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Multi-trichothecene mycotoxin exposure activates glutathione-redox system in broiler chicken

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ABSTRACT

Co-occurrence of mycotoxin contamination of feeds is a frequent problem, therefore the purpose of this study was to evaluate the combined effect of T-2 toxin and deoxynivalenol (DON) on lipid peroxidation, parameters and regulation of the glutathione redox system in broiler chickens in a sub-chronic (7 day) study. The applied doses were: low mix: 0.23 mg T-2 toxin and 4.96 mg DON/kg feed; medium mix: 1.21 mg T-2 toxin and 12.38 mg DON/kg feed; and high mix: 2.42 T-2 toxin and 24.86 mg DON/kg feed. Liver samples were taken on days 0, 1, 2, 3, and 7 of the feeding trial. Lipid peroxidation decreased significantly as compared to the control on days 3 and 7 as effect of low and high doses, which can be related to the activation of the antioxidant system, which is supported by the elevated glutathione peroxidase activity and reduced glutathione concentration as compared to the control on day 3 in the medium and high dose groups. Gene expression of glutathione peroxidase 4 (*GPX4*) elevated on day 1 in a dose dependent manner, and showed continuous elevation in the highest dose group thereafter. The results suggested that common exposure of T-2 toxin and DON induced oxidative stress in the liver of broiler chickens, which activated the enzymatic antioxidant system, and consequently decreased lipid peroxidation.

1. Introduction

Mycotoxins considered as one of the most significant risk in the food chain with high economic impact in animal production (Bryden, 2012). *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* are the most prevalent toxigenic fungi genera (Tsitsigiannis et al., 2012). Trichothecene mycotoxins are secondary metabolites of *Fusarium* spp., which are mostly infect cereal grains (Miraglia et al., 2009). In temperate climates *Fusarium* mycotoxins, including 'B type' trichothecene DON and 'A type' T-2 toxin are the most relevant contaminants of cereal grains (Binder et al., 2007). Trichothecene mycotoxins well studied in farm animals, but despite that common occurrence of mycotoxins are general in the nature (Bertuzzi et al., 2014); the effect of mycotoxin mixtures less described in the literature (Avantaggiato et al., 2007). Combined exposure might cause additive, synergistic or antagonistic toxic effects, but very little is known about the actual multi-mycotoxin risk, yet (Alassane-Kpembi et al., 2015).

Trichothecene mycotoxins are linked to different health effects in farm animals (Awad et al., 2013), however, broiler chicken is relatively resistant to trichothecenes (Swamy et al., 2004), but their toxicokinetic parameters are different. Deoxynivalenol has a low absolute oral bioavailability and T-2 toxin has no plasma levels above the limit of quantification after an oral bolus due to rapid metabolism in the liver (Osselaere et al., 2013). It was described, that T-toxin and DON in single application induce reactive oxygen substances (ROS) formation and cause oxidative stress in rats (Rizzo et al., 1994). It can manifest alterations of many different pathways, such as activation of MAP kinases, HSP 90, and results in ribotoxic stress, apoptosis or necrosis, which may lead to tissue damage (Awad et al., 2014; Wu et al., 2017). However, there are only few data about the combined effect of T-2 toxin and DON (Wu et al., 2014; Assunção et al., 2016). The hierarchical model of oxidative stress (Gloire et al., 2006) describes that the antioxidant defence response first to rising ROS levels at gene and protein expression levels. The redox-sensitive Keap1-Nrf2-ARE (antioxidant response element) pathway drives regulation of the expression of antioxidant genes, where Keap1 represses Nrf2 transcription protein in physiological conditions. The Nrf2-binding cysteine sites are sensitive for oxidative stress, and in elevating ROS formation they oxidise, and

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Nrf2 releases and reaches the ARE in the nuclei (Suzuki et al., 2016).

Previous studies with poultry support that individual T-2 toxin induces oxidative stress either at extreme high dose (14.4 mg kg^{-1}feed) in short-term (Bócsai et al., 2015) or at high (8 mg kg-^{1}) or low dose (1.53 mg kg^{-1} feed) in sub-chronic exposure (Surai, 2002; Weber et al., 2010). DON also induce oxidative stress in long-term exposure at high (10 mg kg^{-1} feed) dose exposure (Awad et al., 2012; Frankic et al., 2006). Trichothecene mycotoxin-induced oxidative stress causes oxidative damages, such as lipid peroxidation. However, the results are controversial in case of T-2 toxin, because lipid per-oxidation, as effect of oxidative stress, did not prove in long-term exposure at 3.4 mg toxin kg^{-1} feed (Balogh et al., 2015) or at extreme high (13.5 mg kg^{-1} feed) dose (Rezar et al., 2007).

The aim of present study was to evaluate the combined effects of graded levels of T-2 toxin and DON on the regulation and activation of the antioxidant defence system and lipid peroxidation in broiler chickens in sub-chronic exposure.

2. Materials and methods

2.1. Production of toxin, artificial mycotoxin contamination of the feed and determination of mycotoxin content in feeds

DON produced by *Fusarium graminearum* (NRRL 5883) and T-2 toxin by *Fusarium sporotrichioides* (NRRL 3299) strains on corn substrate according to Fodor et al. (2006). The production strains validated for mycotoxin production, but the final fungal culture did not check for purity. Appropriate amount of the fungal culture mixed with the complete feed, according to its mycotoxin content. DON content of the complete feed was determined according to Pussemier et al. (2006), and T-2 toxin concentration based on the method of Trebstein et al. (2008) with HPLC by fluorescence detection after immune-affinity clean up.

2.2. Animals and experimental design

A total of 104 Cobb 540 broiler chickens was randomly allotted into 8 groups at the age of 18 days, and after 3 days acclimatization period (21days of age), the 7-day feeding trial was carried out, with control, low, medium and high mixture groups, in two replicates.

Broiler chickens were kept in deep litter condition with continuous light regimen to improve the contaminated feed intake. The trial started after 18 h of feed deprivation.

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^{-1} vitamin E and 0.25 mg kg^{-1} selenium). The nutrient content of the diet met the requirements for broiler chickens.

The basal diet (T-2 toxin: < 0.01 mg kg^{-1}, DON: 0.25 mg kg^{-1}) was artificially contaminated with the mixtures at concentrations of low dose mix: 0.23 mg T-2 toxin and 4.96 mg DON/kg feed, medium dose mix: 1.21 mg T-2 toxin and 12.38 mg DON/kg feed and high dose mix: 2.42 mg T-2 toxin and 24.86 mg DON/kg feed, respectively. The applied doses in low dose mix demonstrate a highly contaminated feed, and it reflects a casual contamination in temperate climates, but medium and high dose mix contain higher than the contamination level in practice for provoking measurable changes within a short period of time. The complete feed not checked for other mycotoxins, but ochratoxin A, zearalenone and fumonisin B1 content of the basic ingredients, corn and soybean meal measured and those were lower than limit of detection.

The experiment regulated by the Hungarian Animal Protection Act, in compliance with the EU rules, except the continous light regimen. The experimental protocol was authorised by the Food Chain Safety, Land use, Plant and Soil Protection and Forestry Directorate of the Pest County Governmental Office (PE/EA/1965-7/2017).

2.3. Sampling and biochemical analyses

Six animals taken randomly at day 0 as absolute control, and from each group at day 1, 2, 3 and 7 after the start of the experiment. After cervical dislocation, *post mortem* liver samples taken into liquid nitrogen, and stored at −70 °C until analysis.

Markers of the initial phase of lipid peroxidation, conjugated dienes (CD) and conjugated trienes (CT) were measured after extraction in 2,2,4-trimethylpentane by the absorbance at 232 nm and 268 nm, due to lack of appropriate standard (AOAC, 1984). Marker of the terminal phase of lipid peroxidation, thiobarbituric acid reactive substances measured according to Mihara et al. (1980) and expressed as malondialdehyde (MDA) which served as standard. Reduced glutathione (GSH) content measured by the method of Sedlak and Lindsay (1968), and the activity of glutathione peroxidase (GPx) based on the method of Lawrence and Burk (1978) in the 10,000 g supernatant fraction of liver homogenates. GSH content and GPx activity calculated to protein content, which was determined with Folin-phenol reagent (Lowry et al., 1951) in the 10,000 g supernatant fraction of liver homogenates.

2.4. RNA isolation, reverse transcription and qPCR

Total RNA purified from liver samples with Trizol reagent (Molecular Research Centre, Cincinnati) in Phase Lock Gel tubes (5Prime GmbH, Hamburg) according to the manufacturer's instructions. Genomic DNA contamination of the RNA isolates eliminated by DN-ase l treatment (Thermo Fisher Scientific, San Jose) according to the standard protocol. The quantity and integrity of RNA were verified by agarose gel electrophoresis (1.5% in Tris-Borate-EDTA buffer pH: 8.0). In addition those samples were accepted for further use when the ratios of absorption 260:280 nm were higher than 2.0 as measured by NanoPhotometer (Implen, Munich). 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* and endogenous control gene (*GAPDH*) were designed with Primer Express 3.0.1 (Thermo Fisher Scientific, San Jose). *GAPDH* selected as a control gene because it has no known interaction with oxidative stress or mycotoxins, as proposed by other studies with mycotoxins in broiler chicken (Yang et al., 2016).

Real time PCR measurements carried out in duplexes (*GAPDH* and *GPX4* gene) using MGB-NFQ TaqMan probes (Thermo Fisher Scientific, San Jose) with pooled cDNA templates. The pools were formed from equal (100 ng) amounts of cDNA per 5 chicken individuals of each sampled group at each sampling point, according to the results of our preliminary studies, when no measurable difference was found if the determination was carried out from pooled, or individual samples. The qPCR was carried out with Maxima Probe qPCR Master Mix (Thermo Fisher Scientific, San Jose) and Step One Plus™ Real Time PCR systems (Applied Biosystems, Foster City). The reaction mix contained 2.5 mM $MgCl_2$, 5 ng cDNA, primers and probes of the target and endogenous control genes (Tables 1 and 2) in a total volume of 12.5 μL per reaction. The PCR profile was 95 °C for 10 min for pre-amplification denaturation, followed by 95 °C 15 s, 58 °C 30 s and 72 °C 30 s for 45 cycles and 72 °C 2 min final elongation, VIC and FAM signals were detected at the

Table 1
Primers used for the quantification of the mRNA transcriptional levels of the target and endogenous control gene (GAPDH).

gene	GenBank accession no.	primer sequence (5′ to 3′)
GAPDH	K01458	forward: TGACCTGCCGTCTGGAGAAA reverse: TGTGTATCCTAGGATGCCCTTCAG
GPX4	AF498316.2	forward: AGTGCCATCAAGTGGAACTTCAC reverse: TTCAAGGCAGGCCGTCAT