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ORIGINAL PAPER

Study of deoxynivalenol effect on metallothionein and glutathione levels, antioxidant capacity, and glutathione-*S*-transferase and liver enzymes activity in rats

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Deoxynivalenol (DON, vomitoxin) is one of several human mycotoxins acquired through residues in foods of plant origin (cereals) and through foods of animal origin (kidneys, liver, milk, eggs). The aim of this study was to investigate the impact of deoxynivalenol-contaminated mouldy grain on the health status of rats and their ability to resist oxidative stress. The rats were fed a diet supplemented with DON along with organic and inorganic Zn(II), and vitamins for 28 days. The study focuses particularly on protective mechanisms and levels of reduced glutathione and metallothioneins (MT) against the adverse effects of xenobiotics. The highest concentrations of MT were detected in the tissues of kidneys ((6.70 ± 0.05) μg g^{-1}) and liver ((6.00 ± 0.05) μg g^{-1}), both of which are involved in detoxifying cationic xenobiotics. Lower concentrations were also detected in heart, brain, testes, and muscle tissues (approximately 3.0 μg g^{-1}). Antioxidant capacity, glutathione-*S*-transferase, and liver enzymes activity were also studied. In conclusion, MT may play an important role in the detoxification of mycotoxins. Even though its role is not fully understood, MT are crucial for the redistribution of ions important for transcription factors and interaction with reactive oxygen species that form mycotoxins.

Keywords: rats, mouldy barley, mycotoxins, deoxynivalenol, metallothionein

Introduction

Mycotoxins are secondary metabolites produced by *Fusarium*. Due to the high toxicity of *Fusarium* mycotoxins and the high occurrence of species of fungi producing the toxins, these mycotoxins have the potential to cause serious harm to humans and other organisms (Luongo et al., 2010). Deoxynivalenol (DON, vomitoxin) is a naturally occurring mycotoxin mainly produced by *Fusarium graminearum* and *Fusarium culmorum* (Kushiro, 2008). Its high occurrence in food and feed (90 %) and its potential marker for the growth of other mycotoxins render its containment even more challenging. DON does not constitute a significant threat to public health. In some cases, short-term nausea and vomiting have been observed. Other effects include diarrhea, abdominal pain, headache, dizziness, and fever (Sobrova et al., 2010). In addi-

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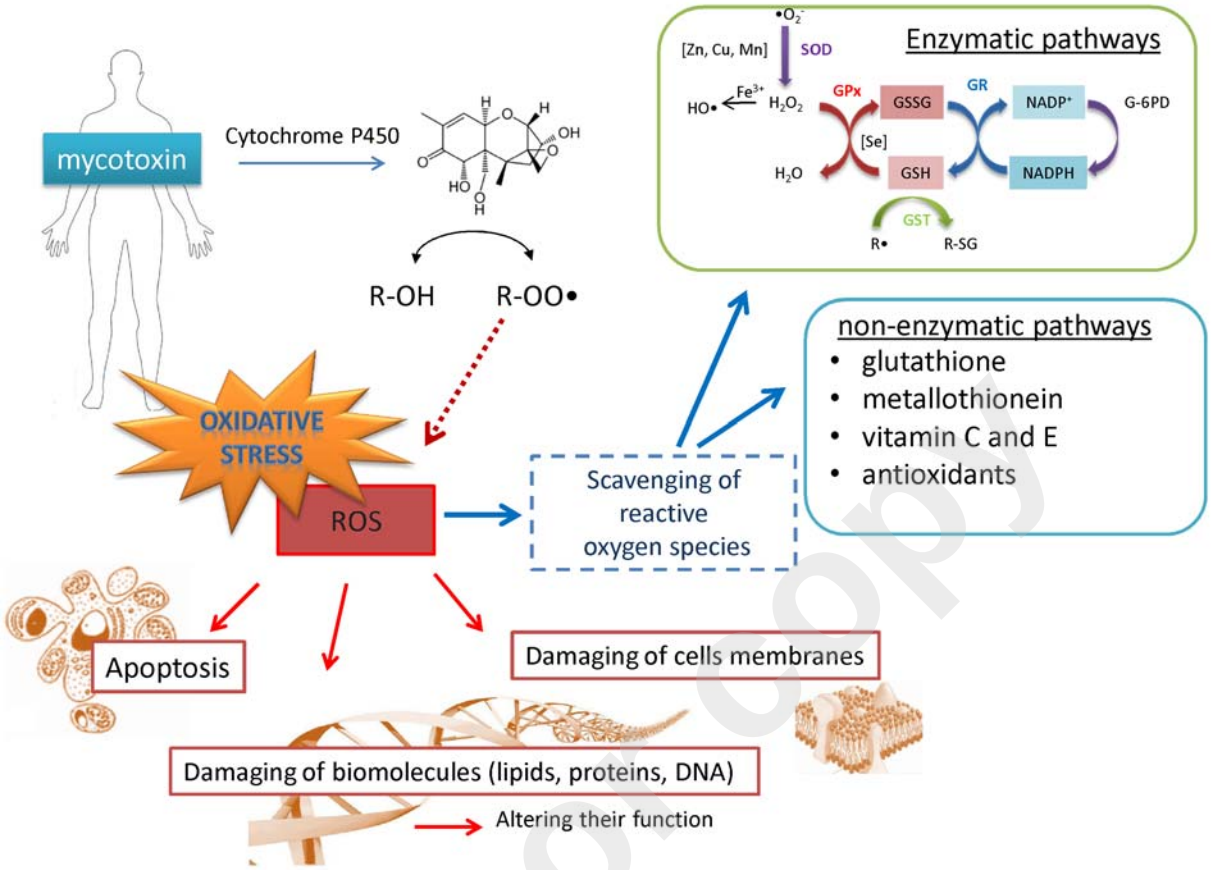

Fig. 1. Scheme of possible way of deoxynivalenol detoxification. The first and one of the most important pathways used for detoxifying DON is cytochrome P450, which serves to catalyse the oxidation of organic substances. This pathway, however, can cleave free hydroxyl groups of DON to produce DON-radical, which can be more dangerous. The DON-radical can be scavenged by enzymatic (glutathione peroxidase (GPx), catalase, superoxide dismutase) or non-enzymatic ways (reduced glutathione (GSH), metallothionein (MT), and vitamins).

tion, DON demonstrates reproductive and teratogenic effects (Debouck et al., 2001), immunotoxicity, cytotoxicity, and genotoxicity (Li et al., 2000; Sun et al., 2002). According to the International Agency for Research on Cancer, DON is classified in the Group 3 category: "not classifiable as to its carcinogenicity to humans" (Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines, and mycotoxins, www.iarc.fr).

Various adverse effects have been described for DON on biochemical pathways (Fig. 1), including those generating reactive oxygen species (ROS) which can damage biomolecules and membranes leading to apoptosis (Desmond et al., 2008). Due to these negative effects, induction of ROS by DON is of great interest (Desmond et al., 2008; Zhang et al., 2009) as well as the possibilities of minimising their negative effects (Krishnaswamy et al., 2010). Thus, it is not surprising that the ingestion of mycotoxins activates numerous mechanisms in the body to reduce their toxicity and induce their excretion. Enzymatic or non-enzymatic reactions can scavenge DON-generated ROS, subsequently preventing oxidative stress (Valko et al., 2006). An interesting pathway for scavenging ROS is based on the expression of low molecular mass proteins rich in cysteine, called metallothioneins (Capdevila et al., 2012; Eckschlager et al., 2009; Maret, 2011; Ryvolova et al., 2011; Vasak & Meloni, 2011). The main function of MTs in the organism is a metal ion transport, maintenance of the oxidative–reducing conditions and regulation of gene expression. Thiols, including MT and glutathione, are known as effective radical scavengers creating optimal oxidative–reducing conditions; as a result, cell compartments and biologically important compounds including cell-cycle-enzymes or DNA are protected.

This work is directed towards investigating the effects of mouldy barley, contaminated with pathogenic fungi producing mycotoxins, on metallothionein levels and on enzyme levels that participate in the protection of the tissues and blood of Wistar albino sewer rats against oxidative stress. In addition, the effects of vitamin and Zn(II) on the treatment of rats with contaminated barley were also studied, due to possible positive interactions between increasing the content of these substances and enhancing the effectiveness of protective mechanisms.

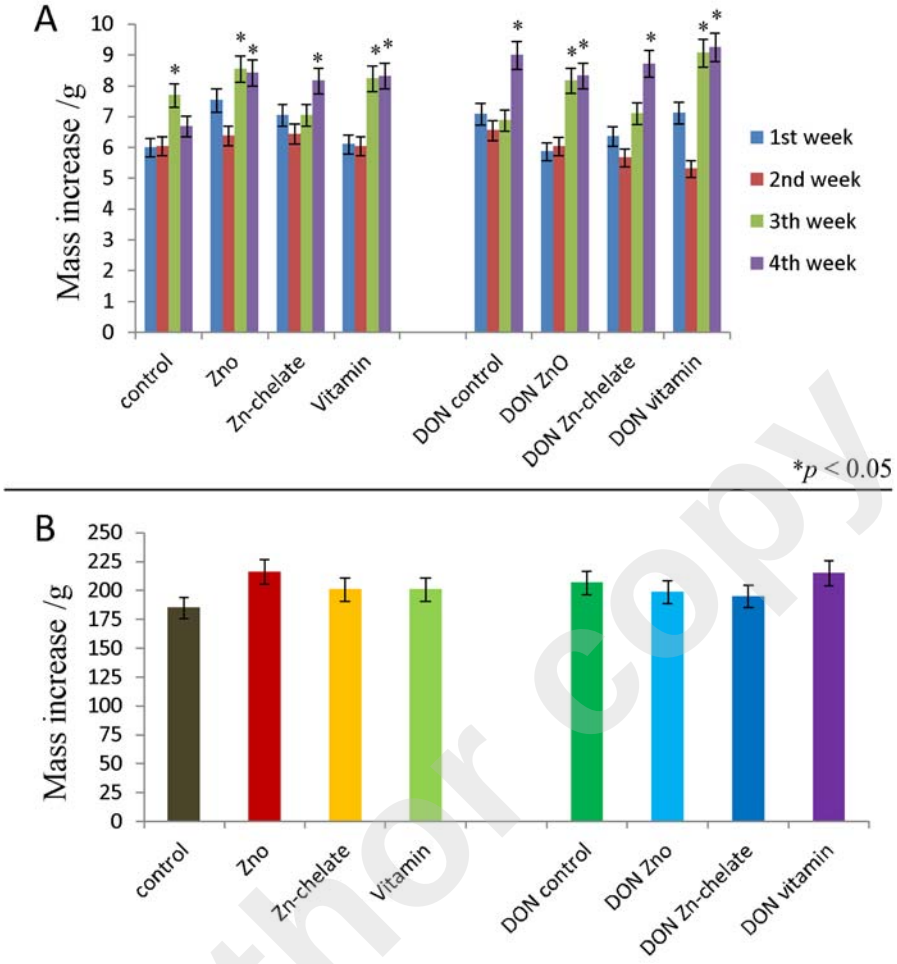

Fig. 2. Influence of DON on rats' mass: Average daily mass increasen (A); average monthly mass increase (B).

Table 1. Composition of feed mixtures; experiments varied in zinc overdose in two forms and vitamins

Group	Barley DON (40 μg kg^{-1})				Mouldy barley DON (3500 μg kg^{-1})			
	1.	2.	3.	4.	5.	6.	7.	8.
Group name	Control	ZnO	Zn-chelate	Vit + 25 %	DON control	DON ZnO	DON Zn-chelate	DON Vit + 25 %
Ingredient	%							
Barley corn	63	63	63	63	–	–	–	–
Barley corn (DON)	–	–	–	–	63	63	63	63
Soybean meal 45 %	19	19	19	19	19	19	19	19
Starch	11.3	11.3	11.3	11.25	11.3	11.3	11.3	11.25
Vitamins mix	0.2	0.2	0.2	0.25	0.2	0.2	0.2	0.25
Minerals mix	3	3	3	3	3	3	3	3
Sunflower oil	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Total	100	100	100	100	100	100	100	100

Experimental

Rabbit liver MT (MW 7143 g mol^{-1}), containing 5.9 % Cd and 0.5 % Zn, was purchased from Sigma–Aldrich (St. Louis, MO, USA). $Co(NH_3)_6Cl_3$ and other chemicals used were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise. The stock standard solution of MT (10 μg mL^{-1}) was prepared with ACS water (Sigma–Aldrich, St. Louis, MO, USA) and stored in the dark at –20 °C. Working standard solutions were prepared daily by dilution of the stock solutions with water of ACS purity,

i.e. chemicals meet the specifications of the American Chemical Society. The pH value was measured using a WTW inoLab pH meter (Weilheim, Germany). All nutrients were purchased from Mikrop Čebín (Czech Republic).

Selected male Wistar albino laboratory rats aged 28 days were used (Fig. 2). The experimental animals were kept in a vivarium with controlled air temperature ((23 ± 1) °C) and photo-period (12 hours day : 12 hours night with maximal intensity of 10800 lx). Temperature-controlled feed mixtures and drinking water were accessible ad libitum. Animals were weighed once per week. The experimental animals were divided into eight groups (eight male rats per group). We used feed mixtures of natural barley and barley contaminated with mycotoxins both with varying quantities of vitamins, Zn(II) in organic and inorganic forms (Table 1). Natural barley contained (40 ± 5) μg kg^{-1} of DON and naturally contaminated barley (3500 ± 5) μg kg^{-1} of DON. Rats were fed these mixtures for 28 days. Subsequently, the animals were killed and tissues and blood samples were collected. The experiment was approved by the Ethics Commission of the Faculty of Agronomy, Mendel University in Brno, Czech Republic and was done in accordance with the Law on Protection of Animals against Cruelty No. 246/1992.

Preparation of biological samples

The rats' tissues (liver, kidneys, spleen, heart, brain, eyes, gonads, and femoral muscle) and blood samples were further analysed. The animal tissues were mixed with an extraction buffer (100 mM potassium phosphate, pH 6.8, 1 mL per 100 mg of tissue) and subsequently homogenised using a semi-automatic homogeniser (Shutt homogen plus, Germany).

Metallothionein. The homogenates as well as the blood samples were centrifuged at 10000g for 15 min at 4 °C (Eppendorf 5402, USA). The samples were then heated at 99 °C in a thermomixer (Eppendorf Thermomixer Comfort, USA) for 15 min with occasional stirring, then cooled to 4 °C. The denatured homogenates were centrifuged at 4 °C, 15000g for 30 min. (Eppendorf 5402, USA). The heat treatment effectively denatured and removed high molecular mass proteins from the samples (Fabrik et al., 2008a, 2008b; Huska et al., 2008; Petrlova et al., 2006; Raspor et al., 2001). The supernatants obtained were diluted 100 × with an extraction buffer (100 mM potassium phosphate, pH 6.8) prior to electrochemical measurements.

Enzymes. The homogenates obtained by homogenisation using a semi-automatic homogeniser were diluted 10 × with a potassium buffer solution (100 mM, pH 6.8) prior to spectrometric measurements. Antioxidant capacity, activity of glutathione-*S*-transferase, and liver enzymes (alanine transaminase (ALT), aspartate transaminase (AST)) levels were determined.

Analytical tools

The analytical tools used for the investigation of mouldy barley contaminated with deoxynivalenol on various rat organs were as follows: electrochemical measurements were used for metallothionein determination from the gonads, liver, spleen, kidneys, heart, muscle, brain, eyes, and blood. The values of metallothionein concentration thus obtained were compared using SDS PAGE. An automated spectrometric analyser was used for monitoring the health status of rats by measuring the total protein content, antioxidant capacity and ALT, AST, and GST activities in blood samples.

Electrochemical measurements were performed with a 747 VA Stand instrument connected to a 746 VA Trace Analyser and 695 Autosampler (Metrohm, Switzerland) using a standard cell with three electrodes and a cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3 M KCl electrode was the reference electrode. A glassy carbon electrode was used as an auxiliary electrode. GPES 4.9 supplied by EcoChemie was employed. Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3 (aq) + NH_4Cl, pH 9.6) was used and changed per analysis. DPV parameters were as follows: initial potential of –0.7 V, end potential of –1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude –250 mV, E_{ads} = 0 V. All experiments were carried out at a temperature of 4 °C (Julabo F12 cooler, Germany) (Fabrik et al., 2008a; Krizkova et al., 2009b).

Electrophoresis was performed (Laemmli, 1970) using a Mini Protean Tetra apparatus with gel dimensions of 8.3 cm × 7.3 cm (Bio-Rad, USA). ACS water was used in the preparation of all solutions. A running solution (15 g of acrylamide per 100 mL of water) was initially poured followed by a stacking gel (5 g of acrylamide per 100 mL of water). The gels were prepared from an acrylamide stock solution (300 g L^{-1}) containing bisacrylamide (10 g L^{-1}). Polymerisation of the running or stacking gels was carried out at room temperature for 45 min or 30 min, respectively. To achieve consistent results, the gels were kept overnight in the dark at 4 °C. The addition of 150 mM dithiothreitol to the sample buffer was chosen for analysis of the biological samples. Prior to analysis, the samples were mixed with a reduction sample buffer (φ_r = 1 : 1). The samples were boiled for 2 min, and then loaded onto a gel. For determination of the molecular mass, the protein ladder "Precision plus protein standards" from Biorad was used. The electrophoresis was run at 150 V for 1 h (Power Basic, Biorad, USA). Silver staining of the gels was performed following a method described

by Oakley et al. (1980). The gels were incubated in a fixing solution of (11 g L^{-1}) acetic acid, methanol (64 g L^{-1}), and formaldehyde (3.7 g L^{-1}) for 1 h. Once removed from SDS, the gels were subsequently incubated with methanol (500 g L^{-1}) three times for 15 min. The gels were then incubated with sensitiser (0.2 g L^{-1} of sodium thiosulphate) for 1 min. After rinsing with deionised water for 20 s, the gels were transferred into a staining solution (5 g L^{-1} of $AgNO_3$ and 0.3 g L^{-1} of formaldehyde). After 20 min the gels were rinsed in deionised water for 20 s and transferred into a developing solution (60 g L^{-1} of sodium carbonate, 0.004 g L^{-1} of sodium thiosulphate, and 0.2 g L^{-1} of formaldehyde). The development of the bands was carried out for 20 min, then they were rinsed with deionised water and fixed in a mixture of 11 g L^{-1} of acetic acid and 64 g L^{-1} of methanol for 10 min.

Spectrometric measurements were carried out using an automated chemical analyser BS-200 (Mindray, China). Reagents and samples were placed on a cooled sample holder (4 ℃) and automatically pipetted into plastic cuvettes. Incubation proceeded at 37 ℃. The mixture was subsequently stirred. The washing steps with distilled water (18 mΩ) were performed between the pipetting intervals. The instrument operated with BS-200 software (Mindray, China).

Weighed tissues (approximately 0.2 g) were transferred into a test-tube. Liquid nitrogen was added to the test tube and frozen to disrupt the cells. The frozen sample was transferred into a mortar and ground for 1 min. 1.000 μL of 0.2 M phosphate buffer (pH 7.2) was added to the mortar then the sample was ground for 5 min. The homogenate was transferred into a new test tube and the frozen samples were homogenised by shaking on a Vortex–2 Genie at 4 ℃ (Scientific Industries, USA) for 5 min. They were then sonicated using a Bandelin Sonopuls HD 2070 at 7 W (Germany) for 10 s. The homogenate was centrifuged (14000g) at 4 ℃ for 15 min using a Universal 32 R centrifuge (Hettich-Zentrifugen, Germany). The supernatant was filtered through a 0.45 μm Nylon filter disc (Millipore, Billerica, MA, USA) prior to analysis.

Total protein content was determined using a biuret solution (15 mM potassium sodium tartrate, 100 mM NaI, 15 mM KI, and 5 mM $CuSO_4$) with albumin (1 mg mL^{-1} of phosphate buffer, pH 7) used as a standard. 180 μL of the biuret solution was mixed with 45 μL of the real or standard sample. After stirring and incubation (at 37 ℃ for 10 min), the absorbance was measured at 546 nm.

Determination of antioxidant capacity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) test was performed following the procedure by Parejo et al. (2000). A stock solution of DPPH (1 mM) with absorbance ($t_0 = 0.20 \pm 0.01$) was briefly mixed with a 0.2 M acetate buffer ($\varphi_r = 1 : 2$). A suspension (10 μL) was added to this solution (190 μL) and the absorbance was measured against methanol at $\lambda =$ 515 nm. The decrease in absorbance (%) was recalculated on Trolox. A calculated amount of DPPH was dissolved in 25 mL of DMSO. After its dissolution, it was made up to a volume of 50 mL with ACS water. The solution may be used for 14 days when stored in the dark at 4 ℃. The measurement procedure for automated analyser was as follows: volume of 200 μL of reagent was incubated with 20 μL of a measured sample (gallic acid, Trolox®). Absorbance was measured at $\lambda = 515$ nm after 1520 s. The value prior to decrease in absorbance (224th second of measurement – A_{224}) and the last measurement (1520th second of measurement – A_{1520}) were used to calculate antioxidant activity. The resulting value was calculated in accordance with the following equation: change in absorbance (%) = $100 - 100(A_{1520}/A_{224})$ (Sochor et al., 2010).

Determination of ALT (AST) activity

250 μL of substrate consisting of 0.2 M DL-α-alanine (L-aspartate) and 2 mM 2-oxoglutarate in 0.1 M phosphate buffer (pH 7.4) at 37 ℃ was added into 50 μL of diluted tissue homogenate. The resulting mixture was incubated at 37 ℃ for 60 min. Subsequently, 250 μL of 2,4-dinitrophenylhydrazine in 1 M hydrochloric acid was added to the mixture. The microtube was carefully stirred and transferred into an automatic biochemical analyser BS-200 (Mindray, China). The BS-200 experimental parameters were as follows: 30 μL of the previously prepared mixture was pipetted into a cuvette and incubated at 37 ℃ for 20 min. 180 μL of 1 M NaOH was added. Ten minutes later, the absorbance was measured at 510 nm.

Determination of glutathione-S-transferase (GST)

The method is based on the GST-catalysed reaction between GSH and GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB), which has the broadest range of isozyme detectability (e.g. alpha-, mu-, pi-, and other GST isoforms). Under certain conditions of temperature, pH, and ionic strength of the solution, the interaction between glutathione and CDNB is wholly dependent on the presence of active GST. The GST-catalysed formation of GS-DNB produces a dinitrophenyl thioether which can be detected by a spectrophotometer at 340 nm. 180 μL of 2 mM CDNB and PBS ($\varphi_r = 1 : 19$) was pipetted into 5 μL of diluted tissue homogenate at 37 ℃. Subsequently, 30 μL of 12.5 mM GSH in 0.1 M phosphate buffer (pH 7.4) was added to the mixture. The microtube was carefully stirred and transferred into an automatic biochemical analyser BS-200 (Mindray, China) and measured at 340 nm.

Descriptive statistics

Data were processed using Microsoft Excel® (USA) and Statistica.cz Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (SD) unless stated otherwise (Excel®). Statistical significance of the differences between MT levels were determined using Statistica.cz. The difference with $p < 0.05$ were considered as significant and were determined using a one-way ANOVA test (the Scheffe test in particular), which was applied for comparison of the means.

Results and discussion

Experimental design

As stated above, a number of experimental studies have focused on mycotoxins' influence on various animals (Sun et al., 2002). In this study, the relationship between the metallothionein level and mycotoxin administration in rats was examined. Sixty-four Wistar albino male rats (28 days old; mass (65 ± 5) g) were divided into 8 different groups and placed in a vivarium. The experimental animals were fed with various mixtures containing different supplements (Table 1). The first four groups were fed with natural barley containing DON (40 μg kg^{-1}). The second four groups were fed with mouldy barley containing DON (3500 μg kg^{-1}). All experimental variations in feed containing unusually high levels of DON were labelled with the suffix "DON". Both the main groups (natural and DON-contaminated barley groups) were divided into 4 subgroups with various mixtures of feed. The first control subgroup was fed only with a vitamin and mineral premix (3 g per 100 g of feed mixtures) which were the minimal requirement stated by the Nutrient Requirements of Rats (NRC). The other three subgroups were fed with mineral premixes supplemented with inorganic (12 mg kg^{-1} feed mixtures, "ZnO") and Zn(II) in organic form (12 mg kg^{-1} feed mixtures, Zn(II) chelate), and vitamin mix ("vit"). Vitamin premixes served to supply vitamins (A, D, E, K, and B groups) not present in the feed.

The influence of mycotoxin on the health of the experimental animals was measured on the basis of daily mass gain. Weekly average day mass increases are shown in Fig. 2A. Data were statistically processed at a probability level of $p < 0.05$. In the last week of the experiment, all the experimental groups, which were fed with DON mixtures had higher week mass increases than the groups, which were not fed with DON mixtures. These results were, however, not significant. In comparison with weekly masses, the results of the last two weeks of the experiment showed higher mass increases ($p < 0.05$) in both groups in comparison with the first two weeks. The significant results were marked "*". The highest growth was observed in rats in the non-DON group fed with Zn(II), Zn(II) chelate, and vitamins. In addition, the average monthly mass increases were observed (Fig. 2B). Monthly mass gain differences between both groups were less noticeable and not significant ($p < 0.05$). The highest mass increase was observed in the non-DON subgroup fed with feed supplemented with inorganic Zn(II). The higher level of Zn(II) ions probably has a positive effect on the growth and development of experimental rats. The feed supplemented with organic Zn(II) chelate and vitamins positively affected monthly mass gains in comparison with the control group. DON-treated experimental groups showed converse results. The highest mass increase was observed in the subgroup supplemented with vitamins followed by the control DON-subgroup. Subgroups with zinc supplementation had the lowest mass gain, which exhibited possible antagonistic effects between DON and Zn(II) ions (Li et al., 2010).

On the 28th day of the experiment, the animals were euthanised and dissected. Necropsy of the animals did not reveal any pathological changes, including damage to liver and kidneys. Tissue extracts from the liver, kidneys, spleen, heart, brain, eyes, gonads, and femoral muscle were obtained, processed and analysed. Tissue homogenates and blood samples used for analysis from all the experimental animals consisted of a total of 576 samples. Changes in protein and metallothionein levels and alterations in its synthesis were dependent on feed mixtures. Enzymes (ALT, AST, GST) and total antioxidant capacity were analysed in blood samples.

Determination of metallothionein level

As stated in the Introduction, metallothioneins are low-molecular mass thermostable proteins that play an essential role in maintaining the metal ions' homeostasis and detoxification (Adam et al., 2007, 2010a, 2010b; Eckschlager et al., 2009; Krizkova et al., 2009c, 2010). Levels of metallothionein were determined to measure oxidative stress. Oxidative stress apparently enhances levels of MT protein in the liver, kidneys, and gonads (Fabrik et al., 2008b; Krizkova et al., 2007). These organs are characterised by higher metabolic activities. Electrochemical detection of metallothionein was used to monitor the MT content in the blood and tissues of the liver, kidneys, spleen, heart, brain, eyes, gonads, and femoral muscle (Fig. 3). The figure clearly shows that the highest level of MT is found in the kidneys and liver and the lowest in the muscle, heart, and eyes. The differences between the MT levels in blood samples from rats fed with natural and mouldy barley were not significant (Fig. 3). Intake of mycotoxin increased the MT levels above 30 % compared with the blood from the control subgroup, with an initial concentration of 4.9 μg mL^{-1} to an increase of 6.16 μg mL^{-1}. The highest level of MT was observed in the non-DON group supplemented with

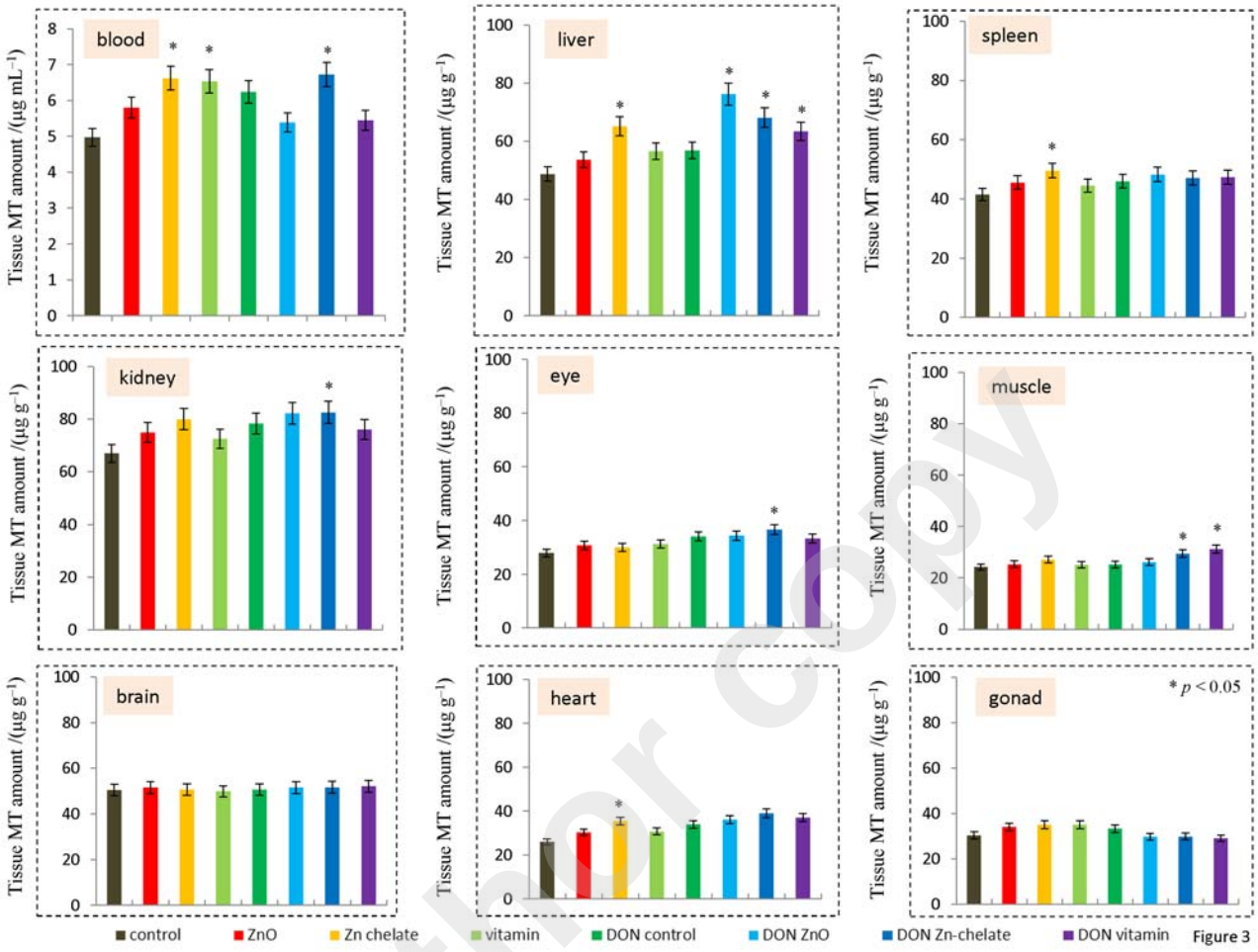

Fig. 3. The experimental-group-average level of MT in blood, liver, kidneys, heart, spleen, gonads, muscle, eyes, and brain. A similar trend was observed in MT level in every tissue sample in individual groups. The highest level of MT was usually observed in DON and non-DON groups in the subgroup fed on Zn-chelate and the lowest in the control non-DON group.

vitamins and organic Zn(II) chelate as well as Zn(II) in organic form in the DON-containing group.

The average MT levels in different tissues show their variability. The highest MT content was found in the kidneys (67.0 μg g^{-1}) and liver (48.7 μg g^{-1}), organs critical to xenobiotics detoxification. The content was twice as high as in other organs. Higher levels were also detected in the brain (50.5 μg g^{-1}) and spleen (41.5 μg g^{-1}). The MT concentrations correspond to isoform MT-3 found mainly in the brain (Vasatkova et al., 2009). The MT levels in the heart (without blood) were similar to the MT levels in muscle. This can be due to the similar physiological activities of both types of muscles. Statistical evaluation of the MT content in organs was carried out. The MT content in the heart was not statistically different from the MT levels in the gonads and eyes. The MT content is also not significant in the gonads in comparison with its level in the eyes. All other tissues showed a significant difference after the application of DON ($p < 0.05$).

The MT levels differed in individual organs and between groups fed with natural and mouldy barley (Fig. 3, $p < 0.05$). The spleen and brain MT levels were not significant. Similar MT level trends were observed in individual groups. The groups containing the highest MT levels were those where the feed was enriched with the organic Zn(II) chelate. Significant ($p < 0.05$) MT levels were observed with the organic chelate in the liver, kidneys, and muscles. The MT levels in groups raised on feed containing DON were slightly elevated in comparison with the non-DON groups. An interaction ($p < 0.01$) was evident in the gonads. The DON-containing groups showed that vitamins, organic, and inorganic zinc supplementation reduced MT levels in comparison with the non-DON groups. Inorganic forms also increased the MT expression but the results were not comparable with organic zinc values. Feed supplemented with vitamins did not indicate any significant effect after consumption. Moreover, organic and inorganic Zn(II) and vitamins supplementation had no significant effect on MT levels in the brain tissues of rats. All significant results are marked "*" in Fig. 3. To verify electrochemical detection, SDS PAGE was used. Electrochemical detection showed re-

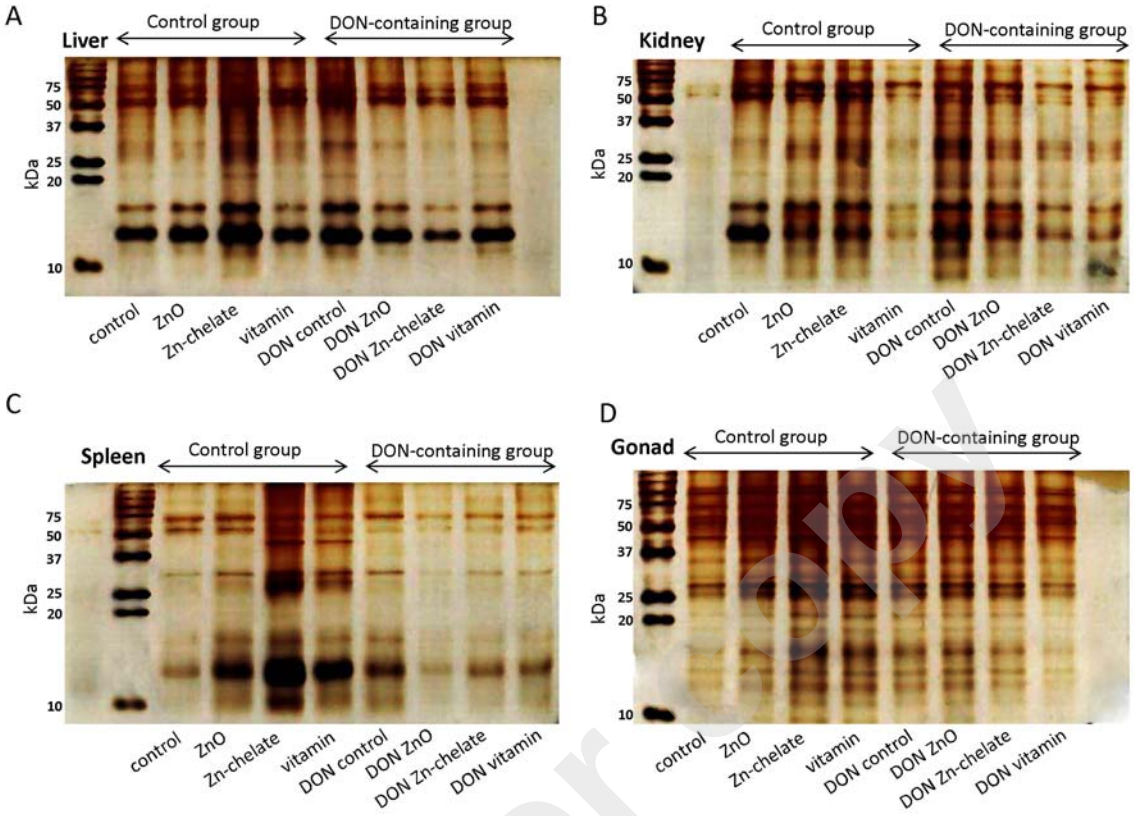

Fig. 4. SDS-PAGE of extracts obtained from liver, kidneys, spleen, and gonads. The content of MT corresponds to results obtained from electrochemical detection. The highest level of MT was demonstrated in the non-DON and DON subgroups fed with mixtures supplemented by zinc in organic form.

sults similar to gel electrophoresis values. The highest level of MT was observed in the subgroups fed on mixtures supplemented with organically bound zinc (Fig. 4). All forms of MT were detected in standards, e.g. 10 kDa, 15 kDa, 25 kDa, and 50 kDa. The most intense band was observed in the tetramer MT with a molar mass of 25 kDa. The intensities observed in the bands were higher in the native samples. However, the MT bands were not as distinctive, due to smearing and aggregation of MT into octamer forms (50 kDa) (Krizkova et al., 2009a).

The correlation of DON occurrence in organs and the MT levels is very interesting. Previous studies have shown varying DON distributions in organs following exposure. DON amounts in the body tissues have been observed within 30 min after exposure in: kidneys (5.68 μg g^{-1}), heart (4.53 μg g^{-1}), blood (4.43 μg g^{-1}), liver (3.90 μg g^{-1}), thymus (3.64 μg g^{-1}), spleen (2.90 μg g^{-1}), brain (0.73 μg g^{-1}) (Yordanova et al., 2003). Values with a high distribution of DON correlated with higher MT levels.

Total content of proteins

MT belongs to a group of thermostable proteins resistant to denaturing temperatures. The enhanced expression of MT caused an increase in levels of all thermostable proteins. The highest levels of these proteins were detected in the gonads ((3.8 ± 0.4) mg mL^{-1}) and the lowest levels were detected in the eyes ((1.1 ± 0.3) mg mL^{-1}). Other values varied from 2.5 mg mL^{-1} to 3.5 mg mL^{-1} (not shown). The results of MT in tissues were recalculated to a protein level. The results show that the trends in MT content and MT-recalculated content are similar. The enhanced MT levels also increased the production of proteins. The lowest ratio was observed in the gonads, which contained MT on an average of only 0.88 mg g^{-1} due to high amounts of thermostable proteins. The highest re-calculated MT content of 3.74 mg g^{-1} was determined in the kidneys. High levels of MT were observed in the eyes in groups with the zinc-supplemented feed mixtures. Numerous papers have demonstrated that eyes are a depository for zinc (Lengyel et al., 2007). Zinc is known for its ability to enter cells, bind to the receptors and activate genes for MT transcription. Its specific abilities may explain the high levels of MT ((3.3 ± 0.5) mg g^{-1}). On the basis of the results obtained, it can be concluded that enhanced levels of MT in one organ correspond to enhanced levels in other organs. This shows that organisms have an effective immune response to the MT levels.

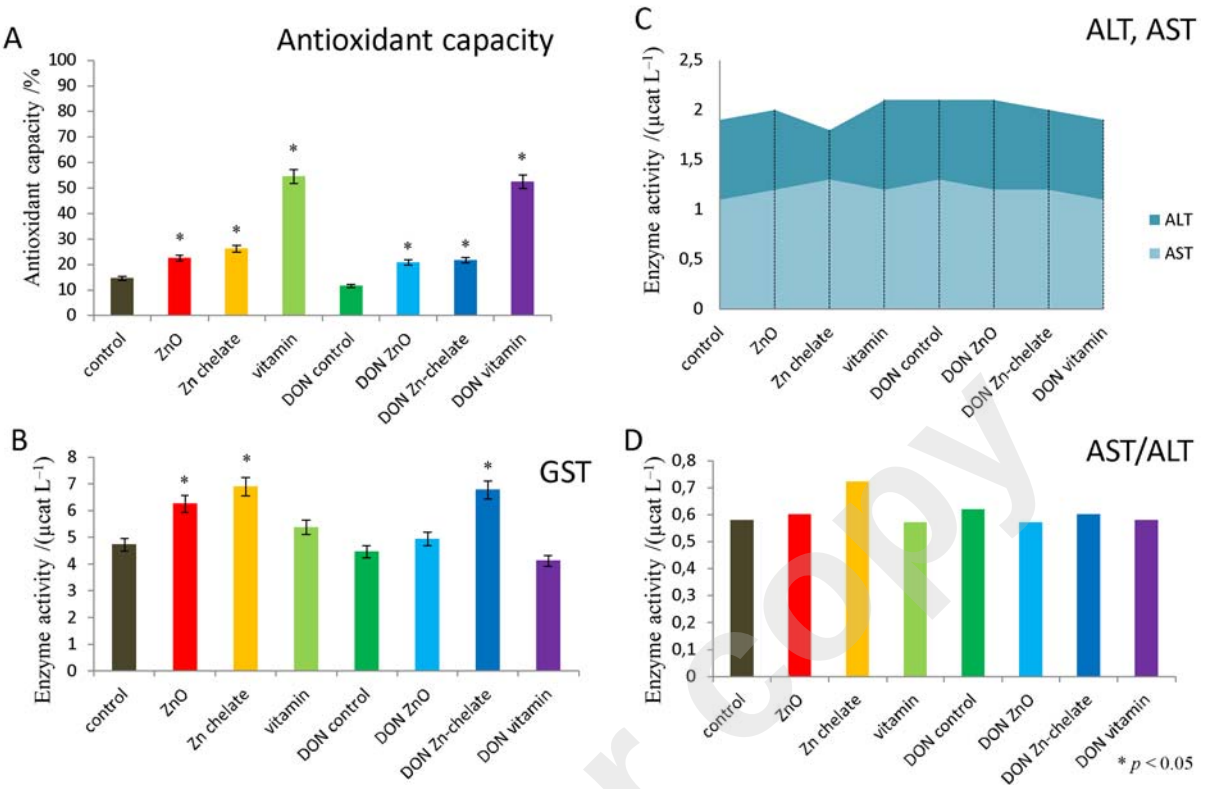

Fig. 5. Antioxidant capacity (A): All groups indicated an enhanced antioxidant capacity in comparison with control groups. The highest response was observed in DON and non-DON groups fed with mixtures containing vitamins. Activity of glutathione-*S*-transferase (B): The highest values were observed in groups which were fed with mixtures supplemented with zinc in organic form. Activity of ALT and AST (C): ALT/AST activities were similar and showed proper liver function. AST/ALT ratio determined in experimental rats (D): A ratio value higher than 1 represents the risk of liver damage. The highest level was observed in the non-DON group fed with zinc chelate but the value did not attain the value of 1.

Antioxidant capacity

Antioxidant capacity provides information on an organism's ability to scavenge free oxygen radicals. DPPH was used to measure antioxidant capacity. The results show an organism's response was highest ($p < 0.05$) in the DON and non-DON groups fed with mixtures containing vitamins (Fig. 5A). This shows that vitamins and their role as cofactors in enzymes influence metabolic activity. The ability to regulate levels of reactive oxygen species is one of their most important functions (Borutova et al., 2008; Doohan et al., 2008). Other groups also exhibited increased antioxidant capacity levels but their values were fractional in relation to the vitamin values. The lowest response was exhibited in the DON-supplemented mixtures control group. This response may be due to the immunosuppressive effects of DON.

Determination of glutathione-S-transferase activity

Glutathione-*S*-transferase (GST) is a family of enzymes that plays an important role in detoxification of xenobiotics. GST supports the formation of a thiol adduct of glutathione to xenobiotics, i.e. it utilises glutathione to scavenge potentially toxic compounds, including those produced by oxidative stress. It is also a part of the defence mechanism against the mutagenic, carcinogenic, and toxic effects of such compounds (Sen & Packer, 1996). An increased activity shows an organism's ability to detoxify xenobiotics. The activities of GST correlated with the MT results (Fig. 5B). The highest values ($p < 0.05$) were observed in groups fed with mixtures supplemented with zinc in organic form (6.9 μcat L^{-1} for non-DON and 6.7 μcat L^{-1} for DON-containing feed mixtures). Although the groups fed with mouldy barley containing high levels of DON showed the lowest activities in relation to the control group without DON, the results were not significant.

Determination of liver enzymes activity

Liver enzymes, alanine transaminase (ALT) and aspartate transaminase (AST), are the most common enzymes found in serum and in various tissues but they are most commonly associated with the liver. These enzymes are used in diagnostic liver function tests to determine liver status. In general, increased levels reflect rates of liver damage. The results did not confirm liver damage in any of the samples

(Fig. 5C). All ALT/AST activities were similar and showed proper liver function (Singh et al., 2010). The aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio, known as the Ritis coefficient, is used in differentiating causes of liver damage (hepatotoxicity) (Gopal & Rosen, 2000; Nyblom et al., 2004). This ratio should not be higher than 1. The ratios of both enzymes are shown in Fig. 5D. The results exhibit that these enzymes and their activities do not represent the risk of liver diseases.

Conclusions

The study of toxicological substances and their influence on animal health is a current and active topic which provides valuable information (Das et al., 2012; Mackuľak et al., 2011, 2012; Takáčová et al., 2012). Numerous studies on toxic substances have been carried out (Reboux et al., 2010) but the effects of Zn(II) and its influence on DON have not yet been examined. The effects of mycotoxin deoxynivalenol were investigated by feeding rats on diets containing mouldy barley for 28 days. Pursuant to a thousand analyses, interesting behavioural and biochemical effects of mycotoxicity were examined. This study shows that metallothionein plays an important role in the process of mycotoxins detoxification. Its exact role remains unclear, but this protein may be involved in the redistribution of significant ions to transcriptional factors. It may also interact with oxygen radicals generated by mycotoxins. This assumption was confirmed with the DON intake results in enhancing synthesis of MT. Zinc-supplemented natural and mouldy feed mixtures showed other interesting results. Synergy as well as antagony is of great interest but has not been extensively studied. The results from this study allowed levels of deoxynivalenol toxicity, its biochemical effects and its role in the detoxification process to be further examined.

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