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SHORT-TERM EFFECTS OF T-2 TOXIN OR DEOXYNIVALENOL ON GLUTATHIONE STATUS AND EXPRESSION OF ITS REGULATORY GENES IN CHICKEN

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Short-term (48-hour) effects of 3.74/1.26 mg kg^{-1} T-2/HT-2 toxin or 16.12 mg kg^{-1} DON in feed were investigated in the liver of three-week-old cockerels (body weight: 749.60 ± 90.98 g). Markers of lipid peroxidation showed no significant changes. At hour 24, glutathione content in the T-2/HT-2 toxin group was significantly higher than in the control. Glutathione peroxidase activity was significantly higher than the control at hour 24 in the T-2/H-2 toxin group and at hour 48 in the DON group. In the DON group, expression of the glutathione peroxidase 4 gene (*GPX4*) was significantly lower than in the control at hours 12 and 14, and higher at hour 48. Expression of the glutathione reductase gene (*GSR*) was significantly lower than in the control at hour 12 in the T-2/HT-2 toxin group, and at hours 12, 24 and 48 in the DON group. However, at hour 36 higher *GSR* expression was measured in the DON group. Due to the effect of both trichothecenes, expression of the glutathione synthetase gene (*GSS*) was significantly lower than in the control at hours 24 and 48. In conclusion, T-2/HT-2 toxin and DON had a moderate short-term effect on free radical formation. T-2/HT-2 toxin induced more pronounced activation of the glutathione redox system than did DON.

Key words: Chicken, deoxynivalenol, glutathione peroxidase, *gpx* genes, T-2/HT-2 toxin

Poultry feeds are often contaminated with *Fusarium* mycotoxins. 'Type A' trichothecene T-2 toxin and its metabolite HT-2 toxin, or 'type B' trichothecene

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deoxynivalenol (DON) are frequent mycotoxins in temperate climates (Binder et al., 2007). Trichothecenes present in feed cause reduced production (Grabarevic et al., 1992) and exert toxic effects, among others causing histopathological changes in the liver of chickens (Konjevi et al., 2004).

Poultry species are more tolerant to DON than other farm animals (Awad et al., 2008). Reviewing the literature on the effects of DON, Dänicke et al. (2001) came to the conclusion that only concentrations beyond 5 mg kg^{-1} diet cause detrimental effects. This relatively high tolerance of poultry is possibly due to the high rate of de-epoxidation of the 12,13-epoxide group of DON by the gut microbiota before absorption (Awad et al., 2008) and the consequent low overall rate of absorption from the small intestine (Greiner and Applegate, 2013). The T-2 and HT-2 toxin tolerance of chicken is much lower, about 0.5 mg kg^{-1} feed (Eriksen and Pettersson, 2004).

The results of previous studies show that the effects of T-2 toxin on lipid peroxidation and the glutathione redox system depend on the applied dose and the duration of exposure (Hoechler and Marquardt, 1996). Low-dose and long-term exposure resulted in significantly increased lipid peroxidation and a decreased amount and activity of the glutathione redox system in chicken (Mézes et al., 1998), but in other studies with higher doses and shorter exposures neither the rate of lipid peroxidation nor the antioxidant parameters showed significant alterations (Frankic et al., 2006; Rezar et al., 2007). The possible cause of the above-mentioned differences is that T-2 toxin can be metabolised via microsomal xenobiotic transformation in the liver, depending on the dose and duration of the exposure (Ványi et al., 1989). Nevertheless, T-2 toxin may reduce the effectiveness of microsomal xenobiotic transformation through a dose-dependent inhibition of the protein expression of cytochrome P450 enzymes and can induce lipid peroxidation in the microsomes (Guerre et al., 2000). DON also caused dose-dependent oxidative stress in a long-term study with poultry (Awad et al., 2014), while another research revealed that a high dose of DON (10 mg kg^{-1} feed) did not induce lipid peroxidation and had no effect on glutathione peroxidase activity (Frankic et al., 2006). In a short-term study, DON consumption was found to induce phase I and II liver biotransformation enzymes (Gouze et al., 2005). However, the exact mechanism of hepatotoxicity of DON *in vivo* or *in vitro* is not known yet (Peng et al., 2017).

Although oxidative stress induced by trichothecene mycotoxins has been reported in several studies, there is a scarcity of information regarding the time and sequence of induction.

The purpose of the present study was to investigate the short-term effect of T-2/HT-2 toxin or DON exposure on lipid peroxidation, amount/activity of the glutathione redox system and the sequence of changes in the expression of glutathione-related genes in broiler chicken. Short-term exposure was used for investigating the primary and direct effects of mycotoxins, while long-term studies

were done to study the potentially modified responses to DON or T-2/HT-2 due to possible adaptation of the xenobiotic transforming system to mycotoxin exposure or liver damage, which impairs the antioxidant defence.

Materials and methods

Experimental design

A total of 72 three-week-old Cobb 540 cockerels (body weight: 749.60 ± 90.98 g) were randomly assigned into experimental groups of 24 birds each. The short-term trial lasted for 48 h, and started after 12 h of feed deprivation. The basal diet was a commercial broiler feed (13.4 MJ kg^{-1} AME, 20% crude protein, 10% ether extract, 3.5% crude fibre, 35 mg kg^{-1} vitamin E and 0.25 mg kg^{-1} selenium). The nutrient content of the diet met the requirements of broiler chickens (Hungarian Feed Code, 2004). Measured mycotoxin concentrations of the commercial diet (1 kg) were: T-2 toxin: < 0.10 mg; HT-2 toxin: < 0.10 mg; DON: 0.25 mg, and the experimentally contaminated diets contained (1 kg): 3.74 mg T-2 toxin and 1.26 mg HT-2 toxin or 16.12 mg DON.

The mycotoxin doses were higher (20 times for T-2/HT-2 toxin and 3.22 times for DON) than the limits stated in the relevant EU proposals, where the maximum allowed value in complete feed is 0.25 mg T-2 + HT-2 toxin kg^{-1} (2013/165/EU), and 5 mg DON kg^{-1} (2006/576/EC).

Production of mycotoxins and experimentally contaminated feeds, mycotoxin analyses

DON was produced by *Fusarium graminearum* (NRRL 5883) and T-2/HT-2 toxin by *Fusarium sporotrichioides* (NRRL 3299) strains on corn substrate according to Fodor et al. (2006). The mycotoxin content of the experimentally infected corn substrate was 16,324 mg kg^{-1} DON, 946 mg kg^{-1} T-2 toxin and 103 mg kg^{-1} HT-2 toxin. For the mycotoxin-treated groups a total of 4.5 kg control feed was contaminated by adding 5 g of the DON-containing and 22 g of the T-2/HT-2 toxin containing corn substrate, respectively.

DON content of the feed was determined according to Pussemier et al. (2006), and T-2 and HT-2 concentration was assayed by the method of Trebstein et al. (2008) using HPLC after immunoaffinity cleanup.

Ethical issues

The experiment was carried out according to the Hungarian Animal Protection Act, in compliance with the relevant EU rules. The experimental protocol was authorised by the Food Chain Safety and Animal Health Directorate of the Pest County Agricultural Office of Hungary (permission number XIV-1-001/1880-5/2012).

Sampling and measurements

The body weight of all birds was measured at the start of the trial, while at samplings at 12, 24, 36 and 48 h of the trial six randomly selected birds from each group were weighed and sampled. Average individual feed intake was calculated from the feed intake of each experimental group, and mycotoxin intake was calculated based on the measured mycotoxin content of the diets.

At the above-mentioned samplings six birds from each group were euthanised. After cervical dislocation, liver samples were collected for biochemical analyses and stored at –70 °C until analysis. For gene expression analyses liver samples were taken into liquid nitrogen and stored at –80 °C until analysis. For biochemical and genetic analyses liver homogenates were made with a nine-fold volume of cold (4 °C) physiological saline (0.65% w/v NaCl).

Conjugated dienes (CD) and trienes (CT), the primary products of lipid peroxidation, were measured according to AOAC (1984). Determination of thiobarbituric reactive substances (TBARS) content was carried out in the native liver homogenates, while the other parameters were determined in their 10,000 × *g* supernatant fraction. TBARS content was determined according to Botsoglou et al. (1994). The concentration of TBARS was calculated using standard curves of increasing concentrations of 1,1,3,3-tetraethoxypropane (Fluka, Buchs), and expressed as malondialdehyde. Reduced glutathione (GSH) content of the 10,000 × *g* supernatant fraction of liver 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 were expressed to protein content of the 10,000 × *g* supernatant fraction of liver homogenates, which was determined by the Folin phenol reagent (Lowry et al., 1951).

RNA isolation, reverse transcription and qPCR

Total RNA was purified with Trizol reagent (Molecular Research Centre, Cincinnati) with Phase Lock Gel (5Prime GmbH, Hamburg) from 5–10 mg liver homogenate, according to the manufacturer's instructions. To avoid genomic DNA contamination, RNA isolates were treated with DNase I (Qiagen, Germantown) according to the supplier's protocol. The quantity and integrity of total RNA were verified with agarose gel electrophoresis [1.5% in Tris-Borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA), pH 8.0] and with NanoPhotometer (Implen, Munich) measurement. All samples with ratios of absorption 260:280 nm higher than 2.0 were accepted. The cDNA was produced with RevertAID Reverse transcriptase and random nanomer primer from 1 μg of total RNA according the recommended protocol.

The primers for the quantification (Table 1) of the mRNA transcriptional levels of *GPX4*, *GSS, GSR* and endogenous control glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) genes were designed with Primer Express 3.0.1

(Thermo Fisher Scientific, San Jose). *GAPDH* does not have any known interaction with oxidative stress or mycotoxins and was used as an internal control gene in several other studies with mycotoxins in broiler chicken (Awad et al., 2011*b*; Yang et al., 2016).

Table 1

Primers used for the quantification of the mRNA transcriptional levels of the target and endogenous control (GAPDH) genes

Gene	Forward primer	Reverse primer
GAPDH	TGACCTGCCGTCTGGAGAAA	TGTGTATCCTAGGATGCCCTTCAG
GPX4	AGTGCCATCAAGTGGAACTTCAC	TTCAAGGCAGGCCGTCAT
GSS	GTACTCACTGGATGTGGGTGAAGA	CGGCTCGATCTTGTCCATCAG
GSR	CCACCAGAAAGGGGATCTACG	ACAGAGATGGCTTCATCTTCAGTG

The real-time PCR was carried out in duplexes (*GAPDH* and one target gene) using MGB TaqMan probes (Thermo Fisher Scientific, San Jose) with pooled cDNA template. The pools were formed from equal (100 ng) amounts of cDNA per 6 birds of each sampled group at each sampling point of treatment. For the qPCR, Maxima Probe qPCR Master Mix (Thermo Fisher Scientific, San Jose) and Step One Plus™ Real Time PCR systems (Applied Biosystems, Foster City) were used. The reaction mix contained 2.5 mM $MgCl_2$ and 5 ng cDNA as well as primers and probes of the target and endogenous control genes (Tables 1 and 2) in 12.5 µL final volume per reaction (Table 2).

Table 2

Final concentration of the primer and probes in the duplex real-time PCR reactions

Duplex	GAPDH primer (pM/µl)	GAPDH probe (pM/µl)	Target gene primer (pM/µl)	Target gene probe (pM/µl)
GAPDH and GPx4	2.4	1.3	2.9	2.1
GAPDH and GSS	0.66	0.66	3.96	3.3
GAPDH and GSR	0.66	0.66	3.96	3.3

The PCR profile was 95 °C for 10 min for pre-amplification denaturation, followed by 95 °C 15 sec, 58 °C 30 sec and 72 °C 30 sec for 45 cycles and 72 °C 2 min final elongation; VIC and FAM signals were detected at the end of the extension period. The amplified products were verified with gel electrophoresis.

The relative expression level of the target genes (*GPX4, GSS* and *GSR*) and endogenous housekeeping control gene, *GAPDH*, was determined by StepOne™/StepOnePlus™ Software v2.2 (Applied Biosystems, Foster City) using comparative Ct method. The delta Ct (ΔCt), delta-delta Ct (ΔΔCt) and rela-

tive quantification (RQ = 2ˆ-ΔΔCt) values were calculated with the formula described by Livak and Schmittgen (2001).

Statistical methods

Statistical analysis of the data (one-way analysis of variance by Student-Newman-Keuls *post-hoc* test, calculation of means and standard deviations) was performed by MedCalc for Windows, version 16.4 (MedCalc Software, Ostend).

Results

There was no mortality during the trial, and no clinical signs of toxicity were observed. Body weight as well as absolute and relative liver weight were similar in each group (data not shown). The calculated feed intake did not show marked differences among the experimental groups in the same periods of the trial. However, lower feed intake was found in all groups in the period between 12 and 24 h, and a much higher intake was noted between 36 and 48 h (Table 3). Changes of feed intake resulted in marked changes in the calculated mycotoxin intake of the treated groups as well in the different periods of the trial (Table 3).

Table 3

Calculated feed and mycotoxin intake of broiler chickens

Calculated feed (g/bird) and mycotoxin (mg/bird) intake	Control group	T-2/HT-2 toxin-treated group	DON-treated group
Feed	0–12 h	68.13	65.42
	12–24 h	42.78	46.39
	24–36 h	75.00	47.92
	36–48 h	87.50	104.17
T-2 toxin	0–12 h	n.d.	0.245
	13–24 h	n.d.	0.174
	25–36 h	n.d.	0.179
	37–48 h	n.d.	0.390
HT-2 toxin	0–12 h	n.d.	0.082
	13–24 h	n.d.	0.059
	25–36 h	n.d.	0.060
	37–48 h	n.d.	0.131
DON	0–12 h	0.017	0.017
	13–24 h	0.011	0.013
	25–36 h	0.019	0.013
	37–48 h	0.022	0.031

n.d.: not detectable (< 0.10 mg/kg feed)

The conjugated diene and conjugated triene levels of the liver did not change as a result of T-2/HT-2 toxin or DON exposure (Table 4). Nor did the content of TBARS (the end-product of lipid peroxidation) change in the liver homogenate as a result of short-term mycotoxin treatment (Table 5). In the group fed a diet contaminated with T-2/HT-2 toxin, reduced glutathione (GSH) content of the liver homogenate was significantly higher than in the control at the 24-h sampling (Table 5), while DON had no significant effect. Glutathione peroxidase (GPx) activity was significantly higher than that of the control at hour 24 in the T-2/HT-2 toxin treated group, and at hour 48 in the group fed the DON-contaminated diet (Table 5).

Table 4

Effect of T-2 toxin or DON on conjugated diene (CD) and conjugated triene (CT) levels in the liver of broiler chickens (mean ± SD; n = 6)

Time	Control	T-2/HT-2 toxin	DON
		CD (OD 232nm)	
12th hour	0.28 ± 0.02	0.26 ± 0.02	0.28 ± 0.01
24th hour	0.34 ± 0.11	0.36 ± 0.12	0.28 ± 0.02
36th hour	0.24 ± 0.01	0.23 ± 0.01	0.23 ± 0.02
48th hour	0.25 ± 0.02	0.24 ± 0.02	0.25 ± 0.02
		CT (OD 268nm)	
12th hour	0.15 ± 0.01	0.14 ± 0.02	0.15 ± 0.01
24th hour	0.17 ± 0.02	0.18 ± 0.02	0.15 ± 0.01
36th hour	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
48th hour	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.01

The expression of genes encoding the glutathione redox components showed changes at all sampling times in the liver (Table 6). Expression of the glutathione peroxidase 4 gene (*GPX4*) was lower than in the control at hours 12, 24 and 36 in both mycotoxin-treated groups, but the difference was statistically significant only in the DON-treated group at hours 12 and 24. However, at hour 48, *GPX4* expression was found to be significantly higher in the DON-treated group (Table 6). The expression of the glutathione reductase gene (*GSR*) was significantly lower than in the control at hour 12 in the group treated with T-2/HT-2 toxin, and at hours 12, 24 and 48 in the DON-treated group. However, at hour 36 the opposite effect was found, as the highest gene expression was measured in the DON-treated group. The expression of the glutathione synthetase gene (*GSS*) was significantly lower in both mycotoxin-treated groups than in the control at hours 24 and 48.

Table 5

Effect of T-2/HT-2 or DON treatment on lipid peroxidation and glutathione redox parameters in liver homogenates (mean ± SD; n = 6)

Time	Control	T-2/HT-2 toxin	DON
		MDA (µmol/g)	
12th hour	10.99 ± 2.15	10.12 ± 3.02	9.36 ± 0.54
24th hour	17.95 ± 3.69	12.62 ± 1.88	15.00 ± 4.94
36th hour	10.05 ± 1.37	11.37 ± 2.31	10.04 ± 2.12
48th hour	12.57 ± 3.47	15.51 ± 2.68	12.03 ± 3.24
		GSH (µmol/g protein content)	
12th hour	3.03 ± 0.75	4.03 ± 1.12	3.55 ± 0.89
24th hour	2.84 ± 1.07	4.56^{*} ± 0.74	3.00 ± 1.17
36th hour	3.46 ± 0.83	3.60 ± 0.58	3.08 ± 0.60
48th hour	2.62 ± 0.40	2.92 ± 0.51	3.15 ± 0.58
		GPx (U/g protein content)	
12th hour	3.01 ± 0.66	3.99 ± 0.91	3.48 ± 0.68
24th hour	3.10 ± 1.24	4.78^{*} ± 0.67	2.89 ± 0.96
36th hour	3.23 ± 0.88	3.44 ± 0.61	3.16 ± 0.73
48th hour	1.99 ± 0.37	2.54 ± 0.48	2.84^{**} ± 0.49

MDA: malondialdehyde; GSH: reduced glutathione; GPx: glutathione peroxidase; Means designated with different superscripts in the same row indicate significant difference as compared to the control $^{*}P < 0.05$; $^{**}P < 0.01$

Discussion

No clinical signs of toxicity and mortality were observed at the applied mycotoxin doses, which supports that broiler chickens have relatively high tolerance to DON (Dänicke et al., 2001) and moderate tolerance to T-2/HT-2 toxin (Eriksen and Pettersson, 2004). Absolute or relative liver weight did not change as a result of mycotoxin exposure, which is supported by a previous study in which 1 or 5 mg DON/kg feed showed the same results (Awad et al., 2011*a*). For T-2/HT-2 toxin no data are available regarding these parameters. Due to the varying feed intake during to trial there were some differences in the calculated mycotoxin intake.

Conjugated diene and conjugated triene levels in the liver (the early markers of lipid peroxidation) did not change after short-term mycotoxin exposure, which means that the early effects of mycotoxins did not induce measurable lipid peroxidation. This is also supported by the lack of significant alterations in the TBARS content. These results are different from those reported in long-term studies with low trichothecene mycotoxin doses (Mézes et al., 1998; Awad et al., 2014), while they are similar to the findings of other studies (Frankic et al., 2006;

Rezar et al., 2007) in which high doses of DON or T-2 toxin were used. In the present study, the lack of marked oxidative stress in the liver suggested that this effect of trichothecenes might depend not only on the dose and duration of the treatment, but also on some other factors such as the antioxidant content of the feed.

Table 6

Effect of T-2/HT-2 toxin or DON treatment on the expression of glutathione peroxidase 4, glutathione reductase and glutathione synthetase genes in the liver (mean ± SD; n = 6; equal amounts of cDNA)

Time	Control	T-2 toxin	DON
	Glutathione peroxidase 4 (*GPX4*)		
12th hour	1.05 ± 0.11	0.88 ± 0.12	0.77^{*} ± 0.07
24th hour	1.17 ± 0.10	1.03 ± 0.05	0.96^{**} ± 0.08
36th hour	1.03 ± 0.05	0.92 ± 0.05	1.02 ± 0.07
48th hour	1.02 ± 0.06	1.01 ± 0.11	1.16^{*} ± 0.05
	Glutathione reductase (*GSR*)		
12th hour	1.01 ± 0.12	0.50^{***} ± 0.12	0.17^{***} ± 0.02
24th hour	0.98 ± 0.01	1.07 ± 0.10	0.39^{***} ± 0.07
36th hour	0.63 ± 0.02	0.67 ± 0.03	1.57^{***} ± 0.41
48th hour	1.34 ± 0.32	1.58 ± 0.27	1.07^{*} ± 0.01
	Glutathione synthetase (*GSS*)		
12th hour	0.92 ± 0.43	1.23 ± 0.25	0.90 ± 0.31
24th hour	1.57 ± 0.42	0.70^{**} ± 0.16	0.66^{**} ± 0.13
36th hour	1.99 ± 0.74	1.37 ± 0.13	2.46 ± 0.63
48th hour	2.11 ± 0.52	0.47^{***} ± 0.06	0.99^{**} ± 0.21

Means designated with different superscripts in the same row indicate significant difference as compared to the control $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

GSH content in the liver, the main site of its biosynthesis (Shelly and Lu, 2013), showed higher values as compared to the control when a diet contaminated with T-2/HT-2 toxin was fed. This can indicate the activation of glutathione biosynthesis due to a moderate oxidative stress induced in the liver by T-2/HT-2 toxin (Zimniak et al., 1997). This result is supported by the moderately higher expression of the glutathione synthetase gene (*GSS*), in particular during the first period of the trial. It is also in line with the results of a short-term study with rats, where a single oral dose of T-2 toxin (2 mg kg^{-1} b.w.) increased the expression of oxidative stress-related genes in the liver, 6 and 9–12 h after treatment (Sehata et al., 2005).

Due to DON exposure, the *GSS* gene expression was also increased, but only in the late period of the trial (between 36 and 48 h), which coincided with

the higher feed and mycotoxin intake in that period. This result is possibly due to the higher feed, including amino acid, intake which might cause activation of GSH synthesis through the expression of the genes encoding its *de novo* synthesis. In addition to this, GSH concentration depends on the rate of reduction of its oxidised form, glutathione disulphide, by the glutathione reductase enzyme. As a result of the mycotoxin exposure, the expression of its gene (*GSR*) was significantly lower in the DON-treated group than in the control, except at hour 36 when mycotoxin intake was the lowest during the trial. This result suggests that the moderate increase of GSH concentration in the liver was not caused by the higher rate of reduction of glutathione disulphide by glutathione reductase, but probably by the higher rate of its *de novo* biosynthesis.

GPx4 activity in the liver homogenates was lower in both mycotoxin-treated groups, in particular during the first period of the trial, when mycotoxin intake was high. This means that during this period trichothecene mycotoxins did not induce oxidative stress, which is required for the activation of the antioxidant defence, either at the gene expression level or at the posttranslational activation of GPx. However, DON caused significantly higher GPx4 activity at hour 48, which means that a longer exposure and appropriate mycotoxin intake are required for the activation, as found also in another long-term study with T-2 toxin (Balogh et al., 2015). This delayed effect was also revealed in a short-term trial with trichothecene mycotoxins in common carp (Pelyhe et al., 2016), which might be due to the rate of absorption (Greiner and Applegate, 2013) or the metabolism of mycotoxins in the liver (Awad et al., 2014).

In conclusion, the results revealed that at the applied doses of T-2/HT-2 toxin or DON had a modest effect on oxygen free radical formation, therefore they caused only a moderate activation of the glutathione redox system in the liver of chicken during a short period (48 h) of exposure.

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