

# Individual and combined effects of feed artificially contaminated with with fumonisin B<sub>1</sub> and T-2 toxin in weaned rabbits

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## RESEARCH ARTICLE

### Abstract

Co-contamination of feed and feed raw materials with two or more mycotoxins is frequently reported, however, only a few studies have investigated the combined effects of low doses of multiple mycotoxins. In the present study the individual and combined effects of 10 mg/kg fumonisin B<sub>1</sub> and 2 mg/kg T-2 toxin (n=12/group) were investigated in weaned rabbits. Mycotoxin contaminated feed was produced by adding fungal cultures of *Fusarium verticillioides* and *Fusarium sporotrichioides*, and fed to 40 days old rabbits during 28 days. Feed intake and body weight were measured weekly, serum biochemistry and antioxidant parameters on day 0, 14 and 28, while histopathological examination and comet assay were performed at the end of the experiment. T-2 exposure both alone and in combination resulted in 15-18% less final body weight compared to the control and FB<sub>1</sub> treatment. There was a significant increase in the concentration of plasma total protein, albumin, fructosamine and creatinine in the group treated with FB<sub>1</sub> compared to the control. The liver and the kidney of most animals treated with T-2 toxin, FB<sub>1</sub> and their combination showed pathological changes, occurring more frequent in animals exposed to both toxins. T-2 resulted in depletion of lymphocytes in the spleen. FB<sub>1</sub> and T-2 exerted synergistic effect on the antioxidant/oxidative parameters after 2 weeks of exposure, manifesting in less glutathione and glutathione peroxidase, while more malondialdehyde was produced. Both toxins caused DNA damage in the lymphocytes, which was more pronounced in the group fed T-2 toxin and T-2 combined with FB<sub>1</sub>, without additive or synergistic effects.

**Keywords:** fumonisin B<sub>1</sub>, T-2 toxin, interaction, rabbits

### 1. Introduction

Mycotoxins are fungal toxic secondary metabolites contaminating agricultural commodities and are present in most feeds and feed raw materials (Bennett and Klich, 2003). Mycotoxicosis in rabbits can cause severe reduction in feed intake (up to 60%), which delays growth, thus resulting in lower productivity and economic loss. This can be of great concern as the worldwide production and consumption of rabbit meat exceeds a million tons annually (Greco *et al.*, 2012).

As it is a common practice to use multiple grain sources in animal feeds, there is an increased risk of exposure to several mycotoxins at the same time (Grenier and Oswald, 2011). Multitoxin studies on animal feed reported that 75-100% of samples analysed contained more than one mycotoxin (Schatzmayr and Streit, 2013; Streit *et al.*, 2012). Feed contaminated with multiple mycotoxins – even below the limit values – could still cause adverse effects due to possible synergistic interactions between them (Speijers and Speijers, 2004). Although, co-occurrence of mycotoxins in animal feeds is very common, data on their combined toxicity is limited. It is difficult to predict the toxicity of co-occurring mycotoxins as it could differ qualitatively

and quantitatively from what would be predicted based upon their individual toxicity (Grenier and Oswald, 2011; Speijers and Speijers, 2004); hence it is necessary to carry out experiments to investigate interactions between different mycotoxins. Therefore, the aim of this study was to determine the individual and combined effects of fumonisin B<sub>1</sub> (FB<sub>1</sub>) and T-2 toxin (T-2) in rabbits.

The occurrence of mycotoxins in rabbit feed is high in some parts of the world, e.g. 77% of rabbit feed samples was reported to be contaminated with aflatoxins in India (Mohanamba *et al.*, 2007), while the occurrence of ochratoxin A was 78% in Argentina (Dalcerro *et al.*, 2002). In a survey in Argentina, feed for finishing rabbits was analysed for mycotoxin contamination (n=17). All samples contained both T-2 and fumonisins (also co-contamination with aflatoxin, deoxynivalenol, ochratoxin A and zearalenone was found). The average concentrations were 63 and 1,010 µg/kg, while the maximum levels measured were 130 and 6,000 µg/kg for T-2 and fumonisins, respectively (Greco *et al.*, 2012). In Europe, according to the scientific opinion of the EFSA Panel on Contaminants in the Food Chain (2014) T-2 and HT-2 toxin exposure corresponds to 7% of the LOAEL for rabbits, which is not of concern. In case of fumonisins the estimated occurrence level in feed is below 5 mg/kg (the guidance value; EC, 2006) and exposure is well below the LOAEL set for pigs and horses, considered as the most sensitive species to fumonisins. On the other hand according to the latest mycotoxin survey report from 2015 (<https://info.biomin.net>) 57 and 52% of feed samples were contaminated with T-2 and FB<sub>1</sub>, respectively, in Central Europe, which cannot be ignored.

FB<sub>1</sub> is the most prevalent and toxic among the fumonisins (EFSA, 2005). It is mainly found in maize and maize-based animal feeds (Soriano and Dragacci, 2004; Waskiewicz *et al.*, 2012) and it was present in all rabbit feed samples analysed by Greco *et al.* (2012). Fumonisins inhibit ceramide synthase leading to the accumulation of free sphinganine and sphingosine in the cellular membranes (Riley, 1996). FB<sub>1</sub> is nephrotoxic and hepatotoxic in rabbits (Gumprecht *et al.*, 1995). In the kidney mainly nephrosis of the proximal tubuli is observed, while the liver shows centrilobular lipid infiltration and necrosis. Acute toxicity of a single of FB<sub>1</sub> dose results in significantly altered serum biochemical parameters also indicating liver and kidney damage (Orsi *et al.*, 2009). According to Ewuola (2009) and Gbore and Akele (2010) FB<sub>1</sub> above 5.0 mg/kg in rabbit reduced growth performance, significantly altered haematological parameters and serum enzyme activities in the animals.

T-2 has only recently become subject to EU regulations (EC, 2013) although it is one of the most toxic trichothecene compounds (Schlatter, 2004). It was present in all investigated rabbit feed samples studied by Greco *et al.* (2012). The most frequent general toxic effects

characteristic to T-2 are reduced feed intake and weight gain, growth depression, gastrointestinal disturbances, dermal necrosis, impairment of the immune system and reproductive disorders (Schuhmacher-Wolz *et al.*, 2010). T-2 was thoroughly studied in rabbits by Fekete *et al.* (1989), who described emaciation, gastritis and necrosis in the lymphoid organs (intestinal mucosa, spleen and lymph nodes). Changes in haematological parameters, hepatocellular necrosis and tubulonephrosis have also been observed (Glávits *et al.*, 1989). Sublethal T-2 intake caused reproductive disorders both in female (Fekete and Huczenicza, 1993) and male rabbits (Kovács *et al.*, 2013). Although T-2 is one of the most toxic members of the trichothecene group as mentioned above, toxicity data of combined toxic effects with other mycotoxins are limited (Speijers and Speijers, 2004).

Individual and combined effects of FB<sub>1</sub> and T-2 were investigated in turkeys and broiler chicks and it was shown in both experiments that the consumption of both toxins had an additive effect on the body weight gain depression (Kubena *et al.*, 1995, 1997). Another study carried out by Szabó *et al.* (2014), investigating red blood cell Na<sup>+</sup>/K<sup>+</sup> ATPase activity in weaned rabbits, found antagonism between the two toxins. The present study investigates the individual and combined effects of subclinical doses of FB<sub>1</sub> and T-2 on feed intake, body weight, DNA damage, histopathology, serum biochemistry and antioxidant capacity in weaned rabbits. The toxins were provided by adding fungal cultures of *Fusarium verticillioides* strain MRC 826 and *Fusarium sporotrichioides* strain NRRL 3299 into the feed between 40 and 70 days of age, i.e. for 28 days.

## 2. Materials and methods

### Chemicals

All chemicals were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

### Experimental design

The experiment was carried out with 35-day old weaned Pannon white rabbits, caged individually. The experimental animals were randomly assigned to 4 groups, each group containing 12 individuals. Each group was fed the same commercially available rabbit feed for a week. From 42 days of age (experimental day 0) the feed of the experimental groups was supplemented as follows: Group 1 received the feed contaminated with 2 mg T-2/kg feed; Group 2 was fed with contaminated diet containing 10 mg FB<sub>1</sub>/kg feed; the diet of Group 3 was supplemented with 2 mg T-2 and 10 mg FB<sub>1</sub> per kg feed; while Group 4 was used as a control group and therefore the basal diet had no toxin supplementation. Feed and water were offered *ad libitum*. The feed intake of

the rabbits was recorded daily, while the body weight was measured twice a week.

Blood samplings from the marginal ear vein were performed at the end of the 2<sup>nd</sup> and 4<sup>th</sup> week of the experiment. Samples for clinical chemistry were taken into native tubes. Heparinized blood was used for comet assay. Plasma was obtained by centrifugation (Janetzky T23, VEB, Leipzig, Germany) at 900×g for 15 min, and was used for the determination of antioxidant parameters. All samples were stored deep-frozen (-27 °C) till analyses.

On the 28<sup>th</sup> day when the rabbits were 70 days old, 6 animals from each group were sacrificed by bleeding after sedation. Pathological examination was performed on the liver, kidney and spleen. The research protocol was reviewed by the Animal Use and Care Administrative Advisory Committee and approved by the Agricultural Administrative Authority (Protocol No. SOI/31/254-3/2013).

### Production of fumonisin B<sub>1</sub> and T-2 toxin

FB<sub>1</sub> and T-2 were produced by *F. verticillioides* strain MRC 826 and *F. sporotrichioides* strain NRRL 3299, respectively (Fodor *et al.*, 2006). The homogenised fungal cultures contained FB<sub>1</sub> at a concentration of 3,440 mg/kg and T-2 at 1,338 mg/kg. These fungal cultures were mixed into the basal feed of the experimental animals, so as to provide contaminated feeds with 2 mg T-2/kg feed and/or 10 mg FB<sub>1</sub>/kg feed, respectively. The final mycotoxin concentration of the control and the experimental feeds was determined with LC-MS (Shimadzu, Kyoto, Japan). The limit of detection (LOD) for FB<sub>1</sub> was 3 µg/kg, while for T-2 it was 10 µg/kg. The diet fed to the control group did not contain detectable amounts of T-2 and FB<sub>1</sub>. None of the diets contained detectable amounts of deoxynivalenol and zearalenone.

### Clinical-pathological analysis

The plasma total protein (TP), albumin (ALB), total cholesterol (TCHOL), triglyceride (TG), urea, fructosamine (FA), creatinine (CREA) concentrations, and the activity of creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALKP) were determined in a veterinary laboratory (Vet-med Laboratory, Budapest, Hungary), using a Roche Hitachi 912 Chemistry Analyzer (Hitachi, Tokyo, Japan) with commercial diagnostic kits (Diagnosticum Ltd., Budapest, Hungary).

### Determination of antioxidant parameters

Lipid peroxidation was determined by the quantification of malondialdehyde (MDA) levels using the 2-thiobarbituric acid method in blood plasma (Placer *et al.*, 1966). The

amount of reduced glutathione (GSH) was measured by the method of Sedlak and Lindsay (1968) and the activity of glutathione peroxidase (GSHPx) according to Lawrence and Burk (1978).

### Comet assay

50 µl heparinised blood was added to 1 ml 1% low melting agarose gel in Eppendorf tubes at 37 °C. The suspension was mixed gently and two drops (140 µl) were transferred to slides previously coated with 1% normal melting point agarose. The slides were covered with cover slips and allowed to set. The cover slips were then removed and cell membranes were lysed with lysis buffer solution (1% Triton X-100, 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, pH 10) for 1 h at 4 °C. Following the lysis the slides were placed in alkaline electrophoresis buffer (pH 13) in an electrophoresis tank (Cleaver Scientific Ltd., Warwickshire, UK) for 40 min at 4 °C, followed by electrophoresis at 25 V (300 mA) for 30 min at 4 °C. The slides were then placed in neutralising buffer (pH 7.5) and washed three times for 5 min, followed by a final wash in double distilled water for a further 5 min. The slides were then left to dry overnight and stained with ethidium bromide (30 µl) and covered with cover slips. For visualisation, an Optika fluorescent microscope (Ponteranica, Italy) was used where 200 cells per sample were scored. Manual scoring was carried out as described by Horvatovich *et al.* (2013). Comet were classified into scores of '0', '1', '2', '3' and '4' according to DNA damage and head/tail migration. Each comet was scored visually and assigned into an arbitrary unit from 0 to 4 depending on the relative intensity of DNA fluorescence in the tail.

### Histopathology

After registering the macroscopic pathological signs on the internal and external organs, liver, kidneys and spleen were stored in 10% neutrally buffered formalin and were embedded into paraffin. For light microscopic analysis microtome slides of 5 µm were prepared and stained with haematoxylin-eosin (HE). The histopathological analysis was performed according to the Act 9/2011 (03.30) of the Hungarian Ministry of Agriculture and Rural Development and was in accordance with the ethical guidelines of the OECD Good Laboratory Practice for Chemicals (OECD, 1997).

### Statistical analysis

Statistical analyses were carried out using SPSS 19 software (SPSS Inc., Chicago, IL, USA). Differences among groups were investigated using one-way ANOVA followed by post hoc Tukey's test for group-wise comparisons. Data obtained from the comet assay was not normally distributed; therefore the non-parametric chi-squared test was used to compare the different groups.



### Determination of type of interaction

The type of interaction was determined according to the characterisation of the different interactions between mycotoxins in the meta-analysis of Grenier and Oswald (2011). Synergistic effect was identified when effect of mycotoxin combination was greater than expected from the sum of the individual effects of the two toxins. Synergistic effect was determined also if one of the toxins did not display any effect, but the effect of T-2 and FB<sub>1</sub> in combination was greater than the effect of the other toxin alone. Additive effect means that the effect of combination can be calculated as the sum of the individual effects of the two toxins.

## 3. Results

### Body weight and feed consumption

Differences in body weight among the experimental groups are shown in Table 1. Rabbits fed with the diet complemented with T-2 alone and combined with FB<sub>1</sub> showed slightly reduced body weight compared to the group fed with diet containing only FB<sub>1</sub>, although these differences were not significant at any of the measurement events. On the last 2 weeks FB<sub>1</sub> treated rabbits were significantly

heavier compared to those fed T-2. When comparing body weight gain (0 to 28 days) of toxin treated rabbits to the control, T-2 resulted in reduction by 15 and 14%, alone and in combination, respectively.

In respect to feed intake (Table 2) there were no significant differences among the experimental groups, except in the first week of the experiment, when rabbits fed with diet containing FB<sub>1</sub> consumed significantly more feed than those fed with diet containing both toxins. Although differences were not significant, the effect of treatments was in accordance with body weight, namely T-2 and T-2+FB<sub>1</sub> consuming animals had the lowest feed intake.

### Blood chemical parameters

There were no significant differences among the experimental groups in the following parameters: TCHOL, TG, urea, AST, ALT, ALKP, CK, LDH. TP and ALB concentration increased due to FB<sub>1</sub> treatment compared to control, and reached the highest level in FB<sub>1</sub> group at both sampling dates (Table 3). FA concentration was also higher in FB<sub>1</sub> group compared to T-2 and control groups at the 2<sup>nd</sup> and 4<sup>th</sup> week, respectively. FB<sub>1</sub> treatment increased CREA level significantly, as compared to T-2 treatment and control at both sampling dates.

**Table 1. Mean body weights (g) and standard deviation of rabbits belonging to the different experimental groups (n=12/group).<sup>1,2</sup>**

Experimental day	Control	T-2	FB <sub>1</sub>	T-2 + FB <sub>1</sub>
0	948±62	948±80	949±73	948±76
7	1,541±206 <sup>ab</sup>	1,512±151 <sup>ab</sup>	1,648±111 <sup>b</sup>	1,420±184 <sup>a</sup>
14	1,905±182	1,732±175	1,961±141	1,728±149
21	2,165±211 <sup>ab</sup>	1,972±172 <sup>a</sup>	2,245±193 <sup>b</sup>	1,997±177 <sup>ab</sup>
28	2,523±231 <sup>ab</sup>	2,286±157 <sup>a</sup>	2,579±169 <sup>b</sup>	2,298±198 <sup>a</sup>
Body weight gain (0 to 28 d) (g)	1,575	1,338	1,630	1,350
Change to control		-15%	+3%	-14%
Change to FB <sub>1</sub>		-18%		-17%

<sup>1</sup> Different letters indicate significant differences among groups ( $P < 0.05$ ).

<sup>2</sup> T-2 = T-2 toxin; FB<sub>1</sub> = fumonisin B<sub>1</sub>.

**Table 2. Mean feed intake (g) and standard deviation of rabbits belonging to the different experimental groups (n=12/group).**

Experimental day	Control	T-2	FB <sub>1</sub>	T-2 + FB <sub>1</sub>
0-7	94.0 <sup>ab</sup> ±30.7	81.2 <sup>ab</sup> ±29.9	105.4 <sup>b</sup> ±35.4	65.4 <sup>a</sup> ±37.1
7-14	116.5±38.4	103.9±20.4	129.4±27.1	96.9±38.6
14-21	135.6±19.0	120.1±14.5	136.8±28.1	124.1±21.4
21-28	154.4±27.3	147.2±13.5	166.2±19.6	147.6±27.1

<sup>1</sup> Different letters indicate significant differences among groups ( $P < 0.05$ ).

<sup>2</sup> T-2 = T-2 toxin; FB<sub>1</sub> = fumonisin B<sub>1</sub>.

**Table 3. Comparison of blood chemical parameters among the four experimental groups (n=12/group) two and four weeks after the start of the experiment.<sup>1,2</sup>**

Parameters	Experimental day	Control	T-2	FB <sub>1</sub>	T-2 + FB <sub>1</sub> <sup>1</sup>
Total protein (g/l)	14	56.5±6.6 <sup>a</sup>	56.9±6.8 <sup>ab</sup>	64.3±6.4 <sup>b</sup>	57.1±7.2 <sup>ab</sup>
	28	55.0±5.8 <sup>a</sup>	55.3±4.9 <sup>a</sup>	63.0±3.7 <sup>b</sup>	55.7±2.1 <sup>a</sup>
Albumin (g/l)	14	35.0±2.7 <sup>a</sup>	36.3±3.9 <sup>ab</sup>	38.7±1.6 <sup>b</sup>	35.3±3.1 <sup>ab</sup>
	28	34.9±2.9 <sup>a</sup>	35.3±2.9 <sup>ab</sup>	38.7±1.5 <sup>b</sup>	35.0±0.9 <sup>a</sup>
Fructosamine (μmol/l)	14	175.2±18.0 <sup>ab</sup>	155.8±29.6 <sup>a</sup>	195.9±27.6 <sup>b</sup>	178.8±30.2 <sup>ab</sup>
	28	171.1±19.2 <sup>a</sup>	179.6±20.8 <sup>ab</sup>	202.6±18.5 <sup>b</sup>	179.1±9.4 <sup>ab</sup>
Creatinine (μmol/l)	14	73.3±4.9 <sup>a</sup>	74.3±14.7 <sup>a</sup>	98.0±11.1 <sup>b</sup>	99.8±8.9 <sup>b</sup>
	28	79.8±5.5 <sup>a</sup>	84.5±9.8 <sup>a</sup>	102.5±12.6 <sup>b</sup>	110.6±7.9 <sup>b</sup>

<sup>1</sup> Different letters indicate significant differences among groups ( $P<0.05$ ).

<sup>2</sup> T-2 = T-2 toxin; FB<sub>1</sub> = fumonisin B<sub>1</sub>.

### Antioxidant parameters

GSH concentration was significantly higher compared to the control after two weeks by the T-2 treatment, and significantly lower by the combination of T-2 and FB<sub>1</sub> (Table 4). After four weeks of treatment the GSH values of all groups treated with toxin were significantly lower compared to the control group. Very similarly, the group whose diet was supplemented with T-2 combined with FB<sub>1</sub> had significantly lower GSHPx levels after two weeks of treatment compared to the group whose diet was supplemented with T-2 alone. After four weeks of treatment the GSHPx activities of all groups treated with toxin were significantly lower compared to the control group. The combined effect of the two toxins resulted in significantly more MDA production after two weeks of exposure compared to the control and to the group treated with T-2 alone, but after four weeks of treatment there were no significant differences in MDA concentration among the toxin treated groups.

### Comet assay

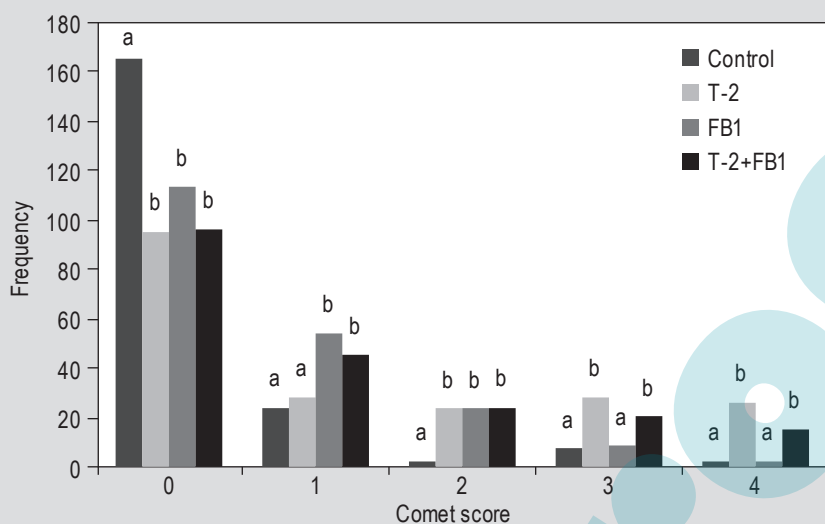
All toxin treatments resulted in significant reduction of the number of intact lymphocytes (comet score 0) compared to the control (Figure 1). Treatment with FB<sub>1</sub> and FB<sub>1</sub> combined with T-2 toxin resulted in a significant frequency increase of slightly damaged lymphocytes (comet score 1) compared to the control and to the group on T-2 toxin alone. All groups treated with toxin had significantly higher frequencies of category 2 (comet score 2) lymphocyte damage, as compared to the control. There was a significantly higher frequency of category 3 damaged lymphocytes (comet score 3) in the T-2 toxin combined groups, as in the control and to the pure FB<sub>1</sub> group. This was also the case in the frequency of extensive DNA damage (comet score 4) where once again there were significantly higher frequency of damage in the T-2 group alone and in combination with FB<sub>1</sub>, as compared to the control and to the group on FB<sub>1</sub> alone.

**Table 4. Comparison of blood antioxidant parameters among the four experimental groups (n=12/group), two and four weeks after the start of the experiment.<sup>1,2</sup>**

Parameters	Experimental day	Control	T-2	FB <sub>1</sub>	T-2 + FB <sub>1</sub>
GSH (μmol/g protein)	14	6.6±0.6 <sup>b</sup>	8.3±0.7 <sup>c</sup>	5.8±2.2 <sup>b</sup>	3.7±0.2 <sup>a</sup>
	28	6.1±0.8 <sup>b</sup>	5.4±0.1 <sup>a</sup>	4.9±0.2 <sup>a</sup>	5.4±0.5 <sup>a</sup>
GSHPx (E/g protein)	14	5.0±0.3 <sup>ab</sup>	5.2±0.3 <sup>b</sup>	4.8±0.3 <sup>ab</sup>	4.6±0.5 <sup>a</sup>
	28	4.3±0.5 <sup>b</sup>	3.9±0.4 <sup>a</sup>	3.9±0.2 <sup>a</sup>	3.9±0.3 <sup>a</sup>
MDA (μmol/ml)	14	10.8±1.8 <sup>ab</sup>	9.7±1.8 <sup>a</sup>	12.6±2.1 <sup>bc</sup>	14.4±1.7 <sup>c</sup>
	28	12.5±1.7	11.4±1.5	11.7±1.3	11.5±0.7

<sup>1</sup> Different letters indicate significant differences among groups ( $P<0.05$ ).

<sup>2</sup> T-2 = T-2 toxin; FB<sub>1</sub> = fumonisin B<sub>1</sub>; GSH = glutathione; GSHPx = glutathione peroxidase; MDA = malondialdehyde.



**Figure 1.** Differences in the frequencies of comet scores between the experimental groups. Comet scores 0-4 indicate the intensity of DNA damage (0 = no DNA damage, 4 = heavily damaged DNA). Frequency indicates the number of cells within the different comet score categories (200 cells per sample were scored). Letters indicate significant differences among groups ( $P < 0.001$ ).

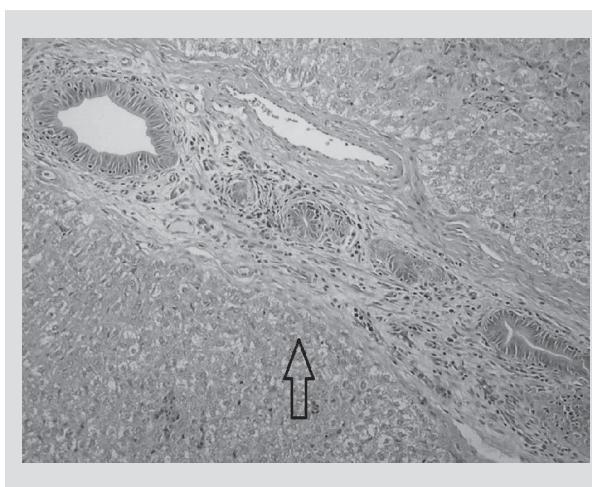
**Table 5.** Frequency of occurrence (%) of histopathological alterations in the liver, kidney and spleen of rabbits belonging to the four experimental groups (n=6/group).<sup>1</sup>

Organ	Alteration	Control	T-2	FB <sub>1</sub>	T-2 + FB <sub>1</sub>
Liver	Thickening of the bile duct walls	0	0	17	83
	Bile duct proliferation	0	33	33	83
	Fibrosis	0	67	67	100
Kidney	Vacuolisation of the tubular epithelial cells	0	67	67	100
	Detachment of tubular epithelial cells	0	67	67	83
	Fibrosis	0	0	17	83
Spleen	Depletion of lymphocytes	0	100	0	100

<sup>1</sup> T-2 = T-2 toxin; FB<sub>1</sub> = fumonisin B<sub>1</sub>.

## Histopathology

The macroscopic investigation of the different organs revealed only a few and slight alterations: centrolobular infiltration in the liver in four animals (two in FB<sub>1</sub> and two T-2+FB<sub>1</sub> rabbits) and pale kidney in the same four animals (Table 5). The most common histopathological alterations observed in the liver and kidney of the T-2 treated animals were liver fibrosis, bile duct proliferation, vacuolisation and detachment of the tubular epithelial cells in the kidney. Besides these, FB<sub>1</sub> caused thickening of the bile duct walls and fibrosis in the kidney. Only T-2 resulted in depletion of lymphocytes in the spleen in all toxin-exposed animals. The occurrence frequency of the above mentioned alterations was higher in those animals which were exposed to both toxins (Figure 2-4).



**Figure 2.** Proliferation of the connective tissue (fibrosis) and the wall of the bile ducts in the liver after combined exposure of T-2 and FB<sub>1</sub> (HE, ×200).



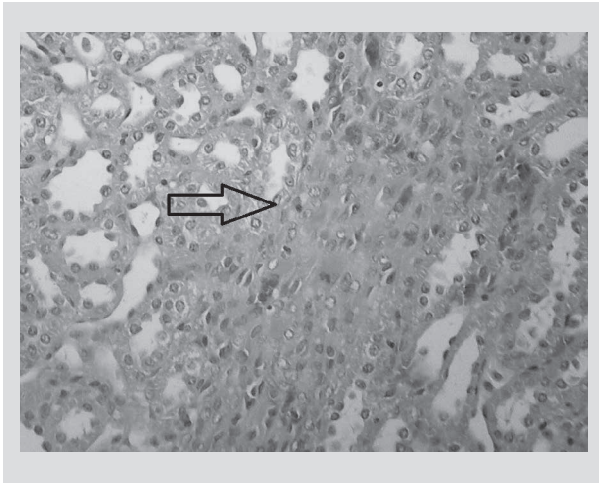


Figure 3. Proliferation of the intertubular connective fibres in the kidney of rabbits exposed to both T-2 and FB<sub>1</sub> (HE, ×400).

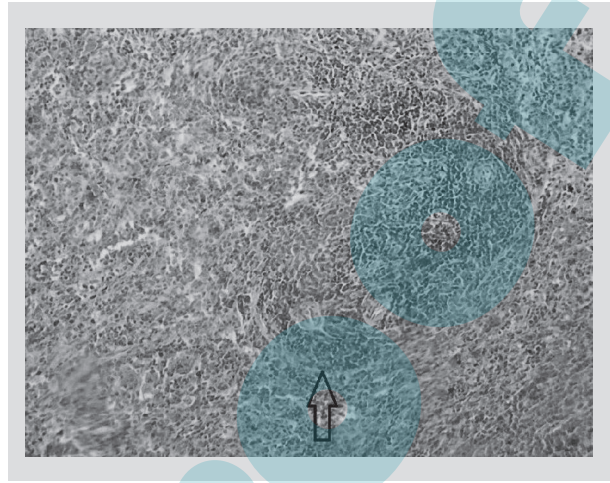


Figure 4. Depletion of lymphocytes (atrophy) observed in the Malpighi bodies of T-2 exposed rabbits' spleen (HE, ×200).

#### 4. Discussion and conclusion

Co-contamination of feed and feed raw materials by mycotoxins is frequently reported. Therefore, special attention needs to be paid on the combined effects of mycotoxins. While the effect of various doses of T-2 and FB<sub>1</sub> is widely studied in rabbits (Fekete and Huszenicza, 1993; Glávits *et al.*, 1989; Kovács *et al.*, 2013; Szilágyi *et al.*, 1994 for T-2 and Ewuola, 2009; Ewuola *et al.*, 2008; Orsi *et al.*, 2007, 2009 for FB<sub>1</sub>), their combined effects are not well documented. Therefore, this study investigated the effects of 10 mg/kg FB<sub>1</sub> and 2 mg/kg T-2 and their combination in feed on weaned Pannon white rabbits. The recommended maximum concentration of FB<sub>1</sub> in rabbit feed is 5 mg/kg (EC, 2006) which is half of the concentration used in the present study. In terms of T-2 no recommendations for animal feeds are available, only for cereal products for feed and compound feed (EC, 2013), which is 0.25 mg/kg. Less than 0.02 mg/kg body weight/day T-2 concentration was reported as the no observed adverse effect level (NOAEL) for rabbit bucks (Kovács *et al.*, 2013), which is again much lower compared to the concentration used in this study.

##### Production parameters and general health

In the present study the experimental animals did not show any clinical signs of disease. T-2 decreased feed intake (not significantly) which was not surprising, since feed refusal is the most well-known characteristic effect of trichothecene mycotoxins (Schuhmacher-Wolz *et al.*, 2010). As a result of this, body weight was less in T-2 treated animals during the whole period of exposure compared to control, while FB<sub>1</sub> fed rabbits grew more intensively. FB<sub>1</sub> did not decrease feed intake and weight gain; on the contrary, it caused a slight improvement in the production parameters. Ewuola *et al.* (2008), observed the effect of diets containing 12.3 and 24.5 mg/kg FB<sub>1</sub> for 5 weeks in male rabbits. While 12.3 mg/kg

FB<sub>1</sub> in feed did not have a negative effect on feed intake, the contamination of feed with 24.5 mg/kg FB<sub>1</sub> reduced weight gain (not significantly), dry matter intake (by 18%,  $P < 0.05$ ) and increased feed conversion ratio (by 16%,  $P < 0.05$ ). When rabbits were fed 1.5 mg FB<sub>1</sub>/kg body weight for 21 days, it had no effect on body weight (Orsi *et al.*, 2007). FB<sub>1</sub> decreased body weight only in extreme high concentration in rabbits, i.e. by 16%, when 630 mg/kg FB<sub>1</sub> was administered in feed by gavage for 7 days (Orsi *et al.*, 2009).

No interaction between the two toxins concerning body weight and feed intake could be detected except for the first week, when the two toxins in combination resulted in less feed consumption than FB<sub>1</sub> alone. These findings are different from that observed in poultry by Kubena *et al.* (1997), according to which the consumption of both toxins for 19 days in high doses (5 mg T-2 and 300 mg FB<sub>1</sub>/kg diet) had additive effect on the body weight gain depression. These results support the conclusion of Grenier and Oswald (2011), according to which interactions are influenced by several factors and may manifest from synergism to antagonism. These discrepancies can be explained by the different sensitivity of animal models to mycotoxins, age, sex, nutrition, time and dose of exposure, mode of administration of the toxin, etc.

##### Blood chemistry, liver, kidney and spleen damage

Although there was a significant increase in the concentration of TP, ALB and CREA in the group treated with FB<sub>1</sub> compared to the control, these levels were still within the physiological range (which is 54-75 g/l for TP, 27-50 g/l for ALB and 44-229 μmol/l for CREA, according to Harcourt-Brown, 2002). Although significant liver morphological damage could be demonstrated by histopathology, none of the liver enzymes (AST, ALT, GGT) showed increased activity in the serum. No effect of liver

damage was observed on clinical chemical parameters in case of binary exposure as well, despite the higher incidence of the pathomorphological findings. This suggests that hepatocytes were not affected by the toxins, T-2 and FB<sub>1</sub> caused mainly proliferation of the connective tissue fibres (thickening of the bile duct walls, bile duct proliferation, fibrosis). Dietary fumonisin in 12.3 and 24.5 mg/kg feed did not adversely affect serum enzyme activities probably because of the short exposure (Ewuola *et al.*, 2008). After a significantly higher FB<sub>1</sub> exposure (1.5 mg/kg body weight compared to the 0.65-0.85 mg/kg body weight exposure in our experiment) Orsi *et al.* (2007) found also only moderate vacuolar degeneration of toxin exposed rabbits' liver. In our previous study with rabbit bucks 0.05-0.2 mg/animal/day T-2 caused mild regressive alteration and centro-lobular infiltration in the liver, however, this adverse effect did not influence liver enzyme activities (Kovács *et al.*, 2013).

This was not the case for kidney injury. The increased level of creatinine indicated the nephrotoxic effect of FB<sub>1</sub>, which was supported by the histopathological findings revealing vacuolisation and detachment of tubular epithelial cells both in T-2 and FB<sub>1</sub> treated animals, and besides these, fibrosis in 17% of FB<sub>1</sub> exposed rabbits. The prevalence of kidney damages was higher when the rabbits fed both toxins, while creatinine concentration did not differ between FB<sub>1</sub> and T-2+FB<sub>1</sub> groups. A synergistic effect between the two toxins on the kidney can be presumed.

These findings are in accordance with previous observations, namely in rabbits the main target organ of FB<sub>1</sub> is the kidney, especially the epithelium of the proximal tubules (Gumprecht *et al.*, 1995) and the liver (Ewuola, 2009).

The increased TP and ALB within the physiological ranges may indicate metabolic alterations in the fumonisin treated animals. Some kind of metabolic effect of the toxin is supported also by the elevated fructosamine concentration in the blood serum. Fructosamine is formed via the binding of fructose to total serum protein, mostly albumin (glycated protein) and reflects mean blood glucose concentrations over the previous two to three weeks (Lee, 2015). It is useful in monitoring metabolic status of the animals, as a close retrospective correlation exists between FA and the 1 week earlier measured blood plasma glucose concentration in rabbits (Temesváry and Opperl, 2001). The probable metabolic effect of FB<sub>1</sub> and its influence on body weight gain should be further investigated.

The effect of T-2 on the spleen, e.g. depletion of lymphocytes from the Malpighi-bodies, narrowing of the T- and B-cell dependent zones without changes in the cytomorphology of lymphocytes and lymphoblasts reveals slight immunosuppression, which is typical for trichothecene mycotoxins (Gutleb *et al.*, 2002).

## Antioxidants and genotoxicity

FB<sub>1</sub> and T-2 exerted additive effect on the antioxidant parameters after 2 weeks of exposure, manifesting in less GSH and GSHPx, while more MDA (terminal-phase oxidation product) production. By the end of the experiment, i.e. after 4 weeks treatment only GSH and GSHPx activities were lower in the toxin treated groups. The 2<sup>nd</sup> week data clearly indicate oxidative damage induction, since GSH level decrease is generally associated with the emerging production of reactive oxygen species and the concomitant scavenging (and 'up-oxidation') of them by GSH. The role of GSHPx is to reduce back oxidised glutathione to GSH, thus, its lower activity may refer to a more intense substrate supply (Mary *et al.*, 2012). MDA is a cytotoxic end-product of lipid peroxidation, produced from fatty acids having at least three double bonds (Mead *et al.*, 1986). Since these are generally cellular membrane components, increased MDA production is generally a marker of membrane lipid oxidative damage (Szabó *et al.*, 2016, personal communication). However, the marked rate of lipid peroxidation, namely significantly higher MDA levels in the toxin fed groups was not present after 4 weeks, meaning a possible antioxidant adaptation mechanism.

Both toxins caused DNA damage indicated by the lower number of intact lymphocytes (comet score 0). DNA damage was more pronounced in the group fed T-2 and T-2 combined with FB<sub>1</sub>, compared to the control and to the group fed by FB<sub>1</sub>, indicated by the increase in the number of strongly damaged lymphocytes (comet scores 3 and 4). No additive or synergistic effects were observed of the two toxins.

The genotoxic nature of T-2 has been demonstrated in several *in vitro* and *in vivo* studies as cited in Van der Fels-Klerx and Stratakou (2010). Already after 24 h of a single dose of 0.5 mg/kg body weight T-2 for chicken, DNA damage of lymphocytes was detected by comet assay (Sokolovic *et al.*, 2007). T-2 induced DNA-fragmentation was successfully prevented by adding antioxidants indicating, that oxidative stress could be involved in DNA damage (Jaradat, 2006). DNA damage caused by T-2 can be due to free radical production by the toxin (Frankić *et al.*, 2008), as underpinned by the MDA and GSH results above.

FB<sub>1</sub> can also cause oxidative stress. It has been shown that subacute treatment with FB<sub>1</sub> (100 mg/kg FB<sub>1</sub> in feed fed for 21 days) had significant negative effect on oxidative stress parameters in the liver of broiler chicks. It increased hepatic thiobarbituric acid reactive substance (TBARS) levels and catalase activity, however the superoxide dismutase and GST level were not altered (Poersch *et al.*, 2014). Wang and Groopman (1999) reported that fumonisins are the only carcinogenic mycotoxins not having a direct DNA damage inducing effect, so genotoxicity is an indirect



consequence of the oxidative damage caused by the toxin. On the other hand, according to Galvano *et al.* (2002) FB<sub>1</sub> induced DNA damage without increased reactive oxygen species (ROS) production and may modulate some gene expression directly.

According to El Golli-Bennour and Bacha (2011) T-2 and FB<sub>1</sub> generate different amounts of ROS through their cellular mechanisms and they belong among the moderate oxidant mycotoxins. In the absence of oxidative stress these toxins are still reactive and may cause DNA damage.

## 5. Conclusions

The results of this study indicate that subclinical doses of FB<sub>1</sub> and T-2 ingested for four weeks had a negative effect on the health status of rabbits. Both toxins altered the antioxidant status, induced DNA damage and pathological changes in the liver and kidney. While pathological changes were more pronounced when the two toxins were fed together, lymphocyte DNA damage was not affected by the combination of these two toxins. Our results confirm the hepato- and nephrotoxic effect of FB<sub>1</sub> and T-2. The combined administration of the two toxins resulted in synergistic / additive effect on the antioxidant status, liver and kidney damage.

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