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Effects of Long-term Feeding of Graded Levels of T-2 Toxin-contaminated Diets on Performance, Some Lipid Peroxide and Glutathione Redox Status Parameters of Broiler Chickens

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The effect of two different contamination levels of T-2 toxin (1.5 or 3.4 mg/kg feed) was investigated in a 28-days feeding trial on body weight, relative weight of liver and spleen, and some lipid peroxidation and glutathione redox parameters of 14-days old broiler chicken. The results showed that T-2 toxin decreased significantly the body weight at both contamination levels and showed a dose-dependent tendency. Relative weight of liver increased till the end of the trial, while relative weight of spleen was lower at both samplings at lower level of T-2 toxin exposure. Initial phase of lipid peroxidation (conjugated dienes and trienes) was not detected in the liver, but as product of later phase, thiobarbituric acid reactive substances increased significantly, except in the liver. Glutathione content on day 14 was higher in liver homogenate as compared to the control at the lower T-2 toxin contamination level. On day 28 it was higher in blood plasma at the higher and in liver homogenate at both levels of T-2 contamination. Glutathione peroxidase activity on day 14 was significantly higher in liver and spleen homogenates as compared to the control at the lower level of T-2 toxin contamination. On day 28, significantly higher activity was found at both T-2 toxin contamination levels in liver homogenate, and at the lower contamination level in spleen homogenate as compared to the control.

The results revealed that T-2 toxin exposure initiates lipid peroxidation and activates the glutathione redox system as well, but the changes were irrespective of the dose- and partly duration of the exposure.

Key words: broiler, glutathione, glutathione peroxidase, lipid peroxidation, T-2 toxin

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Introduction

Cereals are the main components of complete feeds that may contaminated with mycotoxins, secondary metabolites of moulds. The presence of mycotoxins in feed cause loss of production and toxic effects in animals in a dose dependent manner (Diaz, 2005). Fusarium moulds may produce a number of mycotoxins with different chemical structures, depending on temperature and humidity in the environment (Wood, 1992). The presence and quantity of substrates in the grains (e.g. starch), and the presence of oxygen are needed for the growth of the mold (Sokolovic *et al*., 2008). T-2 toxin is one of the most frequently occuring trichothecene mycotoxin in temperate climate (Binder *et* al., 2007), which inhibits protein and DNA synthesis in eukaryotic cells (Holladay *et al*., 1995). Due to the inhibitory effect on protein synthesis, blood and lymphoid tissues are in particular sensitive to these mycotoxins (Ueno, 1983). T-2 toxin has an 12, 13-epoxy group in its chemical structure, which makes it a reactive compound. Poultry species are sensitive for this mycotoxin, and the T-2 toxin contamination of the feed above 1 mg/kg can result in economic loss, because of lower meat producion, as well as adverse effects on egg production, related to reduced feed intake and inhibited protein synthesis (Schuhmacher-Wolz *et al*., 2010).

Oxygen free radical formation and consequently lipid peroxidation increased in farm animals because of long-term exposure of trichothecenes in context of biochemical changes in cells, which affect the amount and/or activity of the biological antioxidant system, as well (Mézes *et al*., 1998; Surai *et al*., 2002). However, results published on the effects

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of T-2 toxin on lipid peroxidation are contradictious. T-2 toxin-induced oxidative stress was found *in vitro* in chondrocytes (He *et al*., 2012) and in human cervical cancer cells (Chaudhari *et al*., 2009a) and *in vivo* in mice (Chaudhari *et al*., 2009b), rats (Rizzo *et al*., 1994; Rocha *et al*., 2005) and broiler chickens (Mézes *et al*., 1998; Dvorska *et al*., 2007) in a dose dependent manner. Most of the previous studies also revealed that T-2 toxin has marked effect on the antioxidant status of animals (Atroshi *et al*., 2002, Surai, 2002) because of its pro-oxidant properties (Rizzo *et al*., 1994). However, in other studies neither the rate of lipid peroxidation nor antioxidant parameters showed significant alterations even at high T-2 dose (Frankic *et al*., 2006; Rezar *et al*., 2007). The possible causes of the above-mentioned differences are that T-2 toxin can be metabolised by the microsomal enzyme system in the liver, through the xenobiotic transformation, depending on the dose and the duration of the exposure (Ványi *et al*., 1989). Nevertheless, T-2 toxin may reduce the effectiveness of the liver microsomes through the lipid peroxidative processes (Guerre *et al*., 2000), which in turn also affects the activity of the xenobiotic transformation (Mézes *et al*., 1996).

During the evolution, an efficient enzymatic and non-enzymatic antioxidant defense system has evolved in the biological systems to protect the cells and cell organs against the damage of peroxidation (Davies, 1995). The chain reaction initiated by the reactive oxygen species is terminated by the scavenging with antioxidant molecules, such as reduced glutathione, or in enzymatic way, such as glutathione peroxidase (Davies, 1995). In addition to traditional quantitative parameters such as reduced body weight, biochemical parameters have been used as endpoints to determine oxidative stress and lipid peroxidation in mycotoxin studies. Glutathione is an essential component of the biological antioxidant defense and xenobiotic transforming systems, therefore enzymes of glutathione synthesis and metabolism and their catalytic activity play a significant role in the antioxidant defense and xenobiotic transformation. Hence, the measuring of antioxidant enzymes and antioxidant molecules provide a potential possibility to characterize the antioxidant status of the organism, and thus can be useful biomarker of oxidative stress. The initial stage of lipid peroxidation processes can be detected by the analysis of conjugated dienes and trienes (Pegg, 2001), while TBARS, which is a widely used endpoint of the lipid peroxidation can be examinated to determinate as a meta-stable end product of the process (Draper *et al*., 1993).

Our hypothesis, and thus, the purpose of the study was to investigate the long-term effect of different doses of T-2 toxin on lipid peroxidation and some glutathione redox parameters in broiler chickens.

Materials and Methods

Experimental Design

A total of 30 two-week old Cobb 540 broiler chickens was randomly assigned into three experimental groups with two replicates of 5 chickens in each. The feeding trial lasted for 28 days.

The basal diet was a commercial broiler feed (13.4 MJ/kg AME, 20% crude protein, 10% ether extract, 3.5% crude fibre, 35 mg/kg vitamin E and 0.25 mg/kg selenium). The nutrient content of the diet met the requirements for broiler chickens (Hungarian Feed Code, 2004). The basal diet (T-2 toxin content $<$0.02 mg/kg) was experimentally contaminated with T-2 toxin at concentrations of 1.5 and 3.4 mg/kg feed. The contamination level of T-2 toxin was 3- or 7-fold than tolerable level as proposed by Eriksen and Pettersson (2004), and 6 or 14-fold as the proposed maximum level of the European Commission (2013).

Production of Toxin and Determination of Mycotoxin Content in Feeds

T-2 toxin was produced experimentally on maize by *Fusarium sporotrichioides* strain NRRL 3229 (Agricultural Utilization Research, Peoria, IL, USA) according to the method of Fodor *et al.* (2006). T-2 toxin content of the diets was determined with HPLC method after immunoaffitiny clean up according to Trebstein *et al*. (2008).

Ethical Issues

The experiment was carried out according to the regulations of the Hungarian Animal Protection Act, in compliance with the EU rules. The experimental protocol was authorised by the Food Chain Safety and Animal Health Directorate of the Pest County Agricultural Office, under permission number XIV-1-001/1880-5/2011.

Sampling and Analytical Measurements

Average feed intake was measured weekly (n=2) in each group, therefore cannot evaluate statistically.

Individual body weight of birds was measured weekly, and relative weight of liver and spleen was measured on day 14 and day 28 of the trial.

Five randomly selected birds were exterminated from each group on days 14 and 28 of the trial. After cervical dislocation, blood samples were collected into EDTA-Na_2 containing tubes. Blood plasma and blood cells were separated by centrifugation (2,500×g, 20 min). Red blood cells (RBC) were haemolysed with 1:9 volume of redistilled water and stored at −18℃ until analysis. Liver and spleen samples were taken and stored at −18℃ until analysis. Liver and spleen homogenates were made with 9-fold cold (4℃) physiological saline (0.65% w/v NaCl).

Primary lipid oxidation products, conjugated dienes (CD) and trienes (CT) were measured by spectrometry at 232 nm (dienes) and 268 nm (trienes) according to AOAC (1984) in the liver. Determination of thiobarbituric acid reactive substances (TBARS) content was carried out in the native homogenate, while the other parameters were detemined in the 10,000×g supernatant fraction of the homogenate. TBARS content of blood plasma and RBC haemolysate was determined using the 2-thiobarbituric acid method according to Placer *et al*. (1966), while in liver and spleen homogenates according to Botsoglou *et al*. (1994) and expressed as malondialdehyde. Reduced glutathione (GSH) content of blood plasma, RBC haemolysate and 10,000×g supernatant fraction of liver and spleen homogenates was measured as

described by Sedlak and Lindsay (1968). Glutathione peroxidase (GPx) activity was determined according to Lawrence and Burk (1976). GSH content and GPx activity was expressed to protein content in the sample, which was determined in blood plasma and RBC haemolysate by the biuret method (Weichselbaum, 1948) or with Folin-phenol reagent in tissue homogenates (Lowry *et al*., 1951).

Statistical Analysis

Statistical evaulation of the data, treatment groups vs. control was performed using Fisher's Least Significance Difference post-hoc test of the one-way ANOVA after calculating the means and standard deviation with Statistica for Windows 4.5 (Statsoft, 1993) software.

Results

Body weigth of animals was significantly lower from day 14 of the trial and showed dose-dependent tendency (Table 1). Relative liver weight differed significantly on day 28, when it was higher as compared to the control, while relative weight of spleen was lower on day 28 at lower level (1.5 mg/kg) of T-2 toxin exposure (Table 1).

Initial phase of lipid peroxidation was not detected in the liver on day 14 or day 28 of the trial because the level of conjugated dienes and conjugated trienes did not differ significantly as compared to control (Table 2). Late-phase of the lipid peroxidation process, as measured by the concentration of TBARS increased significantly on day 14 in blood plasma, red blood cell haemolysate and spleen homogenate at the higher level (3.4 mg/kg) of T-2 toxin exposure, while on day 28 only in blood plasma at the same T-2 toxin contamination level (Table 3). GSH content on day 14 differed only in liver homogenate as compared to the control where it was higher at the 1.5 mg/kg T-2 toxin contamination level. At the end of the trial, on day 28, it was higher in blood plasma at the 3.4 mg/kg T-2 toxin contamination level and in liver homogenate at both levels of T-2 contamination (1.5 and 3.4 mg/kg), but did not differ in red blood cell haemolysate and spleen homogenate (Table 4). GPx activity on day 14 was significantly lower in red blood cell haemolysate and higher in liver homogenates as compared to the control at the lower level (1.5 mg/kg) of T-2 toxin contamination. On day 28 significantly higher GPx activity was found at both T-2 toxin contamination levels in liver homogenate, and at the lower contamination level in spleen homogenate as compared to the control (Table 5).

Discussion

The results of present study showed that T-2 toxin exposure caused lower body weight, as consequence of lower feed intake, which support its well-known growth depression effect between 2 to 5 mg/kg feed (Schuhmacher-Wolz *et al*., 2010). Relative weight of liver increased significantly as effect of T-2 toxin at day 28, which is different than the results of Wyatt *et al* (1973) who did not found changes in

Table 1. **Effect of T-2 toxin contaminated feed on body weight and relative weight of liver and spleen of broiler chickens**

		T-2 toxin concentration (mg/kg)			
		0	1.5	3.4	*p*-value
Body weight	day 0	335.67±45.79	333.33±52.60	325.00±61.77	$p=0.356$
(g)	day 7	674.02±89.73^{a}	552.00±77.39^{b}	551.92±95.38^{b}	$p=0.032$
	day 14	1125.35±151.72^{a}	882.67±164.12^{b}	844.23±132.02^{b}	$p=0.001$
	day 21	1719.06±226.28^{a}	1188.75±319.80^{b}	1164.29±102.32^{b}	$p=0.001$
	day 28	2113.13±212.55^{a}	1425.00±94.74^{b}	1553.75±432.47^{b}	$p=0.001$
Liver weight	day 14	2.12±0.28	2.36±0.29	2.12±0.25	$p=0.310$
(g/100 g BW)	day 28	1.80±0.15^{a}	2.12±0.23^{b}	1.91±0.32^{ab}	$p=0.026$
Spleen weight	day 14	0.09±0.02	0.07±0.02	0.10±0.04	$p=0.307$
(g/100 g BW)	day 28	0.10±0.03^{a}	0.06±0.01^{b}	0.08±0.02^{ab}	$p=0.031$

Values are mean±SD, and those with different superscripts in the same row are significantly different

Table 2. **Effect of T-2 toxin contaminated feed on conjugated diene (CD) and conjugated triene (CT) levels of liver**

		T-2 toxin concentration (mg/kg)			
		0	1.5	3.4	*p*-value
CD	day 14	0.54±0.04	0.57±0.06	0.52±0.08	$p=0.533$
($A_{232\,nm}$)	day 28	0.56±0.04	0.55±0.11	0.55±0.04	$p=0.783$
CT	day 14	0.13±0.01	0.13±0.01	0.14±0.01	$p=0.794$
($A_{268\,nm}$)	day 28	0.13±0.01	0.13±0.02	0.13±0.01	$p=0.826$

Values are mean±SD ($n=5$ each)

Table 3. **Effect of T-2 toxin contaminated feed on thiobarbituric acid reactive substances concentration**

		T-2 toxin concentration (mg/kg)			
		0	1.5	3.4	*p*-value
Blood plasma	day 14	9.03±1.83[a]	9.27±1.44[ab]	17.15±7.16[b]	$p=0.041$
(μmol/L)	day 28	8.71±1.64	9.11±1.58	9.35±1.89	$p=0.525$
RBC haemolysate	day 14	11.31±1.39[a]	11.72±1.25[ab]	9.60±1.21[b]	$p=0.034$
(μmol/L)	day 28	11.43±2.22	11.73±0.98	12.68±1.31	$p=0.176$
Liver homogenate	day 14	8.02±3.51	8.05±1.58	8.78±1.36	$p=0.983$
(μmol/g)	day 28	10.14±1.49	9.96±1.76	11.71±2.94	$p=0.216$
Spleen homogenate	day 14	8.50±1.47[a]	8.74±0.94[ab]	10.28±1.66[b]	$p=0.039$
(μmol/g)	day 28	13.09±3.20	12.79±2.66	12.21±1.79	$p=0.568$

Values are mean±SD ($n=5$ each), and those with different superscripts in the same row are significantly different

Table 4. **Effect of T-2 toxin contaminated feed on reduced glutathione (GSH) concentration**

		T-2 toxin concentration (mg/kg)			
		0	1.5	3.4	*p*-value
Blood plasma	day 14	12.57±1.07	13.36±0.47	13.12±1.19	$p=0.154$
(μmol/g protein)	day 28	13.12±1.32[a]	13.35±0.83[ab]	15.47±2.69[b]	$p=0.030$
RBC haemolysate	day 14	13.04±1.47	11.53±2.61	12.20±1.87	$p=0.212$
(μmol/g protein)	day 28	12.03±1.87	12.98±1.07	13.35±2.24	$p=0.202$
Liver homogenate	day 14	2.23±0.34[a]	2.90±0.42[b]	2.59±0.40[ab]	$p=0.007$
(μmol/g protein)	day 28	2.37±0.30[a]	3.69±0.84[b]	3.27±0.72[b]	$p=0.036$
Spleen homogenate	day 14	1.20±0.23	1.32±0.11	1.23±0.16	$p=0.253$
(μmol/g protein)	day 28	1.07±0.17	1.13±0.11	1.02±0.26	$p=0.567$

Values are mean±SD ($n=5$ each), and those with different superscripts in the same row are significantly different

Table 5. **Effect of T-2 toxin contaminated feed on glutathione peroxidase activity**

		T-2 toxin concentration (mg/kg)			
		0	1.5	3.4	*p*-value
Blood plasma	day 14	11.54±0.63	11.97±0.37	11.82±0.96	$p=0.481$
(U/g protein)	day 28	12.65±1.58	12.74±0.90	12.86±2.50	$p=0.833$
RBC haemolysate	day 14	7.34±1.05[a]	6.36±0.91[b]	7.05±0.33[ab]	$p=0.049$
(U/g protein)	day 28	7.02±0.64	7.55±0.70	7.65±1.54	$p=0.295$
Liver homogenate	day 14	1.98±0.45[a]	2.72±0.14[b]	2.03±0.76[ab]	$p=0.026$
(U/g protein)	day 28	1.87±0.19[a]	2.44±0.23[b]	2.47±0.73[b]	$p=0.033$
Spleen homogenate	day 14	1.19±0.11	1.32±0.28	1.08±0.04	$p=0.238$
(U/g protein)	day 28	1.34±0.09[a]	1.48±0.16[b]	1.30±0.09[ab]	$p=0.048$

Values are mean±SD ($n=5$ each), and those with different superscripts in the same row are significantly different

relative weight of liver. On the contrary, relative weight of spleen reduced, which may be explained with the increase of apoptotic cells in spleen as effect of feeding T-2 toxin contaminated diet (Venkatesh *et al*., 2005). Apoptosis of spleen cells can cause detrimental effect of the immune response because spleen is a lymphatic organ that plays a fundamental role in protecting the body from invading pathogens (Jeuirssen, 1991). This suggest with those results that exposure of chicken to T-2 toxin caused increased mortality by *Salmonella* infection (Ziprin and Elissalde, 1990) and lower antibody titres after vaccination against Newcastle disease (Weber *et al*., 2006).

Conjugated dienes and trienes are the early markers of lipid peroxidation (Sugiyama, 1994). In the present study,

there were no significant changes in their level in the liver, which means, that initial steps of lipid peroxidation occurred earlier than the first sampling on day 14. TBARS content, as end-product of lipid peroxidation, did not change significantly at the lower level (1.5 mg/kg feed) of T-2 toxin contamination, and also not in the liver at both (1.5 or 3.4 mg/kg feed) contamination levels. This result may be explaining those previous results where significant differences in TBARS concentration in the liver were found only at higher dose of exposure (Dvorska *et al*., 2007). This may cause by the activation of antioxidant defense after T-2 toxin exposure, which eliminate oxygen free radicals effectively. The results suggest that at higher dose of T-2 toxin exposure the antioxidant system is not able to eliminate free radicals, therefore the process can be terminated. However, at the higher level of contamination (3.4 mg T-2 toxin/kg feed) TBARS content increased not only in the liver and spleen but also in blood plasma, which may be explained by the route of excretion of TBARS from liver through blood circulation to urine (Moser *et al*., 1993). On the contrary, red blood cell haemolysate showed significantly lower TBARS content at the same sampling. This result is contradictory as described by Rizzo *et al*. (1992) who found membrane lipid peroxidation in rat red blood cells in *in vivo* and *in vitro* systems, but may be explained with the difference of fatty acid composition, and therefore different sensitivity of mammalian and poultry red blood cell membrane to oxidative damage.

GSH content changed significantly in blood plasma and liver homogenate. Blood plasma reflects the increased GSH synthesis in the liver, as its main site (Shi *et al*., 1996), but it increased even at lower level of contamination and earlier in liver, than in plasma. GSH synthesis regulated, among other factors, by the gene expression of g-glutamyl-cysteine-synthase, which is activated by oxidative stress (Rahman and Macnee, 2000). This result may be supported by the not significant changes in liver TBARS content, because the T-2 toxin-induced oxidative stress possibly activates the antioxidant response, in this case glutathione synthesis through the activation of gene expression of glutathione synthetising enzymes.

GPx activity did not change significantly in blood plasma, but it was lower than the control at the lower level of contamination (1.5 mg/kg feed) at day 14 in red blood cell haemolysate. This result can be explained with those findings that antioxidant defense of red blood cells depends only partly on GPx but also on catalase (Nicholls, 1972). In GPx acticity in liver and spleen homogenates increased in both tissues at lower level of contamination on day 14 and at both levels on day 28 in liver. These results support our hypothesis that T-2 toxin exposure caused oxygen free radical formation and oxidative stress. The effect of T-2 toxin on oxidative stress possibly mediated through a nuclear factor (Nrf2) which constitutes the main oxidative stress response and drives the transcription of genes involved in glutathione synthesis and recycling (Jennings *et al*., 2013) , including glutatione peroxidase (Köhle and Bock, 2007). T-2 toxin down-regulates the Nrf2 expression *in vivo* in mice brain (Chaudhary and Rao, 2010), and up- or down-regulates some glutathione recycling enzymes expression as was found in a proteomic analysis *in vitro* in primary chicken hepatocytes (Mu *et al*., 2013). However, according to our knowledge the exact mechanism of T-2 toxin on oxidative stress mechanism is not yet known.

In conclusion, the results of present study showed that T-2 toxin may be involved in the generation of free radicals in broiler chicken tissues, but the changes are not dose-dependent, however they depend on the period of exposure. The results also support our hypothesis that antioxidant defense system activated as early event of T-2 toxin exposure, therefore it decreased the rate of lipid peroxidation, but was unable to eliminate other effects of T-2 toxin, such as lowering the growth rate.

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