} FB_1, while in primary astrocytes, its cytotoxicity was observed only after eight days of treatment with the highest concentration (50 μmol L^{-1}). The authors concluded that the glial cells and not the neuronal ones may be the primary targets of FB_1 neurotoxicity. In our recent study on primary rat astrocytes and human SH-SY5Y neuroblastoma cells (35), we confirmed low FB_1 cytotoxicity to cells of neural origin *in vitro*; twenty-four hour treatment with up to the concentration of 200 μmol L^{-1} did not affect cell viability.

All these findings on FB_1 effects on cell viability indicate that FB_1 has low cytotoxicity and that its toxicity is greatly determined by a combination of length of exposure, concentration, cell type/brain region, and species (mouse or rat). For example, FB_1 cytotoxicity was observed in human SH-SY5Y neuroblastoma cells after treatment with 100 μmol L^{-1} for 48 h (34), but not after treatment with 200 μmol L^{-1} for 24 h (35). In mouse primary astrocytes cell death was observed after incubation with 50 μmol L^{-1} for 8 days, but in that study FB_1-treatment began on day 3 *in vitro* (21). On the another hand, incubation with 50 μmol L^{-1} for 6 days resulted in no change in the viability of rat primary astrocytes, but treatment in that study started after two weeks *in vitro*, when astrocytes were well mature and differentiated (30). Therefore, it is hard to compare *in vitro* results of FB_1 cytotoxicity. Using several parameters, Stockmann-Juvala et al. (36) compared sensitivity of neural cells to FB_1 and determined the following order (high to low): human U-118MG glioblastoma cells > mouse GT1-7 hypothalamic cells > rat C6 glioblastoma cells > human SH-SY5Y neuroblastoma cells. Just by comparing human SH-SY5Y neuroblastoma cells and human U-118MG glioblastoma cells the authors concluded that glial cells were more sensitive to FB_1 than neuronal cells.

FB_1 EFFECT ON NEUROTRANSMITTERS

Changes in neurotransmitters and their metabolites can be used as an index of toxic effects of chemicals in the CNS (37, 38). Several studies have looked into the effects of FB_1 on brain neurochemistry and imbalance in neurotransmitters and their metabolism (39-41). Porter et al. (39) studied the levels of several neurotransmitters and their metabolites in whole brain of rats fed *F. verticilloides*-contaminated corn (ELEM-associated) and rodent chow supplemented with *F. verticilloides* and established an increase in 5-hydroxyindoleacetic acid (5-HIAA), a major metabolite of serotonin, and subsequently an increase in 5-HIAA to serotonin ratio. As the level of serotonin, a 5-HIAA precursor, did not change, the authors concluded that fumonisins may have affected either serotonin metabolism or 5-HIAA elimination from rat brain. However, another study by the same authors in which rats were fed with purified FB_1 (15, 50 and 150) mg kg^{-1} in feed for 4 weeks, showed no change in brain serotonin metabolism, but a decrease in norepinephrine to dopamine ratio. This suggests that FB_1 disturbs brain norepinephrine and/or dopamine homeostasis (40). Since the rats in the first study received *F. verticilloides*-contaminated diet and in the second study purified FB_1, the authors contributed the discrepancy to the effects of other mycotoxins produced by *Fusarium* spp. Imbalance in neurotransmitter metabolism was confirmed by Tsunoda et al. (41) in a study on mice treated with

purified FB_1. Subcutaneous FB_1 treatment (6.75 mg kg^{-1} b.w.) in the cervical region for 5 days significantly increased homovanillic acid, a dopamine metabolite, in most brain regions, as well as neurotransmitter metabolite to neurotransmitter ratio (such as homovanillic acid to dopamine and 5-HIAA to serotonin). Tsunoda et al. explain this accumulation of neurotransmitter metabolites after FB_1 treatment with increased neuronal activity or changes in their efflux from cells due to disrupted membrane transport.

Recently, Gbore (42) studied the catalytic activity of acetylcholinesterase (AChE) in several brain regions of weanling pigs fed with FB_1 (0.2 mg kg^{-1} to 15.0 mg kg^{-1} diet) for 6 months. AChE activities in the pons, amygdala, hypothalamus, and medulla oblongata declined significantly with increase in dietary FB_1 concentrations, indicating that FB_1 has a potential to change brain neurochemistry in growing pigs. In an earlier study, Pepeljnjak et al. (23) found no significant changes in AChE activity in the brain of adult female Fischer rats fed with FB_1 (1 mg kg^{-1} diet) and *Ustilago maydis* contaminated diet for 17 days, even though the catalytic activity was lower than in control animals. This result should be taken with reserve, since animals were not receiving FB_1 alone.

Although the findings on neurotransmitters are not consistent, FB_1 was able to disturb brain neurochemistry in rats, mice, and pigs, indicating that further studies are needed. All the more so, as Banczerowski-Pelyhe et al. (43) have shown that FB_1 modifies signal transmission in the brain. Electrophysiological recordings *in vivo* and in cortex slices *in vitro* showed changes in excitability in the neocortex of rats fed with FB_1 (calculated to be 30 mg kg^{-1} b.w.) for 5 days and their greater susceptibility to seizure. The authors concluded that this type of neuronal stress may stem from altered membrane processes and neurotransmitter level imbalance that could even be related to systemic toxic effects of FB_1.

MECHANISM OF FB_1 NEUROTOXICITY

Impairment of sphingolipid metabolism

The chemical structure of FB_1 is similar to sphingosine and sphinganine, the backbones of sphingolipids (SLs), a class of complex lipids (Figure 2). As a result of this similarity, FB_1 competitively inhibits a key enzyme in SL metabolism ceramide synthase [sphingosine (sphinganine) *N*-acetyltransferase] (44, 45). Inhibition of ceramide synthase results in a decrease of *de novo* biosynthesis of ceramide and complex SLs and in blocking the reuse of sphingosine liberated during SL turnover (Figure 3). The blocking of these pathways by FB_1

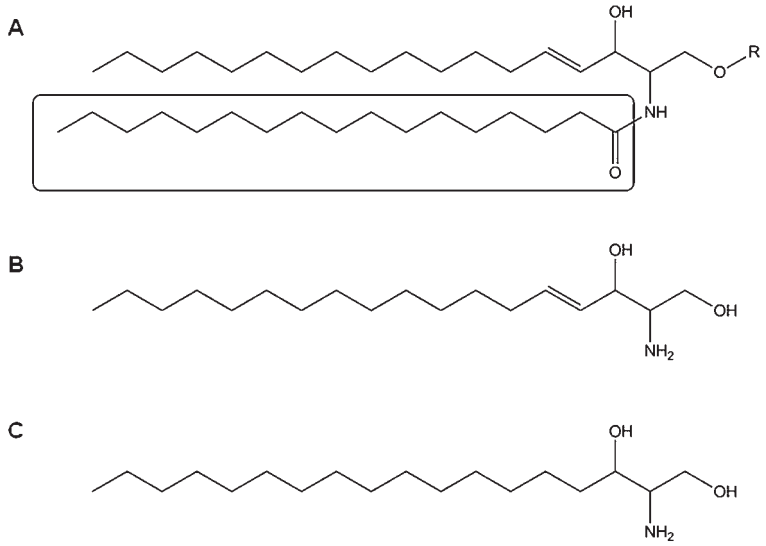


Figure 2 *Structural formula of sphingolipids and sphingoid bases. A) General formula - sphingoid base sphingosine is the backbone of sphingolipids to which fatty acids tail (framed) is linked. Specific sphingolipid molecule depends on the substituent (R) linked to sphingosine [–such as hydrogen (ceramide), phosphocholine (sphingomyelin), and sugar (glycosphingolipid)]. B) structural formula of sphingosine and C) of sphinganine.*

leads to an increase in free sphinganine and to a lesser extent in sphingosine, and consequently to increase in sphingoid base 1-phosphate metabolites, while ceramide and complex SL levels drop (2, 45). Increased levels of sphinganine in blood, plasma, and tissues and the accompanying increase in sphinganine to sphingosine ratio can be used as biomarkers of exposure to FB_1, but only in experimental conditions (2, 46). The use of sphinganine to sphingosine ratio as a biomarker of exposure in epidemiological studies is not reliable (47), as the human population is exposed to much lower levels of FB_1 than experimental animals and since changes in sphingoid base concentrations are reversible (2, 47).

Animal studies and studies of neuronal and glial cells have confirmed that FB_1 treatment alters SL metabolism in the CNS. In developing rats treated with multiple subcutaneous doses of FB_1 (0.4 mg kg^{-1} or 0.8 mg kg^{-1} b.w. per day) from postnatal day 3 to 12, brain sphinganine and sphinganine to sphingosine ratio significantly increased with the highest dose (24). In another study (25), the same group of authors showed that brain SL metabolism disturbed by a single subcutaneous dose of FB_1 (8 mg kg^{-1} b.w.) in developing rats was a consequence of direct action of FB_1 on the brain, rather than of transport of sphingoid bases from blood. In cultured hippocampal neurons, treatment with FB_1 (up to 10 μmol L^{-1} for 24 h) inhibited ceramide synthase in a dose-dependent manner (>90 % inhibition at 2 μmol L^{-1}) and decreased the level of complex SLs (gangliosides) (at 10 μmol L^{-1}) (27). In hippocampal neurons treated with 10 μmol L^{-1} of FB_1 for 66 h, Schwartz et al. (48) observed a decrease in complex SLs ganglioside, sphingomyelin, and glucosylceramide. In primary glial cultures of rat cerebrum, FB_1 treatment (in the range of 0.5 μmol L^{-1} to 75 μmol L^{-1}) increased sphinganine level and sphinganine to sphingosine ratio after 5 days of treatment and decreased sphingosine level after 10 days (29). In a study by Osuchowski and Sharma (21), FB_1 (50 μmol L^{-1}) increased sphinganine and decreased sphingosine level in murine BV-2 microglial and murine N2A neuroblastoma after 4 days of treatment, and in primary astrocytes and primary cortical neurons after 8 days.

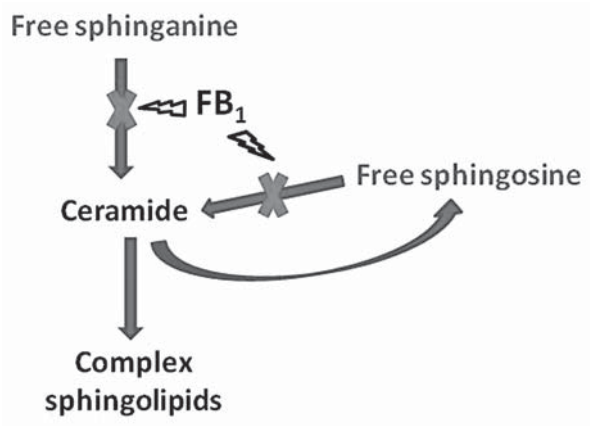


Figure 3 *Simplified diagram of sphingolipid metabolism; de novo synthesis of ceramide and complex sphingolipids, and sphingolipid turnover (adapted from ref. 2). X presents inhibition of ceramide synthase by FB_1. The consequences are lower de novo biosynthesis of ceramide and complex sphingolipids, accumulation of sphinganine and to a lesser extent of sphingosine (due to blocked reutilisation of sphingosine released during complex sphingolipid turnover), and increased level of the sphingoid base 1-phosphate metabolites.*

Disturbed SL metabolism is an immediate event after FB_1 treatment that appears before any toxic effects of FB_1 (44, 45). This suggests that disturbed SL metabolism is behind FB_1 toxicity (21, 29, 49). However, the mechanism by which changes in SL biosynthesis induce cell death is not clear. Since SLs are major structural components of cell membranes, one of the certain consequences of disturbed SL metabolism is a change in cellular membrane function. Several studies on FB_1-treated cells of CNS origin attributed the disruption of neuronal growth, hypomyelination, and folic acid transport to altered SL metabolism (24, 27, 50).

In a study by Harel and Futerman (27), treatment of cultured rat hippocampal neurons with FB_1 (10 μmol L^{-1} for 24 h) reduced the synthesis and level of complex SL ganglioside and inhibited axonal outgrowth. Incubation of cells with the short-acyl chain analogue of ceramide (C6-NBD-Cer) (5 μmol L^{-1}) together with FB_1 (10 μmol L^{-1}) for 24 h, however, reversed the effect of FB_1 on axonal outgrowth. This suggests that SLs, more specifically ceramide, are important for normal neuron development. Another study by the same group has confirmed that SLs play an important role in the formation and stabilisation of axonal branches (48). In that study, cultured rat hippocampal neurons incubated with FB_1 (10 μmol L^{-1}) for 66 h had a shorter axonal plexus and less axonal branches, which correlated with a decrease in complex SLs ganglioside, sphingomyelin, and glucosylceramid.

In a study by Kwon et al. (24) on developing rats treated with multiple subcutaneous doses of FB_1 (0.8 mg kg^{-1} b.w. per day) from postnatal day 3 to 12,

increased sphinganine level and sphinagnine to sphingosine ratio in the CNS were accompanied by hypomyelination. In addition to lower myelin deposition, the authors observed a decrease in the activity of 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP), a myelin-associated enzyme. Similarly, a ten-day FB_1 treatment (3 μmol L^{-1} to 40 μmol L^{-1}) impaired myelin formation and deposition, and delayed oligodendrocyte maturation in aggregating brain cell cultures prepared from foetal rat telencephalon (28). However, CNP did not change in that study.

Altered complex SL metabolism by FB_1 can disrupt cell membrane integrity, affecting receptor function such as folate receptor, thereby affecting the transport of folic acid (50, 51). In a study by Stevens and Tang (50) on Caco-2 cells, FB_1 depleted complex SLs, which brought cellular uptake of folate by its receptor to a complete halt. This clearly shows that FB_1 treatment disrupts folate receptor function. It is well known that folate deficiency is a risk factor for NTD. In a study of Sadler et al. (51) reduced folate uptake by FB_1 was connected to embryotoxicity and to NTD. Whole mouse embryo cultures treated with FB_1 (50 μmol L^{-1} for 2 h) showed a high incidence of NTDs (66.7 %), but when treated with a combination of FB_1 and folic acid (1 mmol L^{-1}) this incidence dropped to 34.3 %. In a long-term experiment within the same study, mouse embryos treated with 1 μmol L^{-1} to 100 μmol L^{-1} FB_1 for 26 h showed a dose-dependent increase in NTD together with an increase in sphinganine levels and the sphinganine to sphingosine ratio, but this increase in biomarkers was not dose-dependent. Folic acid (1 mmol L^{-1}) decreased NTD incidence, but did not reduce the inhibition of SL synthesis.

SLs and their metabolites are important messengers involved in signal transduction, leading to either cell survival or cell death (45, 52). Findings about FB_1 effects on the cell cycle and cell death are not consistent. Some report that FB_1 stimulates cell growth and inhibits apoptosis (29, 53), and some claim the opposite (21, 30). After FB_1 treatment, some observed apoptosis as well as necrosis.

Kwon et al. (29) reported a proliferative effect of FB_1 in rat primary astrocytes and oligodendrocytes. An increase in the number of process-bearing cells after FB_1 treatment (0.5 μmol L^{-1} to 75 μmol L^{-1}, for 5 or 10 days) was noted, which suggests that FB_1 may modify the proliferation or differentiation of glial cells (29). On the other hand, Galvano et al. (30) observed no changes in the astrocyte cytoskeletal marker known as glial fibrillary acidic protein (GFAP, largely distributed in astrocyte processes) after FB_1 treatment with 10 μmol L^{-1} to 100 μmol L^{-1} for up to 6 days. This indicates that FB_1 did not disrupt the astrocyte structure. The same study reported DNA damage and increased caspase-3 activity after FB_1 exposure. As increased caspase-3 activity was recorded at lower FB_1 concentrations and at earlier time points (10 μmol L^{-1}, 48 h) than DNA damage (10 μmol L^{-1}, 72 h or 100 μmol L^{-1}, 48 h), the authors concluded that DNA damage was of the apoptotic type. Similarly, Osuchowski and Sharma (21) did not observe any change in GFAP in mouse primary astrocytes treated with FB_1 (25 μmol L^{-1} and 50 μmol L^{-1}) for 4 and 8 days. Of all cell types tested (murine BV-2 microglial, mouse primary astrocytes, mouse primary cortical neurons, and murine N2A neuroblastoma), only murine BV-2 microglial cells showed a dose-dependent decline in proliferation after FB_1 treatment. As FB_1 treatment did not alter the ratio of microglia cells in different phases of the cell cycle, the authors concluded that the observed anti-proliferative effect of FB_1 did not relay on the cell cycle. In the same study, FB_1 (50 μmol L^{-1} after 6 h and 24 h) suppressed TNFα and IL-1β signalling in microglial and primary astrocytes. Additionally, the authors observed necrosis but not apoptosis in murine BV-2 microglial cells and primary astrocytes and linked the absence of apoptosis to the down-regulation of TNFα and IL-1β. Moreover, the authors suggested that pro-apoptotic signalling is dysfunctional in cell lines less sensitive to FB_1 and that FB_1-induced cell loss is primarily caused by necrosis. In contrast, Mobio et al. (31) showed that FB_1 modified the distribution of rat C6 glioblastoma cells in different cell cycle phases. FB_1 treatment (3 μmol L^{-1} to 18 μmol L^{-1} for 24 h) increased the number of cells in G2/M phase and decreased the number of cells in S phase. Arrest in G2/M phase was not dose-related but was induced by a concentration of FB_1 as low as 3 μmol L^{-1} that was not cytotoxic to C6 glioblastoma cells. Since in this study FB_1 also induced hypermethylation of the DNA, the authors connected cell cycle arrest after FB_1 treatment with the genotoxic potential of FB_1. In the next study by the same authors (53) on C6 glioblastoma cells treated with FB_1 (3 μmol L^{-1} to 27 μmol L^{-1} for 24 h) DNA fragmentation parameters such as DNA laddering, DNA damage verified by the comet assay, and evident apoptotic bodies (revealed by chromatin staining with acridine orange and ethidium bromide) pointed to the pro-apoptotic effect of FB_1. To check the involvement

of p53 gene in FB_1-induced apoptosis and cell cycle, the same group of authors used two cell types, rat C6 glioblastoma with normal p53 status, and p53-null MEF mouse embryonic fibroblasts (32). FB_1 treatment (3 μmol L^{-1} to 36 μmol L^{-1} for 24 h, 48 h, and 72 h) induced DNA fragmentation in both cell types. However, the number of cells in different phases and increased number of apoptotic bodies were evident only in C6 glioblastoma cells, and no change in the number of cells in different phases and apoptosis were noted in p53-null MEF cells. Therefore, the results of this study indicate that FB_1-induced DNA damage (observed in both cell types) in cells that lack the p53 function is carried over to the next cell generation, and, as authors concluded, loss of p53 tumour suppressor gene could be responsible for FB_1-induced tumour promotion. In a study of Stockman-Juvala et al. (36) four different cell types were used, mouse GT1-7 hypothalamic, rat C6 glioblastoma, human U-118MG glioblastoma, and human SH-SY5Y neuroblastoma and were treated with 0.1 μmol L^{-1} to 100 μmol L^{-1} FB_1 for 24 h to 144 h. DNA fragmentation was observed in all cells, and increased caspase-3 activity was noted in all cells, except human SH-SY5Y neuroblastoma. However, the expression of p53 and its target proteins from the pro- or anti-apoptotic Bcl-2 family were not affected even with the highest concentration and prolonged exposure, indicating that the p53 dependent mitochondrial Bcl-2 pathway is not important for FB_1-induced apoptosis in these cells. Loss of viability observed in this study suggests, according to the authors, that FB_1 toxicity involves both apoptosis and necrosis.

The discrepancies between these studies may be owed to different effects of various SL products accumulating after ceramide synthase inhibition by FB_1. For example, free sphingoid bases, sphinganine and sphingosine, as well as ceramide exert pro-apoptotic, cytotoxic, and growth inhibitory effects, while sphingoid 1-phosphate metabolites inhibit apoptosis, and promote mitosis and regeneration. Keeping that in mind, the ability of FB_1 to block cell cycle and induce apoptosis depends on various parameters such as cell type, toxin concentrations, and exposure time. As suggested by Desai et al. (52), it is possible that toxicity occurs in cells that accumulate sphinganine (and sphingosine), and mitogenesis in cells that accumulate sphingoid 1-phosphates. Numerous regulatory pathways involving SLs have been identified that can influence apoptotic processes. Control of apoptosis is a complex, non-linear process that is cell-specific and dependent on a variety of feedback mechanisms. Thus, as observed by Stockman-Juvala et al. (36), it could be that in different cell lines, different signalling pathways may be involved in the effects of FB_1.

A recent study by Myburg et al. (54), where human SNO oesophageal carcinoma cells were treated with FB_1 (1 μmol L^{-1} to 32 μmol L^{-1} for 24 h), confirmed that FB_1 induced cell death by apoptosis and necrosis. Moreover, it pointed to the importance of FB_1 concentration applied. At lower FB_1 concentrations (4 μmol L^{-1} and 8 μmol L^{-1}), morphological changes were observed such as blebbing or vesiculation of the plasma membrane and smaller membrane-bound apoptotic bodies, and the treated cells broke up into smaller bodies with no swelling. Swelling as an indicator of necrosis appeared at higher concentrations (16 μmol L^{-1} and above). The authors concluded that apoptosis was the likely mechanism of cell death at lower concentrations, whereas necrosis was the mechanism of death at higher concentrations of FB_1.

However, it is important to emphasise that regardless of how the cell dies (by apoptosis or necrosis), loss of cells initiates compensatory mitosis, and increased proliferation stimulates DNA replication that can lead to cancer development.

Induction of oxidative stress

Yin et al. (55) investigated FB_1-induced lipid peroxidation and oxidative stress. Treatment of egg yolk phosphatidylcholine (EYPC) bilayers with FB_1 (1 mmol L^{-1} to 10 mmol L^{-1}) increased oxidation, promoted free radical intermediate production, and accelerated chain reactions associated with lipid peroxidation. The authors suggested that FB_1 induced oxidative stress and cell damage through enhanced oxygen transport in cell membranes, which in turn increased membrane permeability. Lipid peroxidation was confirmed as a mechanism of FB_1 toxicity in a study of Abel and Gelderblom (56). They observed higher lipid peroxidation in the liver of rats fed with FB_1 (10 mg kg^{-1} to 500 mg kg^{-1} diet for 21 days). In primary hepatocytes, treatment with FB_1 (75 μmol L^{-1} to 500 μmol L^{-1} for 44 h) increased the level of lipid peroxidation in a dose-dependent manner and was associated with an increase in cytotoxicity. Combined treatment of primary hepatocytes with FB_1 (75 μmol L^{-1} to 250 μmol L^{-1}) and vitamin E (10 μmol L^{-1}) for 44 h prevented FB_1-induced lipid peroxidation, but vitamin E could not completely prevent FB_1-induced cytotoxicity. The authors

concluded that lipid peroxidation was not solely responsible for the cytotoxic effects of FB_1 and played a secondary role as a result of cell injury.

Several studies investigated lipid peroxidation and oxidative stress as the mechanism of FB_1-induced cell death in cells of CNS origin (30-34, 53). However, the results of these studies are somehow inconclusive. Mobio et al. (31) found increased lipid peroxidation in rat C6 glioblastoma cells incubated with FB_1 (10 μmol L^{-1} to 20 μmol L^{-1} for 24 h) that was efficiently reduced by adding vitamin E (25 μmol L^{-1}) to FB_1. In the same study, pre-treatment of the cells with vitamin E (25 μmol L^{-1} for 24 h) prevented cell death induced by FB_1 (3 μmol L^{-1} to 54 μmol L^{-1} for 24 h) as well as inhibition of protein and DNA synthesis after FB_1 treatment (10 μmol L^{-1}). This study suggests that lipid peroxidation is involved in FB_1 cytotoxicity. The same group confirmed this observation in their subsequent study (32). In rat C6 glioblastoma and p53 null mouse MEF embryonic fibroblasts, FB_1 (3 μmol L^{-1} to 36 μmol L^{-1} for 24 h) induced lipid peroxidation that was prevented by pre-incubation of cells with vitamin E (25 μmol L^{-1} for 24 h). Furthermore, in both cell lines the authors observed a dose-related increase in 8-oxodG, a marker of oxidative DNA damage. In another study on rat C6 glioblastoma cells by these authors (53), DNA laddering, DNA fragmentation, and apoptotic bodies indicated that FB_1 treatment (3 μmol L^{-1} to 27 μmol L^{-1} for 24 h) stimulated the apoptotic type of cell death. Effects such as DNA fragmentation and apoptotic bodies were prevented by pre-incubation of the cells with vitamin E (25 μmol L^{-1} for 24 h), which clearly implicates oxidative stress in FB_1-induced apoptosis. Stockman-Juvala et al. (34) confirmed that oxidative stress played a role in FB_1 neurotoxicity. In human rat C6 glioblastoma and mouse GT1-7 hypothalamic cells FB_1 (in the dose range of 0.1 μmol L^{-1} to 100 μmol L^{-1} and exposure time from 48 h to 144 h) increased reactive oxygen species (ROS) and lipid peroxidation, and decreased glutathione levels. These effects were not dose-dependent and were in some instances observed only after treatment with the highest dose and prolonged exposure. Interestingly, in human SH-SY5Y neuroblastoma FB_1 did not increase ROS. In a study that followed (33), this group of scientists established higher ROS production and lipid peroxidation, and lower glutathione levels in human U-118MG glioblastoma cells after treatment with 0.1 μmol L^{-1} to 100 μmol L^{-1} FB_1 for 24 h to 144 h. They also observed DNA laddering and higher caspase-3 activity and concluded, similarly to Mobio et al. (32), that oxidative stress may be involved in FB_1-induced apoptosis. Moreover, they suggested a possible sequence of events, as follows: activation of caspase-3 (that was observed at the earliest time point) increases ROS production leading to lipid peroxidation and reduction of intracellular glutathione level, and finally to DNA fragmentation and cell death. In contrast, Galvano et al. (30) did not find any change in ROS production, even though they treated primary cultures of rat astrocytes with FB_1 doses as high as 50 μmol L^{-1} for as long as six days. They however did find increased DNA damage, caspase-3 activity, and expression of HSP70. The authors concluded that FB_1 first modulates the expression of some genes, rather than activate the pathways involving ROS production.

In our recent study (35), FB_1 did increase ROS production in primary rat astrocytes and human SH-SY5Y neuroblastoma cells, but the effect was not dose-dependent. Using live imaging and specific inhibitors of ROS sources within the cell, we established that the source of cytosolic ROS after FB_1-treatment is impaired mitochondrial function. Treatment of astrocytes with FB_1 did not alter the level of lipid peroxidation. Glutathione increased dose-dependently, clearly indicating that oxidative stress is not involved in FB_1 toxicity. Moreover, these data suggest that cells responded to FB_1-increased ROS production by increasing glutathione synthesis to protect themselves from further damage, including lipid peroxidation. Our results support the opinion of other authors (30, 56, 57) that ROS over-production is rather a consequence than a mechanism of FB_1 toxicity. Dragan et al. (45) noted that the chemical structure of FB_1 does not imply pro-oxidant activity and concluded that cellular oxidative damage may be due to toxicity rather than a direct cellular effect of FB_1. It could be, as Abel and Geldreblom (56) suggested, that FB_1 makes cells more susceptible to lipid peroxidation and cellular damage by disturbing SL metabolism. Nevertheless, these authors continue, oxidative stress may mediate FB_1 carcinogenesis (56). Ongoing cell death, continued production of ROS, and compensatory cellular proliferation, may all favour cancer development.

Recent studies of the mechanisms of FB_1 neurotoxicity

It has already been established that due to its specific chemical structure FB_1 targets membranes

(55, 56), but whether it can enter the cell remains unclear. Myburg et al. (54) have shown that FB_1 can actually enter human SNO oesophageal carcinoma cells. In that study, ultrastructural changes were monitored with transmission electron microscopy, and FB_1 detected within the cells with an imunocytochemical method. Transmission electron microscopy revealed enlargement of the nucleus, microsegregation of the nucleolus, signs of membrane damage, and elongated and/or swollen mitochondria. FB_1 was detected in the cytoplasm, nuclei, and mitochondria. Interestingly, it was also detected, to a limited extent, in the mitochondria with no apparent structural changes. As FB_1 is a large and highly polar molecule, it cannot freely permeate membranes. Therefore, the authors suggested that FB_1 may be using the trans-membrane transport system to cross the membrane, probably by mimicking a sphingolipid-type membrane-binding agent. Once in the cell, FB_1 can exert its toxicity by binding to cellular macromolecules or membrane components of the affected organelles. And indeed, in our study (35) we showed that once FB_1 enters the cell, it targets mitochondria and inhibits complex I of the mitochondrial electron transport chain, which lowers the rate of mitochondrial and cellular respiration. In that study FB_1 depolarised the mitochondrial membrane in a dose-dependent manner and was able to increase cytosolic calcium signal [induced by adenosine-5'-triphosphate (ATP)], which suggests that mitochondrial dysfunction caused by FB_1 makes cells vulnerable to physiological calcium signalling. Using cyclosporine A, an inhibitor of mitochondrial membrane permeability, we showed that the opening of membrane transition pores is not involved in cell death induced by FB_1. This observation is in line with Stockman-Juvala et al. (36), who observed that the Bcl-2 pathway is not important for FB_1-induced apoptosis. Taken together, our results suggest that by inhibiting complex I of the mitochondrial electron transport chain FB_1 inhibits cell respiration and lowers mitochondrial membrane potential, which leads to over-production of ROS in mitochondria and to deregulation of calcium signalling (as outlined in Figure 4). Eventually, this can lead to cell death.

In our recent study on primary rat neuronal cultures (58), we confirmed that mitochondrial dysfunction and calcium signalling deregulation are behind FB_1 cytotoxicity. We used three well-established models: 1) the physiological model that involved treatment of neurons with 5 μmol L^{-1} of glutamate to induce physiological calcium signalling; 2) the glutamate excitotoxicity model (as seen in stroke) induced by treatment of neurons with 100 μmol L^{-1} of glutamate, which brings calcium signalling to a higher plateau; and 3) the low magnesium model of epilepsy characterised by repetitive calcium oscillations. In all three models, FB_1 increased or changed the shape of calcium signal and simultaneously depolarised mitochondrial membrane.

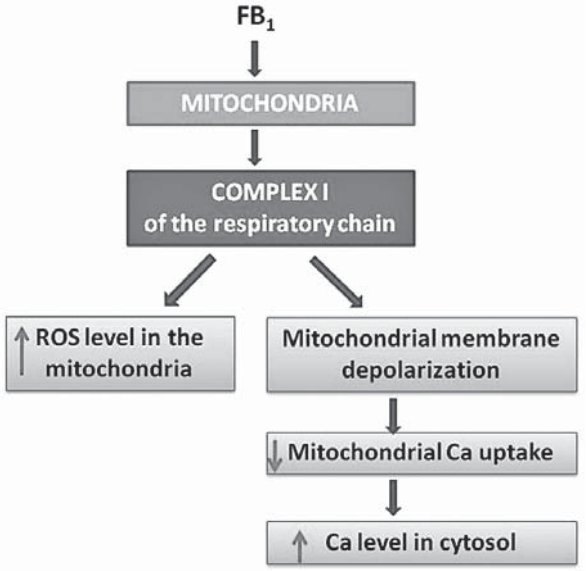


Figure 4 *Proposed mechanism of FB_1 cytotoxicity in neural cells (adapted from ref. 35). Once in the cell, FB_1 targets mitochondria and inhibits complex I of the mitochondrial electron transport chain. This lowers the rate of mitochondrial (and cellular) respiration, depolarises mitochondrial membrane, stimulates over-production of ROS, and deregulates calcium signalling.*

CONCLUSION

FB_1 is a neurodegenerative mycotoxin, and ingestion of FB_1-contaminated food has been associated with a brain disorder in horses, ELEM. However, the mechanism of FB_1 neurotoxicity is still not known. Due to the structural similarity of FB_1 to sphingoid bases, the backbones of SLs, FB_1 inhibits SL metabolism. SLs are important constituents of cell membranes, and by impairing SL metabolism, FB_1 alters membrane functions. One affected function is the folate receptor function, and the effect may be inhibition of folate intake and possibly NTD. SLs are also engaged in signal transduction, and FB_1 may interfere with cell signalling pathways. Experimental evidence suggests that FB_1 has different effects on cell

death/cell growth from pro-apoptotic and growth-inhibitory to anti-apoptotic and growth-stimulatory. It is clear that these effects of FB_1 depend on cell type and origin, route of exposure, concentration, and duration of exposure. Oxidative stress as a mechanism of cell death is not completely ruled out, since together with altered SL metabolism it can contribute to cell death and to cancer development, although it is clear that it is activated as a consequence of other mechanisms triggered by FB_1. Recent studies have shown that FB_1 targets mitochondria. By inhibiting complex I of mitochondrial electron transport chain, FB_1 blocks mitochondrial (and cellular) respiration. This leads to depolarisation of mitochondrial membrane, ROS over-production, and calcium deregulation. Calcium deregulation can lead to cell death. However, this is probably not the only mechanism by which FB_1 can induce cell death. Future studies and new approaches are needed to answer questions about the complex nature of FB_1 toxicity.

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Sažetak

FUMONIZIN B_1: NEUROTOKSIČNI MIKOTOKSIN

Fumonizin B_1 (FB_1) jest mikotoksin koji proizvode plijesni roda *Fusarium* spp. koje nalazimo kao onečišćivače žitarica, ponajprije kukuruza diljem svijeta. Od svih do sada izoliranih fumonizina FB_1 se najčešće može naći na kukuruzu, a i najtoksičniji je fumonizin. FB_1 ima različite toksične učinke ovisno o životinjskoj vrsti. Tako u konja izaziva leukoencefalomalaciju kopitara (ELEM), u svinja plućni edem, a za eksperimentalne je glodavce nefrotoksičan i hepatotoksičan. U ljudi je izloženost FB_1 povezana s razvojem primarnog karcinoma jetre i karcinoma jednjaka koji se učestalo pojavljuju u regijama svijeta (kao Transkeiska regija u Južnoj Africi) u kojima ljudi rabe kukuruz u dnevnoj prehrani. I pojavljivanje defekta neuralne cijevi u nekim je zemljama Srednje Amerike (kao Meksiko i Honduras) povezano s učestalom konzumacijom kukuruzne hrane kontaminirane s FB_1. Ipak se sa sigurnošću ne može povezati razvoj navedenih bolesti u ljudi s izloženosti FB_1. Međunarodna agencija za istraživanje raka (IARC) klasificirala je FB_1 kao mogući karcinogen za ljude (grupa 2B).
Kako je FB_1 uzročnik ELEM-a, poremećaja središnjega živčanog sustava (SŽS) u konja, to upućuje na mogućnost da FB_1 uzrokuje promjene u mozgu. Nekoliko studija na pokusnim životinjama i na staničnim kulturama stanica podrijetlom iz SŽS-a potvrdilo je da je FB_1 neurotoksičan iako mehanizam neurotoksičnosti FB_1, pa tako i mehanizam njegove toksičnosti, još nije razjašnjen. Stoga je cilj ovoga rada dati pregled dostupne literature o neurotoksičnosti i mehanizmu neurotoksičnosti FB_1 kako bi se omogućilo bolje planiranje budućih istraživanja.

KLJUČNE RIJEČI: *defekt neuralne cijevi, leukoencefalomalacija kopitara, mitohondrij, neurotransmiteri, oksidativni stres, sfingoidne baze, sfingolipidi*

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