ORIGINAL ARTICLE

The effects of mycotoxin deoxynivalenol (DON) on haematological and biochemical parameters and selected parameters of oxidative stress in piglets

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Abstract

OBJECTIVES: Deoxynivalenol (DON) – trichothecene mycotoxin, is frequently detected in high concentrations in cereals in the temperate region of Europe. The aim of this study was to determine the effect of DON in feed on haematological and biochemical parameters and on oxidative stress in piglets.

METHODS: Two concentrations of DON in feedstuff for pigs were chosen: 0.6 mg/kg (group C) and 2.0 mg/kg (group M). Twelve weaned pigs were used in each group. Pigs were fed with naturally contaminated feed for 4 weeks. On days 14, 21 and at the end of the experiment (day 28) samples of blood were taken to determine haematological parameters, plasma biochemical parameters, ceruloplasmin activity and FRAP (ferric reducing ability of plasma).

RESULTS: The haematological variables did not show changes in response to contaminated diet with exception of the mean corpuscular volume, which was significantly decreased at the end of the experiment in the group M. A significant increase of alkaline phosphatase activity (140%, $p<0.01$) was found in the group M compared to the group C at the end of the experiment. A significant decrease was found on the day 21 in FRAP (85%, $p<0.001$) and on the day 28 in ceruloplasmin (75%, $p<0.01$) in the group M compared to the group C.

CONCLUSIONS: The decrease of FRAP and ceruloplasmin indicate a lowered ability of organism to scavenge reactive oxygen species. The higher concentration of DON in feedstuffs had a negative influence on the antioxidant ability of piglet's plasma.

Abbreviations:

ALP	- alkaline phosphatase
ALT	- alanine aminotransferase
AST	- aspartate aminotransferase
BW	- body weight
DON	- deoxynivalenol
FRAP	- ferric reducing ability of plasma
Hb	- haemoglobin
LDH	- lactate dehydrogenase
MCH	- mean corpuscular haemoglobin
MCHC	- mean corpuscular haemoglobin concentration
MCV	- mean corpuscular volume
PCV	- packed cell volume, haematocrit
PLT	- platelet number
RBC	- red blood cell count
TP	- total protein
WBC	- white blood cell count

INTRODUCTION

The *Fusarium* fungi are probably the most prevalent toxin-producing fungi of the northern temperate regions and are commonly found on cereals grown in the temperate region of America, Europe and Asia (European Commission, Scientific Committee on Food, 1999). A variety of *Fusarium* fungi is a common soil fungus, which produces a number of different mycotoxins from the class of trichothecenes (T-2 toxin, HT-2 toxin, deoxynivalenol, nivalenol and another) and some other toxins (zearalenone and fumonisins). In the Czech Republic the most frequently detected mycotoxins are trichothecenes, especially DON and T-2 toxin (Klem *et al.* 2007). Among farm animals, especially pigs react sensitively to higher concentration of DON (Döll & Dänicke, 2011). The contamination of fusariotoxins in feedstuffs requires establishment of limits in feed, which would not place animals the risk. According the Commission Recommendation 2006/576/EC the guidance value for DON in complementary and complete feedstuff for pigs is 0.9 mg/kg.

The aim of this study was to determine the effect of two-fold increased concentration of DON in feed on haematological and biochemical parameters and on oxidative stress in piglets. Moreover, we tested whether biochemical markers could be used to indicate DON contamination in feed and how they changed during the exposure.

MATERIALS AND METHODS

Test animals, an experimental design and a sample collection

Twenty-four weaned crossbred pigs (Landrace x Czech large white) at 4 weeks of age were used in the experiment. The piglets (castrated males and females) were allocated to four subgroups 6 animals per each (3 males and 3 females) and acclimatized 2 weeks at the experimental facility. The animals were fed ad libitum a diet prepared locally and formulated according to energy and amino acid requirements (Table 1). Initial average body weight of the pigs was 9.44±1.2 kg. After acclimatization, a half of the piglets (two subgroups) continued to be fed the same diet (group C), the other half (two subgroups) was fed diet which contained DON-contaminated corn (group M). The content of DON in the control diet and in the contaminated feed was 0.6 and 2 mg/kg, respectively. After 2 and 3 weeks and at the end of the experiment (4 weeks), samples of blood were aseptically collected from a jugular vein into tubes containing sodium heparin and animals were weighted. Plasma samples were obtained after centrifugation of blood and stored at –80 °C until analyzed.

Experimental procedures were in compliance with national legislation (Act No. 246/1992 Coll., on the Protection of Animals Against Cruelty, as amended, and Decree No. 207/2004 Coll., on the Protection, Breeding and Use of Experimental Animals, as amended).

Haematological measurement and plasma analyses

The packed blood cell volume (PCV), hemoglobin (Hb), red blood cells number (RBC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet number (PLT), white blood cells number (WBC) were determined using an automated haematology analyzer (Nihon Kohden, Celltac Alpha). The differential count of white blood cells (lymphocytes, monocytes, neutrophils, eosinophils and basophils) was determined manually on 100 leukocytes. Blood smears were stained panoptically according Pappenheim using May-Grűnwald and Giemsa-Romanowski stains.

The plasma biochemical parameters (albumin, total protein, urea, creatinine, cholesterol, triglycerides, lactate, calcium, phosphorus, chloride, potassium, sodium, ALP, LDH, AST, ALT) were analyzed using a biochemical analyzer Konelab 20i and commercial test kits (BioVendor, CZ). The ceruloplasmin activity was analysed according to Ceron and Martinez-Subiela (2004) with slight modifications using a Varioskan flash spectral scanning multimode reader (Thermo Scientific). Results were expressed as the amount of the absorbance increase per minute × 10,000. Ferric reducing ability of plasma (FRAP) was measured on a biochemical analyzer Konelab 20i according to Benzie and Strain (1996).

Histological examination

The tissue samples (liver, kidney) were collected from euthanatized animals and fixed in buffered 10% neutral formalin for histopathological analysis. The tissue pieces were dehydrated trough graded alcohols and embedded in paraffin wax. Sections of 3 μm were stained with hematoxylin–eosin. For each organ, three slides per animals were prepared for analysis.

Statistical analysis

The results were analyzed using the statistical package Unistat 6.1. (Unistat Ltd., GB). For all variables tested,

normality and homogeneity of variances was checked by means of a Shapiro-Wilk test. Data were subjected to a one-way ANOVA and subsequently to a Tukey-HSD test for multiple comparisons in order to assess the statistical significance of differences among all possible pairs of groups. Differences were considered significant at $p<0.05$.

Analyses of mycotoxins in the animal diet

The sample preparation of animal feed was based on the method described by Monbaliu *et al.* (2010). The measurement of mycotoxins was based on high–performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry. A Thermo Scientific UHPLC Accela 1250 system was connected to a Thermo Scientific TSQ Quantum Access MAX Triple Quadrupole Instrument (Thermo, San Jose, CA, USA) equipped with heated electrospray ionization (HESI-II) probe. A Thermo Scientific Hypersil C_{18} (2.1×50 mm, 1.9 µm) column was used at a constant flow rate of 300 µl/min. Mobile phase consisted of water/methanol/acetic acid (94/5/1; v/v/v), and 5 mmol/l ammonium acetate (solvent A) and methanol/water/acetic acid (97/2/1; v/v/v) and 5 mmol/l ammonium acetate (solvent B). The gradient used was: 0–8 min linear gradient from 5 to 75% B; 8–10 min held at 75% B; 10–10.5 min from 75 to 5% B and 10.5–11 min held at 5% B. The full loop injection volume of the extract was set at 10 µL. Standards of mycotoxins and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). All solvents were of residual analysis purity (Chromservis, s.r.o., CZ). Detection limits for T-2 toxin, HT-2 toxin, DON and 3-acetyl DON are 35, 65, 95 and 350 µg/kg, respectively. The mycotoxin content in animal feeds is in Table 2.

RESULTS

Higher concentration of DON in diet did not significantly impair animal body weight during the test (Table 3). Haematological variables did not show changes in response to contaminated diet with exception of the mean corpuscular volume, which was significantly decreased (94%, $p<0.05$) at the end of experiment in the group M (Table 4).

A significant increase of ALP activity (140%, $p<0.01$) was found in the group M compared to the group C at the end of the experiment. A significant decrease of the phosphorus concentration (83%, $p<0.05$) and increase of the calcium concentration (105%, $p<0.05$) in plasma was detected in the group M after 3 and 2 weeks, respectively (Table 5). Significant decreases were found on day 21 in FRAP (85%, $p<0.001$) and on day 28 in ceruloplasmin (75%, $p<0.01$) in the group M compared to the group C (Table 6).

The histological examination revealed no changes in control tissue samples and tissue samples from mycotoxin treated animals.

DISCUSSION

The pigs are highly susceptible to the effect of DON. The most common effects of prolonged dietary exposure are decreased weight gain, anorexia, and altered nutritional efficiency (Pestka, 2007). Various adverse effects have been described for DON on biochemical pathways, which can damage biomolecules and membranes leading to apoptosis (Desmond *et al.* 2008).

A significant decrease of daily feed consumption in pigs was observed at a DON concentration from 1.7 mg/kg in naturally contaminated oats (Bergsjø *et al.* 1993). We did not recorded significant changes in body weight of piglets during the 4 week trial fed naturally

Tab. 1. Composition of the experimental diet.

Ingredient	(%)
Corn	50
Barley	22.5
Soybean meal	22.5
Vitamin and mineral premix*	5
Composition (%)	
Crude protein	15.5
Starch	6.5
Crude fiber	4.5
Fat	2.2
Lysin	1.25
Methionin	0.3
Ca	1.0
P	0.68
Na	0.25

* Vitamin A, 6 000 IU/kg; vitamin D_3, 900 IU/kg; iron, 80 mg/kg; iodine, 0.5 mg/kg; cobalt, 0.4 mg/kg; copper, 14 mg/kg; manganese, 35 mg/kg; zinc, 90 mg/kg; selenium, 0.1 mg/kg; D,L-methionine, 0.25 g/kg; lysine, 4 g/kg

Tab. 2. Mycotoxin contamination (mg/kg) in the experimental diets.

Mycotoxin	Group C	Group M
Deoxynivalenol	0.6	2.0
3-acetyl DON	nd*	nd
T-2 Toxin	nd	nd
HT-2 Toxin	nd	nd
Zearalenon	nd	nd
Fumonisin B1+B2	nd	nd
Ochratoxin A	nd	nd

*nd = not detectable

Tab. 3. Body weight (BW) in pigs (n=12) fed diets containing different concentrations of DON after 2, 3 and 4 weeks of exposition.

	Start of experiment			2 weeks			3 weeks			4 weeks		
	Group C	Group M	*p*-value	Group C	Group M	*p*-value	Group C	Group M	*p*-value	Group C	Group M	*p*-value
BW [kg]	9.58	9.31	0.60	17.42	16.43	0.34	22.67	20.70	0.09	28.60	26.65	0.27

Tab. 4. Haematological values in pigs (n=12) fed diets containing different concentrations of DON after 2, 3 and 4 weeks of exposition.

Parameter*	2 weeks			3 weeks			4 weeks		
	Group C	Group M	*p*-value	Group C	Group M	*p*-value	Group C	Group M	*p*-value
RBC [T/l]	5.56	5.59	0.85	5.67	5.86	0.20	5.32	5.31	0.98
Hb [g/l]	100.83	97.90	0.34	105.80	106.90	0.42	101.82	96.50	0.17
PCV [l/l]	31.64	30.86	0.40	32.84	32.55	0.62	30.89	29.12	0.15
MCV [fl]	57.00	55.31	0.22	57.94	55.60	0.13	58.15	54.82	**0.03**
MCH [pg]	18.16	17.54	0.19	18.75	18.26	0.38	19.21	18.16	0.06
MCHC [g/l]	318.75	317.20	0.51	323.36	328.10	0.32	329.91	331.50	0.65
PLT [G/l]	699.92	714.22	0.85	593.80	706.90	0.09	583.54	606.80	0.63
WBC [G/l]	23.62	23.90	0.90	20.61	24.65	0.17	21.05	21.92	0.66
Lymphocytes [%]	70.25	68.70	0.77	73.25	70.40	0.54	41.92	42.30	0.95
Neutrophils [%]	26.83	27.80	0.84	25.58	26.20	0.79	52.58	53.10	0.77

* WBC-white blood cells, RBC-red blood cells, Hb-hemoglobin, PCV-packed blood cell volume, MCV-mean corpuscular volume, MCH-mean corpuscular hemoglobin, MCHC-mean corpuscular hemoglobin concentration, PLT-platelets

Tab. 5. Serum chemical values in pigs (n=12) fed diets containing different concentrations of DON after 2, 3 and 4 weeks of exposition.

Parameter*	2 weeks			3 weeks			4 weeks		
	Group C	Group M	*p*-value	Group C	Group M	*p*-value	Group C	Group M	*p*-value
Albumin [g/l]	32.38	31.86	0.57	33.96	33.63	0.81	26.16	28.30	0.33
TP [g/l]	56.10	53.39	0.11	53.47	53.20	0.85	47.18	49.24	0.47
ALP [µkat/l]	5.95	7.46	0.06	4.60	5.62	**0.05**	3.72	5.19	**0.006**
ALT [µkat/l]	1.11	0.96	0.31	1.00	1.00	0.99	0.89	0.90	0.87
AST [µkat/l]	1.07	0.92	0.36	0.79	0.77	0.82	1.42	1.54	0.64
LDH [µkat/l]	12.46	12.69	0.68	10.59	11.56	0.19	15.31	19.48	0.18
Ammonia [µmol/l]	103.62	83.68	0.17	80.28	65.95	0.21	67.88	82.78	0.30
Bilirubin [mmol/l]	2.27	2.16	0.99	0.86	0.78	0.68	1.73	1.77	0.87
Cholesterol [mmol/l]	2.41	2.37	0.78	2.40	2.46	0.61	1.69	1.90	0.24
Triglycerides [mmol/l]	0.65	0.62	0.77	0.52	0.59	0.29	0.55	0.66	0.17
P [mmol/l]	3.56	3.55	0.85	4.05	3.37	**0.02**	2.42	2.38	0.97
Cl [mmol/l]	106.48	105.69	0.23	107.95	106.72	0.14	105.36	104.82	0.59
K [mmol/l]	4.60	4.25	0.07	5.12	5.36	0.36	3.89	3.58	0.17
Na [mmol/l]	145.05	144.71	0.79	146.00	144.63	0.19	145.67	145.48	0.87
Ca [mmol/l]	2.78	2.93	**0.01**	2.49	2.51	0.85	2.40	2.56	0.16
Creatinine [µmol/l]	101.72	96.69	0.55	102.00	102.22	0.96	107.94	125.48	0.28
Lactate [mmol/l]	14.82	13.51	0.38	14.38	13.36	0.55	7.06	9.33	0.26
Urea [mmol/l]	2.95	2.29	0.36	2.28	2.01	0.34	2.81	2.52	0.30

* TP-total proteins, ALP-alkaline phosphatase, ALT-alanine transaminase, AST-aspartate transaminase, LDH-lactate dehydrogenase

Tab. 6. Ferric reducing ability of plasma (FRAP) and ceruloplasmin concentration in pigs (n=12) fed diets containing different concentration of DON after 2, 3 and 4 weeks of exposition.

Parameter	2 weeks			3 weeks			4 weeks		
	Group C	Group M	*p*-value	Group C	Group M	*p*-value	Group C	Group M	*p*-value
FRAP [μmol/l]	305.44	276.07	0.08	285.38	242.72	**0.0003**	289.16	283.12	0.73
Ceruloplasmin [ΔA/min × 10,000]	357.27	308.57	0.15	388.23	372.70	0.66	351.76	264.37	**0.003**

contaminated feed containing 2 mg/kg of DON compared to 0.6 mg/kg of DON. Although the difference in nominal weight of both experimental groups at the start of experiment was less than a half of a kilogram, after 2 weeks of test it was 1kg and after 3 and 4 weeks it was 2kg. The variability in weight was statistically insignificant due to the insufficient number of experimental animals and the short duration of the experiment. This finding is in agreement with the result of Chaytor *et al.* (2011), who has not observed differences between the treatments of pigs on diets with DON and aflatoxin until after 3 weeks of feeding.

The haematological parameters have been weakly or not affected by DON in the low concentration. The natural contamination of pig feed with 0.28 to 0.84 mg/kg of DON did not influence PCV, hemoglobin concentration, MCHC and MCV in piglets after 28-days (Accensi *et al.* 2006). Grenier *et al.* (2011) did not find an effect on any haematological parameters in piglets after 35-days feeding DON-contaminated diet in concentration 2.8 mg/kg. The temporary fall in PCV was described in concentration 3.5 mg/kg of DON (Bergsjø *et al.* 1993). The decrease of mean corpuscular volume (MCV) at the end of experiment was detected in our experiment whereas other red blood cells indices were not influenced by the treatment. The decline of MCV in pigs is often connected to ferric deficiencies. The decrease of feed consumption in the group with higher concentration of DON in feed could cause a lower intake of iron.

Biochemical variables are sensitive indicators of animal health. The influence of low concentration of DON in biochemical parameters of pigs was described by Accensi *et al.* (2006). A significant diet effect was shown only for phosphorus. Using greater doses of DON in the feed (3–5.8 mg/kg), a decrease in Ca, P, Cl, total serum protein and globulin was observed (Bergsjø *et al.* 1993; Prelusky *et al.* 1994; Rotter *et al.* 1995; Swamy *et al.* 2002, 2003).

A reduction of plasma or serum phosphorus concentration and the influencing of ALP activity were found by several workers (Lun *et al.* 1985; Trenholm *et al.* 1994; Young *et al.* 1983). It is possible that DON may interfere with metabolism of phosphorus in the pig, but the specific mechanism of action is not clear. The significant effect of DON on phosphorus concentration in serum and ALP activity in our experiment was manifested only after 3 and 4 weeks, respectively.

The observed change of serum calcium concentration in our experiment at 2-weeks is of minor importance and probably is not related to the effect of DON.

Recently, it was shown that mycotoxin-containing diet can cause an oxidative stress in a mouse (Hou *et al.* 2013), broilers (Borutova *et al.* 2008), a pig (Jiang *et al.* 2011) and other animals. A diet contaminated by aflatoxin, zearalenon and DON in low concentrations caused a decrease of a catalase activity and an increase of glutathione peroxidase activity and malondialdehyde concentration in a mouse serum (Hou *et al.* 2013). A study of Jiang *et al.* (2011) showed that dietary zearalenone exposure in gilts induced oxidative damage by increasing lipid peroxidation and decreasing of selected antioxidant enzymes activities in the liver. Zbyňovská *et al.* (2013) found that DON influenced anti- and prooxidant indices in porcine blood *in vitro*. To determine whether these mycotoxins could induce the oxidative stress *in vivo*, our study examined ceruloplasmin level in the plasma. Ceruloplasmin has various functions in organism including an antioxidant activity. It has been reported that ceruloplasmin may scavenge reactive oxygen species (Healy & Tipton, 2007).

FRAP represents antioxidant power of plasma generally mediated through non-protein antioxidants. The FRAP method has been used to determine antioxidant capacity changes due to actions of drugs (Pohanka *et al.* 2011), pesticides (Haluzová *et al.* 2011) and other foreign agents. The DON-contaminated diet caused the decrease of the antioxidant capacity of plasma piglets after 4 weeks.

CONCLUSION

The haematological parameters are only weakly influenced by low concentration of DON. ALP is the most apparent biochemical parameter in plasma affected by DON. Although the effect of trichothecene mycotoxins on oxidative stress requires a future study, it is clear that DON strongly decreases the antioxidant capacity of piglets and the animals being fed higher concentration of DON are more susceptible to other toxicants.

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