The Effect of a Single Dose of Fumonisin B₁ on Rat Kidney

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It is known that exposure to mycotoxin fumonisin B₁ (FB₁) causes apoptosis and increases lipid peroxidation in the kidney of experimental animals. In this study, adult male Wistar rats were given a single dose (5, 50, 500 μg per kg of body mass) by gavage and sacrificed 4, 24 and 48 hours after dosing. The parameters of oxidative stress, sphingolipids, DNA lesions and histopathological changes were checked in the kidney of treated and control animals. The metabolism of sphingolipids was significantly affected with the lowest FB₁ dose (sphinganine/sphingosine: 2.53 ± 0.52 vs. control 0.62 ± 0.10), while even the highest dose did not change the parameters of oxidative stress. DNA lesions measured as comet tail length (range (13.6 ± 0.41) μm to (19.83 ± 1.43) μm) and tail intensity ((0.60 ± 0.16) % to (2.74 ± 0.17) %) were FB₁ dose- and time-dependent. Apoptotic cells and mitotic figures were found in the cortical and outer stripe of the medullar part of the kidney.

Keywords
mycotoxin
comet assay
histopathology
sphingolipids

INTRODUCTION

Fumonisins are a group of naturally occurring mycotoxins produced by the Fusarium family of moulds. They contaminate maize and other cereals in the whole world. Fumonisin B₁ (FB₁) is the most common and most toxic fumonisin. It causes leukoencephalomalacia in horses and pulmonary edema in pigs, as well as nephrotoxic and hepatotoxic lesions in rats and mice. Long-term studies have shown that it is more toxic and carcinogenic in male than in female rats, and that kidney is the target organ of FB₁ toxicity in male rats.¹ Although exposure to FB₁ in humans is associated with high incidence of esophageal cancer in some regions of Africa and with primary liver tumors in China, IARC has classified FB₁ as a possible carcinogen for humans (Group 2B) due to the lack of epidemiological evidence.²

Most of the toxic properties of FB₁ are probably related to the disruption of sphingolipid biosynthesis as the consequence of the inhibition of ceramide synthase.³,⁴ Increase in free sphingoid bases, primarily sphinganine (Sa) and sometimes of sphingosine (So), and in their ratio (Sa/So) occurs in the kidney and urine and to a lesser extent in the liver, and it may induce apoptosis.³ The apoptotic effect of FB₁ was noticed in cultured cells and in the kidney and liver cells of experimental animals receiving high oral or intraperitoneal chronic or subchronic doses of the toxin⁶⁻⁸ or of animals exposed to FB₁ contaminated feed.⁹ In the kidney and liver of FB₁-treated rats apoptotic and oncotic necrosis were seen as well as a large variety of pathological changes.

The mechanism of FB₁ genotoxicity is not fully understood. It has been demonstrated on various cultured cell lines, and some authors have associated it with ROS

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production and/or lipid peroxidation. Other authors have shown that ROS follows the production of DNA lesions, thus indicating that the effects of FB1 are not the result of oxidative injury but the response that may occur after modulation of protective genes. In our previous study on rats using the comet assay, we have shown that repeated FB1 dosing causes DNA lesions in rat kidney.

Earlier studies have shown that FB1 is not acutely toxic, and some reversible toxic effects in experimental animals are obtained only with very high doses. The aim of this study was to find out whether a single FB1 dose can produce oxidative stress, disturbance of sphingolipid metabolism, DNA damage and apoptosis in rat kidney shortly after administration.

EXPERIMENTAL

Animal Treatment

Adult male Wistar rats (mass = 230 g) exposed to a light/dark cycle of 12 h and constant temperature of 24 °C were kept in macrolone cages. Animals were fed on a standard diet for laboratory rodents (Mucedola, Settimo Milanese, Italy) and had free access to water.

The rats were randomized in ten (nine exposed plus one control) groups of six animals each. Each group was gavaged a different single FB1 dose (5, 50 or 500 μg/kg of body mass) dissolved in sterile saline (9 g NaCl per 1 L H2O) or solvent only (control animals). FB1 (98 % purity) was purchased from Sigma Chemicals (St. Louis, MO, USA).

The animals were killed by cervical dislocation 4, 24 and 48 hours after dosing. Kidneys were collected and washed in saline. One kidney was immersed into a 4 % buffered formaldehyde solution for histology. The comet assay was performed immediately, while the tissue samples for the biochemical analyses were stored at –20 °C.

The principles of good laboratory animal care were followed throughout, as well as the 2007 Croatian Animal Welfare Act. The Institute’s Ethical Committee approved the study.

Biochemical Assays

Kidney homogenates (10 g per 100 ml buffer) were prepared for biochemical assays in chilled 0.05 mol dm–3 potassium phosphate buffer (pH = 7.4).

Reduced glutathione (GSH) was measured in supernatants of the kidney homogenates using Ellman’s method. In 0.1 ml of sample 0.85 ml of 0.3 mol dm–3 phosphate buffer, pH = 7.4, and 0.05 ml of 10 mmol dm–3 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were added. The reaction was read at 412 nm and the results were expressed as (nmol GSH)/mg).

The concentrations of malondialdehyde (MDA) were measured in kidney homogenates spectrophotometrically at 532 nm using the method of Drury et al. Briefly, 1 % 2-thiobarbituric acid (0.75 ml), 0.6 g thiobarbituric acid per 100 ml H2O (0.25 ml) and redistilled water (0.445 ml) were added to 0.05 ml sample aliquots which were then heated in water bath at 95 °C for 20 min. After cooling, samples and MDA standards were read against blanks at 532 nm. The concentrations were determined using standard MDA curves, and the results were expressed as (nmol MDA/mg) / (mg protein/mg).

Protein concentration was determined according to Bradford.

The mass fraction of sphingolipids were determined in the same kidney homogenates according to method described by Sheppard and van der Westhuizen. Sphingolipids were derivatized with o-phthaldialdehyde. An aliquot was injected onto an HPLC with fluorescent detection (Varian Walnut-Creek, USA), and chromatograms were analyzed using the Varian Star Chromatography Workstation Ver. 5.0 (Varian Walnut-Creek, USA).

Alkaline Comet Assay

Kidney tissue samples for the comet assay were homogenized in a chilled homogenization buffer (pH = 7.5) containing 75 mmol dm–3 NaCl and 24 mmol dm–3 Na2EDTA to obtain a 10 g / 100 ml buffer tissue solution. A Potter-type homogenizer was used.

We used alkaline comet assay protocol according to Sasaki et al. 6 μl of kidney homogenate were suspended in low-melting agarose (0.5 g in 100 ml TBS) and sandwiched between a layer of normal-melting agarose (0.6 g in 100 ml TBS) and a top layer of low melting agarose (0.5 g in 100 ml TBS) on fully frosted slides. The slides were kept on ice during the polymerization of each gel-layer. After the solidification of the normal-melting agarose layer, the slides were immersed in a lysis solution (34.1 mmol dm–3 sodium sarcosinate, 2.5 mol dm–3 NaCl, 100 mmol dm–3 Na2EDTA, 10 mmol dm–3 Tris-HCl, 1 % (vol. fraction) Triton X-100 and 10 % (vol. fraction) DMSO) at 4 °C. After one hour, the slides were placed into an electrophoresis buffer (0.3 mol dm–3 NaOH, 1 mmol dm–3 Na2EDTA, pH = 13) for 10 minutes at 0 °C to allow for the DNA to unwind. Electrophoresis was performed for 10 minutes at 300 mA and 1 V/cm. The slides were neutralized with a Tris-HCl buffer (pH = 7.5) and stained with ethidium bromide (20 μg/ml) for 10 minutes. Each slide was analyzed using the Leitz Orthoplan epifluorescence microscope. One hundred cells were analyzed on each slide using the comet assay II automatic digital analysis system (Perceptive Instruments Ltd., Suffolk, Halstead, UK) to determine the tail length and tail intensity. Tail length (expressed in μm) is the distance of DNA migration from the center of the body of the nuclear core and it is used to evaluate the extent of DNA damage. Tail intensity (expressed in percents) is the fraction of the genomic DNA that migrated during the electrophoresis from the nuclear core to the tail. Both, tail length and tail intensity are measured automatically by image analysis software.
Histopathology

After fixation kidney tissues were dehydrated through an ethanol series, treated with xylene and embedded into paraffin. 3 μm thick sections were stained with Periodic acid Schiff stain (PAS). The number of cells undergoing apoptosis and mitotic figures were determined by counting the number of apoptotic or mitotic cells in the outer stripe of the outer medulla and in the cortex of rat kidney on 10 randomly selected high power microscopic fields (400x).

Statistical Analysis

Differences in biochemical parameters (concentration of Sa, So and their ratio, MDA, and GSH) in kidney tissues of treated and control animals and between different groups were tested using the Student’s t-test for independent samples using Statistica 5.0.

Possible differences in the comet assay endpoints in tissue samples were evaluated using the Kruskal-Wallis non-parametric test. We also analyzed differences between treatment times and doses.

Probability values of $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

In the kidney tissues of all FB1-treated groups, GSH and MDA concentration did not change as compared to controls (data not shown). These results are in accordance with the study of Bondy et al.24 in rats treated with FB1 (7.5 or 10 mg/kg b.m., i.p.) for four consecutive days as well as with our previous study where rats were given FB1 dose of 0.5 mg/kg b.m., i.p. for two days.25 Our results confirm that oxidative stress is the consequence of rather than a step in the mechanism of FB1 toxicity.14,15,26

Numerous studies have demonstrated that FB1 inhibits enzyme ceramide synthase in the kidney, with a consequent increase in Sa concentration and Sa/So ratio.4 Repeated FB1 dosing (50 mg/kg diet for 10 days) increases the Sa concentration over 100 times in the kidney.3 Other authors claimed that the lowest dose of FB1 which induced a change in Sa/So ratio in any tissue was 1.0 mg/kg of b.m., p.o. (5 days a week per 5 weeks).27 However, in our study mentioned above, the increase in Sa and So mass fractions, as well as the increase in Sa/So ratio was noticed even after two treatments with 0.5 mg/kg b.m., i.p.25 In our experiment the mass fraction of Sa and Sa/So ratio were significantly higher than in controls (Table I). This effect was noticed with all applied doses, and appeared in the first four hours after dosing, indicating that this biomarker was more sensitive than was believed earlier.

Studies of FB1 genotoxicity are rather scarce and are mostly performed on cell cultures. A single study performed using a kidney cell line was published by Rumora et al.28 The authors observed concentration- and time-de-
TABLE I. Mass fraction (w) of sphingosine (So) and sphinganine (Sa) and So/So ratio in kidney tissue of rats (mean ± S.E.; N = 6) treated with single dose of FB1 and sacrificed 4, 24 and 48 hours after treatment

<table>
<thead>
<tr>
<th>m(FB1) / μg</th>
<th>t / h</th>
<th>w(So) · 10^6</th>
<th>w(Sa) · 10^6</th>
<th>w(Sa) / w(So)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>8.36 ± 3.33</td>
<td>4.89 ± 1.82</td>
<td>0.62 ± 0.10</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>13.14 ± 5.46</td>
<td>(30.72 ± 11.11) (c)</td>
<td>(2.53 ± 0.52) (c)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>25.08 ± 5.41</td>
<td>(72.03 ± 17.22) (c)</td>
<td>(2.93 ± 0.42) (c)</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
<td>21.40 ± 4.61</td>
<td>(29.65 ± 6.50) (c)</td>
<td>(1.44 ± 0.13) (c)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>11.63 ± 0.70</td>
<td>(35.56 ± 3.72) (c)</td>
<td>(3.14 ± 0.44) (c)</td>
</tr>
<tr>
<td>500</td>
<td>4</td>
<td>11.08 ± 0.42</td>
<td>(32.36 ± 2.92) (c)</td>
<td>(2.95 ± 0.30) (c)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>7.30 ± 1.52</td>
<td>(16.04 ± 3.69) (c)</td>
<td>(2.17 ± 0.35) (c)</td>
</tr>
</tbody>
</table>

(a) w(sphingolipids) = (m(sphingolipids)/ng) / (m(tissue)/mg).
(b) m(FB1): 5, 50 and 500 μg per kg of body mass.
(c) Different from controls.
(d) Different from 4 hours exposure.
(e) Different from 24 hours exposure.
(f) Different from 5 μg dose per kg b.m.
(g) Different from 50 μg dose per kg b.m. (P < 0.05).

Histopathological changes caused by FB1 in this study were milder but consistent with previous studies obtained with higher doses and longer exposure times. In other studies, treatment with high doses of FB1 (15–75 mg/kg b.m., p.o. for 11 days), in addition to apoptotic cells and mitotic figures, also caused dose-related necrosis, sloughing of tubular epithelial cells in the inner cortex, anisokaryosis, cytoplasmic basophilia and atrophy of tubular epithelial cells. When shorter i.p. treatment (4 days) with lower FB1 doses (7.5 and 10 mg/kg b.m.) was applied, minimal renal lesions with rare single-cell necrosis and desquamation of epithelial cells were seen. However, the most prominent finding in the kidney of male rats exposed to FB1-contaminated feed (99, 163, 234, and 484 mg/kg b.m.) for 28 days were apoptosis and mitotic figures in the inner cortex. All treated rats had essentially the same level of apoptosis, and except for the 99 mg/kg dose group, an equivalent severity of kidney degeneration was reported. The lack of the difference in the severity of kidney lesion between groups of rats treated for
four and 13 weeks was also noticed in animals fed with FB₁ contaminated feed (9, 27, 81 mg/kg). In our study, histopathological changes included the appearance of apoptotic cells and mitotic figures in the cortical part of the kidney as well as in the outer stripe of medulla (Figure 1). The number of apoptotic cells and mitotic figures both in the cortical and medullar part of the kidney was higher than in controls, but their appearance was not clearly dose- and time-related, which is similar to the study of Tolleson and co-workers. Although it is known that FB₁ stimulates degenerative and regenerative processes in the kidney, the co-occurrence of apoptosis and mitotic figures both in the cortical and medullar part of the kidney is surprising because they indicate that these processes occur even after a single FB₁ dose.

CONCLUSION

The major finding of this study was that the single dose of FB₁ in rats disturbs the sphingolipid metabolism even after a very short period of exposure. DNA lesions occur after longer exposure to the lowest dose, but they are time- and dose-dependent. Apoptotic cells and mitotic figures seen in all treated groups indicate the high potential of FB₁ to produce degenerative and regenerative processes, probably due to changes in sphingolipid metabolism.

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SAŽETAK

Učinak jednokratne doze fumonizina B1 na bubreg štakora

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Mikotoksin fumonizin B1 (FB1) u pokusnih životinja uzrokuje apoptozu i povećanu lipidnu peroksidaciju u hubrezima i jetri. U ovom istraživanju odrasli mušaci štakora soja Wistar tretirani su jednokratnom dozom FB1 (5, 50, 500 μg/kg tjelesne mase) i žrtvovani 4, 24 i 48 sati nakon tretmana. Parametri oksidacijskog stresa, koncentracija sfingolipida, oštećenja DNA i histopatološke promjene praćene su u bubregu tretiranih i kontrolnih životinja. Tretman s FB1 značajno je poremetio metabolizam sfingolipida već i pri najmanjoj primijenjenoj dozi (Sa/So: 2,53 ± 0,52; kontrola 0,62 ± 0,10), dok ni najveća doza nije uzrokovala promjene parametara oksidacijskog oštećenja. Ovim istraživanjem dokazano je da jednokratna doza FB1 uzrokuje oštećenje DNA koje je ovisno o duljini izloženosti i dozi (dužina repa: (13,6 ± 0,41) μm do (19,83 ± 1,43) μm; intenzitet repa: (0,60 ± 0,16) % do (2,74 ± 0,17) %). Apoptotične stanice i stanice u mitozi nađene su u kortikalnom i medularnom dijelu bubrega svih tretiranih životinja.