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Oral administration of fumonisin B_1 and T-2 individually and in combination affects hepatic total and mitochondrial membrane lipid profile of rabbits

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Weaned rabbits were fed diets contaminated with 2 mg/kg diet T-2 toxin alone, or 10 mg/kg diet fumonisin B_1 (FB_1) alone, and both toxins in combination (2 + 10 mg/kg, respectively) compared to a toxin-free control diet. Samplings were performed after 4 weeks (blood and liver). Bodyweight of T-2-fed group was lower after 4 weeks; the liver weight was increased dramatically (threefold of control). Liver total phospholipids (PLs) provided slight alterations in the fatty acid (FA) composition; all three toxin-treated groups showed a decrease in palmitoleic acid (C16:1 n7) proportion. In the liver mitochondrial PL FA composition, margaric acid (C17:0) proportion decreased in the separated toxin treatments compared to the combined setting. Oleic acid (C18:1 n9) proportion was increased and arachidonic acid (C20:4 n6) was decreased in the FB_1-treated group, while docosapentaenoic acid (C22:5 n3) was decreased in the separated treatments. The total monounsaturation was significantly higher in the FB_1 group's mitochondrial PL FA profile. After 4 weeks, all toxin treatments decreased the blood plasma reduced glutathione and glutathione peroxidase activity, and FB_1 increased the plasma sphinganine/sphingosine ratio. Both mycotoxins seem to cross the hepatocellular and the hepatic mitochondrial membrane, without drastic membrane disruption, as assessed from the PL FA composition, but inducing detectable lipid peroxidation.

Keywords: rabbit, T-2, FB_1, membrane lipids, liver, mitochondria, lipid peroxidation

Introduction

Mycotoxins are secondary metabolites of fungal (mold) metabolism with various biological activities, e.g., protein (T-2) or ceramide (fumonisin B_1, FB_1) synthesis inhibition, but T-2 and FB_1 in high concentrations and prolonged consumption are carcinogens as well (9, 11, 13). Since most fungal strains produce single or multiple mycotoxins, their combined effects are nowadays in focus (12).

T-2 toxin is a trichothecene, produced by *Fusarium* species, occurring mostly in corn and further cereals. T-2 toxin is metabolized (hydrolyzed to HT-2) after ingestion, yielding multiple metabolites with known or less known toxicity and membrane association

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capability (9). Their mode of toxic action is rather divergent, as the inhibition of protein synthesis (specifically in eukaryotes) and inhibition of DNA synthesis (9), or the disruption of the mitochondrial electron transport system (26), augmented lipid peroxidation, affecting ultimately cell membrane integrity. According to Ngampongsa et al.'s study (26), the ATP-linked oxygen consumption rate of cardiomyocyte mitochondria is compromised by T-2 toxin, which is underpinned with our recent results, in which rabbit erythrocyte Na^+/K^+ ATPase activity (the rate of ATP breakdown) was markedly lowered by T-2 toxin (33). It was established that in the rat liver, T-2 toxin inhibits the mitochondrial electron transport system, concluding that mitochondria are possible hepatic targets of T-2 toxin action (27). It is important to add that even in embryonic stem cells (being pluripotent), T-2 toxin induced apoptosis via a reactive oxygen species-mediated mitochondrial pathway (14). In summary, it is proven that T-2 toxin crosses the hepatic cellular and subcellular membranes and disrupts ATP production in mitochondria (28). An essential addition is that hepatic subcellular distribution of labeled $^{[3H]}$T-2 toxin revealed immediate association to the plasma membrane and a concomitant association to the membranes of other cellular compartments, including mitochondria (28). Anyhow, T-2 toxin is less known considering its membrane disruptor effect exerted during its transmembrane movement on which this study was focused. The question is even more intriguing taking the solubility of pure T-2 toxin into account, namely, it is only weakly water soluble (3).

Molecular-compositional changes underlying the hepatic membrane lipid alterations (alteration of the lipid profile of phospholipid (PL) classes in the microsomal, the nuclear, and the cellular membranes) caused by FB_1 were partly elucidated when feeding rats with a drastically toxic, cancer promoting toxin level (250 mg/kg feed) (6). In the same study, FB_1 altered lipid constituent proportions; in particular, phosphatidylethanolamines (PEs) were significantly increased in the mitochondrial and even in the plasma membrane fractions. The fatty acid (FA) profile of the main polar lipid fractions (i.e., P-choline and PE) showed a decrease in polyunsaturated (especially C20:4 n6, arachidonic acid)/saturated (P/S) FA ratio, suggesting a less fluid membrane state. The decreased polyunsaturated FA levels in phosphatidylcholine (PC) along with a marked elevation of oleic (C18:1 n9) and linoleic (C18:2 n6) acids were indicative of an impaired delta-6 desaturase activity. The authors concluded that FB_1 disrupts sphingolipid, PL (the polar lipid fractions interaction with a polar toxin molecule), cholesterol, and FA metabolism in the liver, in a manner to decrease membrane fluidity (ultimately leading to apoptosis). Arachidonic acid has a profound role in this, namely, the outer plasma membrane lipid leaflet is enriched with this acid, being the precursor of inflammatory eicosanoids.

Since both T-2 and FB_1 are unequivocal membrane disruptors even at the subcellular level, their detailed study is reasoned.

The co-occurrence of mycotoxins makes the cytotoxicity assessment more complex, meanwhile Clarke et al. (10) used the EU regulation concentration limits for FB_1, ochratoxin, and aflatoxin B_1 in a tertiary combination (10). In our recent study, it was found that at levels of 2 mg/kg diet T-2 toxin alone, or 10 mg/kg diet fumonisin B_1 (FB_1) alone, and both toxins in combination exerted hepatotoxic effect and acted antagonistically onto the red cell sodium pump ATP breakdown capacity, T-2 markedly lowering its activity (33). However, red cell membrane fatty acyl chain composition was only slightly modified in the cells of the systemic circulation, supposing that both toxins act at the level of polychromatic erythroblasts. Here, we tested the effects of the same toxin exposure onto the hepatic membrane and hepatic mitochondrial membrane composition and investigated possibly emerging lipid peroxidation.

Materials and Methods

Experimental design

Pannon white littermate male rabbits weaned at the age of 35 days were caged as pairs, and four groups were formed, each containing 10 animals. The animals were born, housed, and treated at the rabbitry of the experimental farm of the Kaposvár University. One group was fed a rabbit diet complemented with 2 mg/kg T-2 toxin, another group was fed the same basal diet complemented with 10 mg/kg FB_1, while a further group was given T-2 and FB_1 in a combined form (2 + 10 mg/kg diet). The control group was fed a toxin-free diet containing the same ingredients as the experimental ones, with the exception of fungal cultures. Feed and water were *ad libitum* offered. Bodyweight and feed intake were measured weekly, individually, and the entire feeding period lasted for 4 weeks. All data were recorded on individually marked animals, from six rabbits (chosen out randomly of the total of 10) in each group.

Production of toxins and determination of mycotoxin content in fungal cultures and feeds

FB_1 and T-2 toxin were produced with the application of *Fusarium verticillioides* strain MRC 826 and *Fusarium sporotrichioides* strain NRRL 3299, respectively (15). The homogenized fungal cultures contained FB_1 at a concentration of 3,440 mg/kg, while T-2 toxin concentration was 1,338 mg/kg (HT-2 toxin 285 mg/kg), respectively. These fungal cultures were mixed into the basal feed of the experimental animals, so as to provide contaminated feeds with 2 mg/kg feed T-2 toxin and/or 10 mg/kg feed FB_1.

The mycotoxin concentration of the control and the experimental feeds was determined with LC-MS (Shimadzu, Kyoto, Japan). The limit of detection for FB_1 was 3 μg/kg, while for T-2 toxin, it was 10 μg/kg. The diet fed to the control group did not contain detectable amounts of T-2 and FB_1.

Blood and liver tissue sampling

Fresh venous blood was sampled into heparinized (20 IU/mL whole blood) tubes and was centrifuged for 10 min at 1,000*g*, at the end of the 4 weeks. Plasma was removed and stored at −70 °C for sphinganine and sphingosine analysis (*Determination of the plasma sphinganine* (*Sa*) *to sphingosine* (*So*) *ratio* section).

After exsanguination, whole livers were immediately dissected and weighed. Tissue samples of ca. 5 g were cut with scissors in a medium according to Brand et al. (5). Liver samples were rinsed and resuspended in 10 volumes of ice-cold medium, containing 250 mM sucrose, 5 mM Tris/HCl, and 2 mM EGTA (pH 7.4 at 4 °C), then homogenized using six passes of a motorized Teflon/glass Potter-Elvehjem homogenizer. All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany). The homogenate was centrifuged at 1,000*g* for 3 min, and the supernatant was centrifuged at 12,000*g* for further 10 min at 4 °C. The pellet was then suspended, respun at 12,000*g* for 10 min (MLW K24 Janetzky, Engelsdorf, Germany), and resuspended in isolation medium and kept at −70 °C.

Lipid analysis

Whole tissue samples as well as isolated mitochondria were homogenized (T25 Digital Ultra Turrax, IKA, Staufen, Germany) in a 20-fold volume of chloroform:methanol (2:1, vol:vol) and the total lipid content was extracted (16). Solvents were ultrapure grade

(Sigma-Aldrich, Schnelldorf, Germany) and 0.01% w:v butylated hydroxytoluene was added to prevent FA oxidation. In the frame of lipid fractionation, extracted total lipids were transferred to glass chromatographic columns, containing 300 mg silica gel (230–400 mesh) for 10 mg of lipids (24). Neutral lipids were eluted with 10 mL chloroform for the above fat amount, then 15 mL acetone:methanol (9:1, vol:vol) was added, while 10 mL pure methanol eluted the total PLs. This latter fraction was evaporated to dryness under a nitrogen stream and was transmethylated with a base-catalyzed $NaOCH_3$ method (9). FA methyl esters were extracted into 300 μL ultrapure n-hexane for gas chromatography. Latter was performed on a Shimadzu 2010 apparatus (AOC 20i automatic injector), equipped with a Phenomenex Zebron ZB-WAX Capillary GC column (30 m × 0.25 mm ID, 0.25 μm film, Phenomenex Inc., Torrance, CA, USA) and flame ionization detector (FID, 2×10^{-11}). Characteristic operating conditions were: injector temperature, 270 °C; detector temperature, 300 °C; and helium flow, 28 cm/s. The oven temperature was graded from 80 °C to 205 °C: 2.5 °C/min, 5 min at 205 °C; and from 205 °C to 250 °C: 10 °C/min, 5 min at 210 °C. The makeup gas was nitrogen. To identify the individual FA, an authentic external FA standard (37 Component FAME Mix, Sigma-Aldrich, Cat No: CRM47885) was used. FA results were expressed as weight % of total FA methyl esters.

Unsaturation index (UI) was defined as the number of double bonds in 100 fatty acyl chains. From the FA results, UI was calculated as: $UI = [(1 \times \Sigma \text{ monoenoic FA}) + (2 \times \Sigma \text{ dienoic FA}) + (3 \times \Sigma \text{ trienoic FA}) + (4 \times \Sigma \text{ tetraenoic FA}) + (5 \times \Sigma \text{ pentaenoic FA}) + (6 \times \Sigma \text{ hexaenoic FA})]$ (7). The average fatty acyl chain length (ACL) was calculated from the multiplication of the chain length values and the respective proportion of each FA.

Assessment of antioxidant defense capacity

Lipid peroxidation was assessed in blood plasma with the determination of malondialdehyde (MDA) levels with 2-thiobarbituric acid method (30). The concentration of plasma reduced glutathione (GSH) was measured with photometry (31) and the activity of plasma glutathione peroxidase (GSHPx) according to Lawrence and Burk (22).

Determination of the plasma sphinganine (Sa) to sphingosine (So) ratio

Determination of Sa/So was carried out using acetonitrile (900 μL) addition to the plasma (100 μL) and the mixture was centrifuged at 10,000 rpm for 5 min and filtered through a 0.45 μm filter. Sphinganine and sphingosine levels were determined by LC-MS. Chromatographic separation was carried out using a Waters Symmetry C_{18} column (150 mm × 2.1 mm, ID 5 μm; Waters, Mildford, MA, USA). Quantitative measurement was based on linear calibration to authentic standards.

Statistical evaluation

For the comparison of group means (bodyweight and liver weight FA profile), multivariate analysis of variance was used (evaluating the interactions as well) with the Tukey *post hoc* test for the inter-group differences, preceded with the analysis of normal distribution (Kolmogorov–Smirnov test) (19).

Ethical statement

The experiment was carried out according to the regulations of the Hungarian Animal Protection Act. The allowance number for this study was 656-1/2012.

Results

Bodyweight and liver weight

After 4 weeks of feeding, the T-2 and the T-2+FB_1 groups' bodyweight were significantly lower compared to the control or even to the pure FB_1 data, although the feed intake was not different among the groups (data not shown). The liver weight at the end of the experiment in the group fed with T-2 toxin was significantly higher compared to the other treatment groups and reached ca. three times higher weight than the control (Table I). However, T-2+FB_1 in combination or FB_1 alone did not significantly alter liver weight.

Hepatic total phospholipids

From Table II, showing the total hepatic PL FA composition, it is clear that all three toxin treatments decreased the proportion of palmitoleic acid (C16:1 n7). Arachidic acid (C20:0) proportion in the liver total PL fraction was increased by the combined toxin treatment (T-2+FB_1) compared to the control. Interestingly, the UI was the highest in the combined toxin (T-2+FB_1) exposed animals, differing significantly only from the control, but not from the two individual toxin treatments (T-2 and FB_1 separately). A totally identical alteration was observed in the ACL. Altogether, neither the separated nor the combined mycotoxin treatments induced drastic FA compositional alteration in the hepatic total PLs.

Liver mitochondrial phospholipids

In the isolated mitochondria, the membrane lipids revealed to be more prone towards the mycotoxin exposure. Table III summarizes the results concerning the alterations of the mitochondrial membrane PL FA composition. Margaric acid (C17:0) proportion was decreased in both separated toxin treatments compared to the combined setting. Oleic acid (C18:1 n9) (a major membrane component of the PL fraction) proportion was significantly increased by FB_1. Arachidonic acid (C20:4 n6) was decreased in the FB_1 group compared to the control and the binary mixture treatment. Docosapentaenoic acid (C22:5 n3) proportion was decreased significantly in both separate treatments compared to the combined group, but not differing from the control.

In the calculated FA data, total monounsaturation (MUFA) was significantly higher in the FB_1 group compared to the control. A completely opposite result was found for the total polyunsaturation, while the total n3 FA proportion was increased (on the expense of the total n6 FAs).

Table I. Liver and bodyweight of the four experimental groups (mean ± SD)

Group	Liver weight (g; day 60)	Bodyweight (g; day 60)
	Mean ± SD	Mean ± SD
T-2	219.2 ± 3.37^{b}	2280 ± 82.6^{A}
FB_1	75.8 ± 4.35^{ab}	2695 ± 84.1^{B}
T-2+FB_1	72.8 ± 4.35^{a}	2221 ± 81.7^{A}
Control	91 ± 8.39^{ab}	2653 ± 69^{B}

^{a,b,A,B}Difference between treatments at day 60

Table II. The total hepatic phospholipid fatty acid composition of the control and the toxin-treated groups (all data are mean ± SD of six animals)

Group	T-2	FB_1	T-2+FB_1	Control
Liver PL	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
C14:0	0.06 ± 0.01	0.08 ± 0.02	0.07 ± 0.01	0.12 ± 0.09
C15:0	0.19 ± 0.03	0.17 ± 0.04	0.20 ± 0.02	0.17 ± 0.04
C16:0	19.1 ± 0.97	19.2 ± 1.33	17.5 ± 1.73	20.3 ± 2.81
C16:1 n7	0.39 ± 0.13^{a}	0.45 ± 0.05^{a}	0.31 ± 0.14^{a}	0.66 ± 0.22^{b}
C17:0	0.85 ± 0.12^{b}	0.72 ± 0.14^{ab}	0.91 ± 0.07^{b}	0.63 ± 0.11^{a}
C18:0	24.4 ± 0.96	23.1 ± 1.08	24.1 ± 1.88	22.5 ± 1.64
C18:1 n9c	13.5 ± 2.20	15.2 ± 0.91	13.8 ± 1.13	15.3 ± 1.43
C18:2 n6c	29.2 ± 2.47	29.1 ± 0.74	29.1 ± 0.07	28.4 ± 2.38
C18:3 n6c	0.09 ± 0.09	0.04 ± 0.01	0.05 ± 0.01	0.09 ± 0.08
C18:3 n3	0.50 ± 0.06	0.55 ± 0.09	0.51 ± 0.03	0.49 ± 0.14
C20:0	0.04 ± 0.00^{a}	0.04 ± 0.00^{a}	0.05 ± 0.01^{b}	0.04 ± 0.01^{a}
C20:1 n9	0.27 ± 0.05	0.32 ± 0.16	0.35 ± 0.15	0.31 ± 0.12
C20:2 n6	0.68 ± 0.14	0.89 ± 0.17	0.90 ± 0.29	0.75 ± 0.26
C22:0	0.42 ± 0.35	0.27 ± 0.06	0.34 ± 0.16	0.38 ± 0.22
C20:3 n6	0.93 ± 0.27	0.88 ± 0.11	1.07 ± 0.21	0.95 ± 0.30
C20:3 n3	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.03	0.03 ± 0.01
C20:4 n6	7.96 ± 0.48	7.40 ± 0.98	9.06 ± 1.66	7.41 ± 0.55
C22:5 n3	0.25 ± 0.04	0.23 ± 0.06	0.31 ± 0.07	0.24 ± 0.05
C22:6 n3	0.17 ± 0.04	0.15 ± 0.05	0.19 ± 0.06	0.17 ± 0.05
Saturated	45.0 ± 1.43	43.6 ± 1.05	43.2 ± 1.11	44.1 ± 1.48
Monoenoic	14.1 ± 2.31	16.0 ± 1.02	14.5 ± 1.42	16.3 ± 1.66
PUFA	39.8 ± 2.67	39.3 ± 1.05	41.2 ± 1.72	38. 6 ± 2.73
n3	0.95 ± 0.13	0.96 ± 0.17	1.05 ± 0.11	0.92 ± 0.17
n6	38.9 ± 2.60	38.3 ± 0.93	40.2 ± 1.63	37.6 ± 2.70
n6/n3	41.2 ± 4.26	40.8 ± 6.17	38.7 ± 3.41	42.0 ± 6.58
UI	112.7 ± 3.90^{ab}	112.22 ± 3.40^{ab}	118.39 ± 6.09^{b}	111.1 ± 4.88^{a}
ACL	17.66 ± 0.04^{ab}	17.64 ± 0.05^{ab}	17.70 ± 0.10^{b}	17.62 ± 0.06^{a}

UI = unsaturation index; ACL = average fatty acyl chain length
[a,b]Significant ($P < 0.05$) difference between groups

Table III. The hepatic mitochondrial phospholipid fatty acid composition of the control and the toxin-treated groups (all data are mean ± SD of six animals)

Group	T-2	FB_1	T-2+FB_1	Control
Liver mitochondrial PL	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
C14:0	0.21 ± 0.19	0.28 ± 0.12	0.15 ± 0.10	0.22 ± 0.16
C15:0	0.20 ± 0.10	0.21 ± 0.08	0.27 ± 0.04	0.24 ± 0.06
C16:0	20.6 ± 2.03	21.0 ± 3.73	18.2 ± 1.24	18.4 ± 1.78
C16:1 n7	1.27 ± 0.46	1.20 ± 0.17	0.75 ± 0.23	1.13 ± 0.43
C17:0	0.61 ± 0.18[a]	0.60 ± 0.16[a]	0.92 ± 0.15[b]	0.79 ± 0.17[ab]
C18:0	17.3 ± 1.05	16.5 ± 1.00	18.3 ± 4.39	16.7 ± 4.60
C18:1 n9c	23.4 ± 4.68[ab]	26.8 ± 1.56[b]	20.3 ± 3.63[ab]	19.1 ± 4.99[a]
C18:2 n6c	25.6 ± 5.63	22.0 ± 6.05	28.7 ± 4.24	30.3 ± 3.78
C18:3 n6	0.19 ± 0.06	0.38 ± 0.17	0.09 ± 0.05	0.19 ± 0.24
C18:3 n3	1.61 ± 0.80	2.04 ± 0.60	1.23 ± 0.40	1.92 ± 1.21
C20:0	0.08 ± 0.02	0.09 ± 0.02	0.08 ± 0.01	0.08 ± 0.03
C20:1	0.70 ± 0.37	0.86 ± 0.59	0.51 ± 0.18	0.46 ± 0.12
C20:3 n6	0.90 ± 0.44	0.80 ± 0.37	1.39 ± 0.31	1.28 ± 0.45
C20:3 n3	0.23 ± 0.04[ab]	0.27 ± 0.20[b]	0.07 ± 0.03[ab]	0.23 ± 0.11[ab]
C20:4 n6	4.83 ± 1.05[ab]	4.04 ± 0.34[a]	6.39 ± 1.58[b]	6.43 ± 1.32[b]
C22:5 n3	0.19 ± 0.04[a]	0.18 ± 0.02[a]	0.28 ± 0.05[b]	0.25 ± 0.06[ab]
C22:6 n3	0.83 ± 0.34	1.41 ±1.15	0.63 ± 0.53	0.55 ± 0.24
Saturated	39.0 ± 1.57	38.6 ± 4.12	37.9 ± 4.50	36.4 ± 4.23
Monoenoic	23.5 ± 4.34[ab]	28.7 ± 1.37[b]	21.7 ± 3.66[ab]	20.8 ± 4.97[a]
PUFA	34.4 ± 5.69[ab]	31.1 ± 5.43[a]	38.7 ± 4.87[ab]	41.2 ± 4.55[b]
n3	2.85 ± 1.03[ab]	3.90 ± 0.82[b]	2.20 ± 0.44[a]	2.95 ± 1.55[a]
n6	31.6 ± 6.55[ab]	27.2 ± 6.04[a]	36.5 ± 5.15[ab]	38.2 ± 4.87[b]
n6/n3	12.8 ± 6.71	7.37 ± 2.53	17.6 ± 6.09	16.9 ± 11.5
UI	102.9 ± 20.0	108.7 ± 5.44	118.0 ± 9.68	122.5 ± 9.39
ACL	17.5 ± 0.14	17.5 ± 0.17	17.5 ± 0.13	17.5 ± 0.10

UI = unsaturation index; ACL = average fatty acyl chain length
[a,b]Significant ($P < 0.05$) difference between groups

Antioxidant defense capacity

In the blood plasma, the concentration of reduced GSH was the highest in the control, significantly differing from all toxin-treated groups. Identical finding was detected by GSHPx, but no alteration was detected in the plasma MDA concentration (Fig. 1).

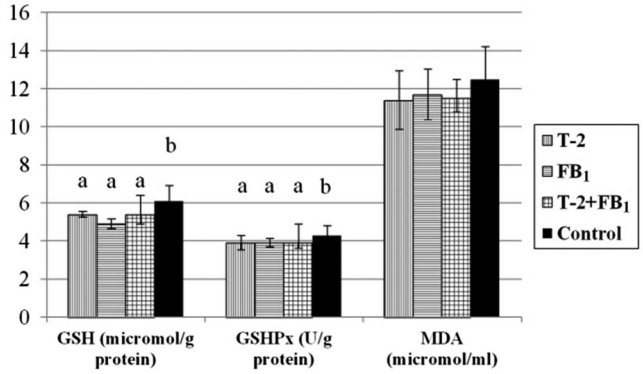

Fig. 1. The extent of antioxidant capacity (GSH, GSHPx) and the concentration of the ultimate lipid peroxidation product, malondialdehyde, in the blood plasma of the rabbits (mean ± SD)

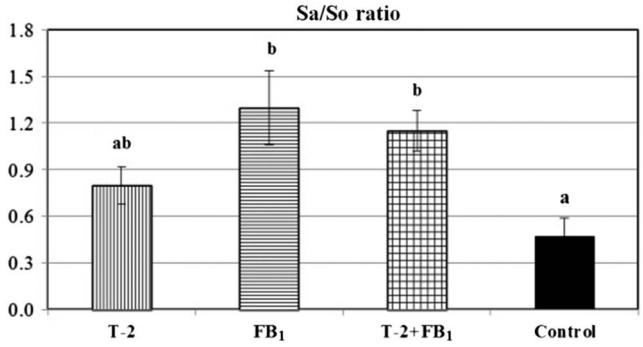

Fig. 2. The plasma sphinganine to sphingosine ratio after 4 weeks of feeding FB_1, T-2 toxin, and T-2+FB_1 compared to the control (mean ± SD)

Plasma sphinganine (Sa) to sphingosine (So) ratio

After 4 weeks of mycotoxin exposure, plasma sphinganine/sphingosine ratio was increased by FB_1 alone, but to a lesser extent, even in combination with T-2 toxin, while T-2 toxin alone did not influence the plasma Sa/So ratio (Fig. 2).

Discussion

Bodyweight and liver weight

The toxin exposure lasted for 28 days until the animals reached the approximate Hungarian conventional slaughter weight. T-2 alone or in combination (with FB_1) appeared as a growth depressing factor. Meanwhile, FB_1 was administered in a relatively high dose (10 mg/kg feed), as a single toxin, it had no detectable effect either on bodyweight or on liver weight compared to the control.

In contrast, T-2 toxin led to a marked growth depression, independently of the administration type (single toxin or co-occurring with FB_1). In a recent study (34), the <0.02 mg/kg bodyweight/day T-2 concentration value was defined as the no observed adverse effect level for rabbit bucks. This present treatment was exceeding this value (ca. 0.96 mg/kg bodyweight/day), leading to well-established compromised growth. The threefold increased liver weight (as in this study) is a well-known consequence even in acute T-2 toxicosis in rabbits, accompanied by

centrilobular dystrophy (34). The most remarkable pathological findings included enlarged, friable, pale, and yellowish liver with spot-like necrotic areas (unpublished data). The results clearly refer to the hypothesis that T-2 toxin, as a type A trichothecene with its characteristic hydroxyl and ester groups [thus being amphipathic (27)], exerts a direct hepatotoxic effect, unequivocally supporting the hypothesis that it – due to its molecular characteristics – effectively crosses the hepatocellular membrane (i.e., the amphipathic PLs) (10).

Fatty Acid Composition

Hepatic total phospholipids

In spite of the dramatically enlarged liver, T-2 toxin alone was less affecting the hepatic total PL fatty acyl chain composition. There was no single FA of which T-2 toxin clearly altered the proportion compared to any of the other (combined or single) toxin treatments. Altogether, one monounsaturated (C16:1 n7, palmitoleic acid) and one odd chain (C17:0, margaric acid) FA showed the altered proportion in the liver total PLs of the T-2 toxin group compared to the control. It was interesting that even on the calculated FA parameters, T-2 toxin only had a slight effect, not differing significantly from the control, even not in the UI value.

The only hint that FB_1 alters hepatic (microsomal) membrane lipid palmitoleic acid proportion has been published by Gelderblom et al. (17). These authors described a disturbed microsomal lipid metabolism with increased delta-9 desaturase activity (not proven in the hepatic total PLs in our study), but it has to be added that in the cited study, 100 mg/kg feed FB_1 was fed to rats (carcinogenic level). Interestingly, the inhibition of ceramide synthase by FB_1 was not reflected in the microsomal membrane lipids (17), even at this high-dose treatment.

As the first-level interpretation, for FB_1, identical results were found to that of T-2 toxin in the liver total PLs. However, compared to the rat microsomal membranes, FB_1 alone was not a very potent modulator/disruptor of hepatic total PL FA composition, neither inducing liver weight alteration.

In contrast, the combined application (T-2+FB_1) of the two mycotoxins compared to the control or even the individual toxin exposures, was "effective;" in that, this treatment reduced the C16:1 n7 proportion (but did not perturb delta-9 desaturation significantly!) and slight, but significant increase was found for arachidic acid, the UI and the ACL. In the multivariate statistical model, there was no interaction for ACL between the treatments (i.e., T-2 and FB_1), but for C16:1 n7, C20:0, and UI, the interaction (i.e., T-2 × FB_1) of the two toxins was significant at the level of $P < 0.001$. We thus suppose a synergism in these cases, the two fusariotoxins augmenting their common effects upon the latter FAs and unsaturation.

Comparing our results with the literature data, it must be emphasized that total hepatic PLs have not yet been analyzed as the targets of combined T-2+FB_1 toxins. It thus seems that at the moderately high levels, neither T-2 toxin nor FB_1 are hepatocellular membrane disruptors in the leporine model, when the exposure is relatively short (4 weeks), but T-2 toxin provides a rather effective translocation across the hepatocellular membrane with major parenchymal consequences.

Liver mitochondrial phospholipids

In isolated mitochondria, the PLs were more strongly modified by the mycotoxin exposure. Margaric acid (C17:0) proportion was decreased by both individual toxin treatments (T-2 and

FB_1) compared to the combined setting, while the combined treatment increased its proportion (but was not different from the control). Their statistical interaction in the multivariate model was significant ($P < 0.001$), suggesting a synergistic effect. In rabbit tissue membranes, margaric acid abundance is clearly dependent on two factors: odd chain FAs are exclusively synthesized by the gut microflora and odd chain saturated FAs are peripherally (position *sn*-1) located in PC and are primary targets of beta oxidation (being quasi-equivalent with paired chain saturated FAs in this respect), when hydrolyzed by the most abundant PLase A_2 (24). The enzymatic cleavage of an odd chain saturated FA at position *sn*-1 may be a part of the Lands mechanism (8, 21), a means to regulate membrane fluidity via the modification of the glycerolipid FA side chain combinations.

Oleic acid (C18:1 n9) proportion was significantly increased only in the FB_1 group, and in the combined treatment, the difference was not significant among individual treatments and control. In our earlier study (33), in the erythrocyte membrane, oleic acid provided the highest proportion in the combined treatment. This partly agrees with the results of Gelderblom et al. (17), who reported that FB_1 at 100 mg/kg feed induced delta-9 desaturase activation and increased membrane monounsaturation. It is important to add that we only fed 4 weeks of this concentration, but it is a necessary addition that rabbits perform coecotrophy (33), while rats perform coprophagy (17); this means that the exact toxin (or mycotoxin metabolite) concentration at the site of action (liver) is with the highest probability higher than that in the feed. The reason why FB_1 seems to be a more potent modulator of delta-9 desaturation may be due to the lower stability of T-2 toxin, which is very quickly enzymatically hydrolyzed to HT-2 (10). Meanwhile, FB_1 is a more stable molecule and there are further factors to be considered in rabbits. In monogastric animals (piglets), the accumulative absorption of FB_1 is only ca. 4% (15). However, as a result of coecotrophy in rabbit, the accumulative absorption may increase, while the hidden proportion, occurring in a matrix associated form may be liberated as well by the digestive enzymes (during the multiplied uptake via coecotrophy), leading ultimately to a higher toxin exposure than determined analytically from the food (34).

The biological background to decrease polyunsaturation in parallel to increase monounsaturation is a means to maintain membrane fluidity by decreasing the sensitivity toward lipid peroxidation (29). Although it was not directly measured from the liver, in the blood plasma, the concentration of reduced GSH was the highest in the control, significantly differing from all other toxin treatments, referring to an effective scavenger role against the emerging reactive oxygen substances (Fig. 1). The same alteration was found for GSHPx, but no alteration was detected in the plasma MDA concentration. MDA is formed in the terminal phase of lipid peroxidation from FAs having at least three double bonds, and is strongly cytotoxic (24). In this study, the extent of lipid peroxidation as assessed from the blood plasma was maintained at a relatively low level.

It thus seems that the cross-membrane transport has a more widespread effect, even detectable in the systemic circulation (4), as published for T-2 (broiler chickens) and for FB_1 in pigs (20). The role of GSHPx is to "up-oxidate" the reduced GSH moiety. Indeed, our results supported this fact, namely, GSHPx was likewise in a more saturated enzymatic phase compared to the control.

Mitochondrial membrane arachidonic acid (C20:4 n6) proportion decreased only in the FB_1 group compared to the control and the binary mixture treatment. In the biological membranes, primarily in PLs, arachidonic acid is the root component of the eicosanoids

liberated via the activity of phospholipase A_2. Although different cell organelles and lipid fractions react with FB_1-induced stimuli divergently, Burger et al. (6) reported supportive results in which FB_1 was liberated from the PC fraction, augmenting prostaglandin E2 series synthesis and inducing sustaining cell proliferation (2). The further fact to take into account is that impaired arachidonic acid metabolism, in particular its proportional decrease in the PC fraction has been implied as a growth stimulus for hepatocellular nodules (1).

Docosapentaenoic acid (C22:5 n3) proportion was decreased significantly in both separate treatments compared to the combined group, but not differing from the control. According to Abel et al.'s study (1), the proportional decrease of C20–C22 FAs is a factor to control/improve antioxidant properties. This is underpinned by the fact that although the pentaenoic FA proportion and the monounsaturation were increased, there was a ca. 4-fold difference in the peroxidation sensitivity between oleic and docosapentaenoic acids (18). In the calculated FA data, total monounsaturation (MUFA) was significantly higher in the FB_1 group compared to the control. A completely opposite result was found for the total polyunsaturation, while the total n3 FA proportion increased.

Summarizing the above findings in the mitochondrial FA composition, Burger et al. (6) reported that the modified FA profile may be coupled with an altered growth pattern of FB_1-intoxicated hepatocytes, including as well the perturbation of the ceramide synthesis. Indeed, we did not measure hepatocellular, but only plasma sphinganine/sphingosine ratio, it was found that FB_1 alone, but to a lesser extent, even in combination with T-2 was able to markedly increase this ratio, while T-2 did not influence the plasma Sa/So ratio (Fig. 2).

In conclusion, it was found that at the predefined mycotoxin concentration values in young rabbits were more prone toward T-2 toxin administration, which slightly alters hepatic plasma and mitochondrial membrane composition. When T-2 toxin was fed in combination with FB_1, synergistic effects were proven for saturated (C16:0, C17:0, C20:0) and monounsaturated FAs. All toxin treatments induced lipid peroxidation and activated the antioxidant defense (GSHPx). Fumonisin was, moreover, proven to perturb membrane ceramide homeostasis, even at a moderate concentration and at a relatively short exposure time.

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